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A STUDY OF SOME ASPECTS OF THE KINETICS, MECHANISM AND LIPID DEPENDENCE OF THE Na^{+}-K^{+}-ATPase.

BY

Ruthven Norbert Alwin Hudson Lewis

B.Sc. (Dunelm)

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Being a thesis presented in candidature for the degree of Doctor of Philosophy of the University of Durham.

December 1978

Hatfield College,
University of Durham

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ABSTRACT

The kinetics of the Na^+-K^+-ATPase with respect to ouabain inhibition, potassium activation and the effect of temperature on its catalytic activity has been studied, and attempts were made to define these kinetic parameters using computer assisted procedures. The pattern of ouabain inhibition of the enzyme was described within the context of the enzyme being a cooperative kinetic dimer. The theoretical basis for the non-ideal Arrhenius temperature kinetics of this enzyme was also studied and its behaviour was described in terms of a thermodynamic equilibrium between a high temperature active state and a low temperature inactive state. The sigmoidal response of the enzyme to increasing potassium ion concentrations was quantified in terms of its stoichiometric requirement for two moles of potassium ion per mole of ATP hydrolysed.

The effects of lipid targeted and protein targeted modulators on the kinetic properties of the Na^+-K^+-ATPase were studied. The kinetic properties of the enzyme was insensitive to the perturbations in the membrane brought about by peroxidisation of membrane lipids, and the detergent extraction procedures used for partial purification of the enzyme provided that the final preparation was not labile. Those kinetic properties were also insensitive to the membrane lipid changes coincident with the adaptation of a given species to different temperatures. The kinetic properties of the Na^+-K^+-ATPase (or its K^+-phosphatase activity) were found to be sensitive to a glycoprotein aimed modulator (Concanavalin A), and a sulphydryl blocking reagent (thimerosal). A partial thermal inactivation of the Na^+-K^+-ATPase was also found to result in significant changes in its kinetic properties.

Studies on the thermal inactivation of the Na^+-K^+-ATPase revealed a biphasic decay pattern which was interpreted in terms of a sequential decay by a dimeric species. A comparative study of the thermal inactivation of the enzyme showed that Na^+-K^+-ATPases prepared from animals that were naturally adapted to function at 'high' temperatures were of greater thermal stability than those prepared from animals that normally function at low temperatures. However, the thermal stability of the enzyme was not affected by acclimation of a species to different temperature conditions. The kinetic stability of the enzyme was not affected by a partial purification by sodium dodecylsulphate extraction, but was significantly reduced on incubation with octanol.

It is proposed that membrane lipids play little (if any) role in the fine control of the Na^+-K^+-ATPase, and that their primary role is that of restricting the Na^+-K^+-ATPase to its biologically active conformational states. It is also proposed that fine control of the Na^+-K^+-ATPase can be brought about by a variety of mechanisms aimed at the protein-protein interactions as well as allosteric modulations by regulatory ligand binding sites.

CHAPTER 1

GENERAL INTRODUCTION

The active coupled transport of sodium and potassium ions has been shown to be a property characteristic of the plasma membranes of animal cells. This has been linked to the activity of an ATPase which is synergestically activated by sodium and potassium ions. The relevant enzyme, the Na^{+}-K^{+}-ATPase (ATP phosphohydrolase EC 3.6.1.3), was first reported by Skou (1957), and a role for this enzyme in the direct or indirect regulation and control of cellular function has been recognised (see Baker, 1972). Such has inevitably resulted in a great deal of research aimed at the total characterisation of this enzyme system. These studies have advanced mainly along structural and kinetic/mechanistic lines of investigation.

(a) Structural studies of the Na^{+}-K^{+}-ATPase.

An early insight into the structure of the Na^{+}-K^{+}-ATPase was given by the demonstration that a severe delipidation of membranes containing the enzyme resulted in a loss of Na^{+}-K^{+}-ATPase activity (Schatzmann, 1962). This suggested that a firm association with membrane lipid was necessary for the retention of biological activity (i.e. the Na^{+}-K^{+}-ATPase is a functional lipoprotein). This has been supported by delipidation and lipid reconstitution studies (Tanaka & Strickland, 1965), and by the apparent inability to obtain pure biologically active Na^{+}-K^{+}-ATPase preparations that is free of membrane lipid. However, the controlled use of surface active agents and high ionic strength has been used to differentially extract proteins

that are less firmly membrane bound than the Na^+-K^+-ATPase, and so obtain Na^+-K^+-ATPase preparations of high specific activity (see Hokin & Dahl, 1972; Schwartz, Lindenmayer & Allen, 1975; Jorgensen, 1974(c); Kyte, 1971; Nakao, Nakao, Hara, Nagai, Yagasaki, Koi, Nakagawa & Kawai, 1974). Further analyses of such preparations have shown that they contain two polypeptide species that are associated with a variety of lipid components (see Jorgensen, 1974(b); Kawai, Nakao, Nakao & Fujiata, 1973; DePont, Van Prooijen-Van Eeden & Bonting, 1978).

The protein components isolated from 'highly purified' Na^+-K^+-ATPase preparations have all been shown to contain a large polypeptide (M.W.≃ 95,000-100,000) and a smaller sialoglycoprotein (M.W. ≃ 45,000-55,000). However, there is some uncertainty as regards the relative molar proprtions of these species present in such preparations. Some workers report evidence supporting a 1:1 molar ratio (Jorgensen, 1974(b); Lane, Copenhaver & Schwartz, 1973), while others have reported evidence supporting a 2:1 molar ratio of large polypeptide to small polypeptide (Perrone, Hackney, Dixon & Hokin, 1975; Hopkins, Wagner & Smith, 1976). These two polypeptides are found even after molecular exclusion chromatography of highly active preparations and their separation has been achieved only by methods which result in a loss of biological activity (Kyte, 1971; Lewis, 1974). This has suggested that the functional Na^+-K^+-ATPase is an aggregate of these two polypeptides. Cross-linking studies (Kyte, 1972; Lewis, 1974; Sweadner, 1977) have supported such suggestions by providing evidence that the two polypeptides are closely aligned in the membrane matrix, and recent electron

microscopic studies on highly purified preparations have also provided further support for such an assumption (Vogel, Meyer, Grosse & Repke, 1977).

Some studies pertinent to the structure of the Na^+-K^+-ATPase have indicated that the polypeptide constituents of highly active preparations of this enzyme contain a relatively high proportion of 'hydrophobic' amino acid residues (Kyte, 1972; Hokin, 1974; Hopkins, Wagner & Smith, 1976). In this respect, these polypeptides are similar to those of other tightly bound membrane proteins (see Guidotti, 1972), and are of the form predicted for such proteins (Fischer, 1964). Those reports are therefore consistent with the structural information which can be inferred from some kinetic studies (i.e. the Na^+-K^+-ATPase protein spans the membrane - see Whittam, 1962; Bolstein & Chu, 1977), and recent electron microscopic studies (Van-Winkle, Lane & Schwartz, 1976; Deguchi, Jorgensen & Maunsbach, 1977; Vogel, Meyer, Grosse & Repke, 1977). These, in turn, suggested that a significant part of the Na^+-K^+-ATPase protein is 'buried' in the hydrophobic interior of the biomembrane. Recent reports of infra red spectroscopic studies (Brazhnikov, Chetverin & Chirgadze, 1978) have provided evidence that this may involve some 45% of the amino acid residues of the large polypeptide.

Finally, other structural studies have presented strong evidence that the functional Na^+-K^+-ATPase is oligomeric. The various reports of molecular weight estimations (Nakao, Nakao, Nagai, Kawai, Fujihara, & Fujiata, 1972; Kyte, 1972; Atkinson, Gatenby & Lowe, 1971), all define a value for the molecular weight of the

biologically active preparations that is at least twice that of the minimum molecular weight of 140,000 (1 large polypeptide + 1 small polypeptide). Other workers have shown that it is possible to cross link two large polypeptides and that the kinetics of such cross linking is consistent with an intra-molecular process (see Kyte, 1975; Giotta, 1976, 1977; Liang & Winter, 1977). Although there is some uncertainty as regards the interpretation of the latter reports (Huang & Askari, 1978), the evidence provided by those studies and the molecular weight estimations, clearly suggest that the functional Na^+-K^+-ATPase is an oligomer of smaller catalytic units. The relevance of such a structure to the mechanistic aspects of this enzyme will be discussed later.

(b) Kinetic and mechanistic studies on the Na^+-K^+-ATPase.

The early characterisation studies on the activity of the Na^+-K^+-ATPase provided strong evidence that it is the molecular species responsible for the active coupled transport of sodium and potassium ions across animal cell membranes (see Skou, 1965). Such was inferred from the matching of the ligand requirements of the enzyme and the sodium pump in intact cells. Those studies showed that the hydrolysis of ATP by this enzyme has an absolute requirement for sodium and magnesium ions, and that the requirement for potassium ion is relative rather than specific since a variety of monovalent cations (except sodium) could be substituted for potassium ion (Skou, 1960). The INFERENCE drawn from those studies (i.e. the Na^+-K^+-ATPase is the Na- pump) was consistent with the demonstration of a specific inhibition of the enzyme by cardiac glycosides (Dunham & Glynn, 1961), and the demonstration that the sodium pump and the Na^+-K^+-ATPase had similar cation activation profiles (Post, Merrit, Kinsolving & Albright, 1960).

The characterisation of the activation of the Na^+-K^+-ATPase by its essential ligands (i.e. Na^+, K^+, Mg-ATP) provided strong evidence of a complex reaction mechanism. Experiments using resealed erythrocyte ghosts and other unbroken membrane systems (e.g. the profused squid giant axon) indicated that a transmembrane assymetry is characteristic of its ligand requirements and inhibition by cardiac glycosides (Caldwell & Keynes, 1959; Whittam, 1962; Whittam & Ager, 1964; Hoffman, 1966). These studies showed that the binding sites for sodium ion and the phosphorylating ligand (i.e. Mg-ATP - see Whittam & Chipperfield, 1975) occur on the inner surface of the cell membrane while those for potassium ion and the cardiac glycosides occur on the outer membrane surface. Other studies using similar membrane sources indicated that the hydrolysis of each mole of ATP is accompanied by the translocation of three moles of sodium and two moles of potassium (Garraham & Glynn, 1967; Sen & Post, 1964). As is consistent with such a stoichiometry, the ligand activation kinetics of the enzyme shows a sigmoidal concentration dependence with respect to sodium and potassium ions (Garray & Garrahan, 1973; Robinson, 1967), and a hyperbolic concentration dependence with respect to the phosphorylating ligand (Hexum, Samson & Himes, 1970; Peter & Wolf, 1972). There is kinetic evidence supporting the existence of two potassium sites and three sodium sites (Lindenmayer, Schwartz & Thompson, 1974; Robinson, 1975; Fukishima & Tonomura, 1975), however, there is some uncertainty as to whether the respective monovalent cation sites are kinetically equivalent or non-equivalent (see Schwartz, Lindenmayer & Allen, 1975).

Other mechanistic studies identified a 'high energy' phosphoprotein as an intermediate in the overall reaction mechanism (Charnock, Rosenthal & Post, 1963; Bolstein, 1966). The intermediate has been identified as an acyl phosphate bound to the β-carboxyl group of an aspartate residue (Post & Kume, 1973; Nishigaki, Chen & Hokin, 1974) which was shown to be part of the large polypeptide identified in highly active Na^+-K^+-ATPase preparations (Kyte, 1971a). In the presence of saturating concentrations of Na^+, Mg^{2+} and ATP, the formation of the intermediate is generally found to be quick and quantitative (Hokin, Sastry, Galsworthy & Yoda, 1965; Post, Sen & Rosenthal, 1965). Although ATP was found to be the only nucleotide capable of initiating significant activation of the Na^+-K^+-ATPase (see Skou, 1965), it was found that the enzyme could be phosphorylated by a variety of non-nucleotide high energy organic phosphates (see Rega & Garrahan, 1976; Gache, Rossi & Lazdunski, 1977), and even inorganic phosphate (Post, Toda & Rogers, 1975). However, unlike the absolute sodium ion requirement for the ATP dependent phosphorylation of the enzyme (Albers, Fahan & Koval, 1963; Rodnight, Hems & Lavin, 1966), the phosphorylation of the enzyme by the other phosphate donors does not require sodium ion, and in some cases phosphorylation may be inhibited by sodium ion (Post, Toda & Rogers, 1975).

The phosphorylated intermediate has been identified as the target of a potassium dependent dephosphorylation reaction (see Charnock & Potter, 1969). Since the phosphorylation reactions have been shown to be quick and quantitative even at low temperatures (0^oC - Israel & Titus, 1967; Bond, Bader & Post, 1971), while

Na^+-K^+-ATPase activity proceeds very slowly at low temperatures (Charnock, Cook & Casey, 1971), the reactions involved in the dephosphorylation of the intermediate and the regeneration of the 'free' enzyme are generally assumed to be inclusive of the rate limiting step in the overall reaction mechanism. The potassium dependent dephosphorylation reactions when coupled to the phosphorylation of the Na^+-K^+-ATPase by high energy phosphate donors other than ATP, is thought to comprise the ouabain inhibitable potassium dependent phosphatase activity (K^+-Phosphatase) first reported by Judah, Ahmed & McLean (1962). This activity is always found in association with the Na^+-K^+-ATPase and there is very strong evidence that it comprises a 'partial' reaction sequence of the Na^+-K^+-ATPase reaction mechanism (see Rega & Garrahan, 1976). The phosphorylated intermediate is also assumed to be the specific target of cardiac glycoside binding reactions. This has been inferred from the observation that the optimal conditions for cardiac glycoside binding are the same as those for the formation of the phosphorylated intermediate (Skou, Butler & Hansen, 1971). Photo-affinity labelling studies have provided strong evidence that the cardiac glycoside binding site is also located on the large polypeptide identified in 'purified' Na^+-K^+-ATPase preparations (Ruoho & Kyte, 1974).

Other lines of kinetic investigation are usually aimed at the definition of the various intermediate states in the reaction mechanism of the sodium pump. Such studies provided evidence that in the absence of potassium ion, the enzyme is capable of catalysing a sodium dependent ATP-ADP exchange, and also suggested that the

phosphorylated intermediate exists in two forms (Fahn, Koval & Albers, 1966). The existence of two forms of the phosphorylated enzyme has been supported in later studies (Mardh & Zetterqvist,1974; Mardh, 1975(a), 1975(b); Kuriki & Racker, 1976). Other studies using rapid mixing techniques indicated that the kinetics of dephosphorylation of the potassium sensitive phosphorylated intermediate is biphasic (Mardh, 1975(a), 1975(b); Froehlich, Albers, Koval, Goebel & Berman, 1976; Lowe & Smart, 1977; Mardh & Lindhall, 1977), consisting of a rapid phase of inorganic phosphate release followed by a slower 'steady state' release of inorganic phosphate. These observations were interpreted as being suggestive of the existence of a dephosphorylated form of the enzyme distinct from the 'free' form which is involved in the sodium dependent phosphorylation reactions. The probable existence of multiple phosphorylated and non-phosphorylated forms of the enzyme suggests that significant conformational changes are involved in the normal functioning of the Na^+-K^+-ATPase. Such is compatible with reports that trypsinolysis of 'purified' Na^+-K^+-ATPase preparations in the presence of different ligand activating conditions (i.e. Na^+, K^+, Mg^{2+}, ATP), resulted in the formation of different peptides (Jorgensen, 1975, 1977; Jorgensen & Klodos, 1978; Lo & Titus, 1978). A recent report of the kinetic detection of a substrate induced conformational change in the Na^+-K^+-ATPase (Grisar, Frere, Grisar-Charlier, Franck & Schoffeniels, 1978) has also supported the view that significant conformational changes are involved in the functioning of this enzyme, and provided some evidence that the Na^+-K^+-ATPase may be a hysteretic enzyme.

Finally, the other major line of kinetic investigation has been aimed at the characterisation of the 'active site(s)' of the enzyme. Reports of such work have provided evidence supporting the involvement of a variety of amino acid residues in the 'active centre' of the enzyme. In addition to the aspartic acid residue identified as the phosphorylation site Post & Kume, 1973; Nishigaki, Chen & Hokin, 1974), such amino acid residues are thought to include cystine (Hart & Titus, 1973; Patzelt-Wenczler, Pauls, Erdmann & Schoner, 1975; Schoot, DePont & Bonting, 1978), arginine (DePont, Schoot, Van Prooijen-VanEeden & Bonting, 1977) and tyrosine (Cantley, Gells & Josephson, 1978). Although the configuration of such residues at the 'active centre' and the parts they play in the reaction mechanism are not yet established, the evidence presented by the above authors suggest that the mentioned amino acid residues are involved in the binding of ATP to the enzyme. Other reports have presented evidence of two ATP binding sites on the Na^+-K^+-ATPase, and suggest that sulphydryl containing amino acid residue may be involved in the binding of ATP to a high affinity non-phosphorylating site on the enzyme (Henderson & Askari, 1976, 1977).

In spite of the advances in the characterisation of several aspects of the structure and mechanism of the Na^+-K^+-ATPase, it is apparent from the above summary of the main lines of experimental investigation into this enzyme system, that there are large areas of uncertainty concerning the mechanism by which this cationic pump works. As a result, there tends to be a considerable speculative

component in most of the molecular and mechanistic models of this enzyme system. Some of the proposals described the system in a manner which required some gross rotation of a significant part of the enzyme relative to the plane of the biomembrane (Opit & Charnock, 1965; Stein, Lieb, Karlish & Eilam, 1973). However, the demonstration that some antibodies can bind to the enzyme and not affect catalytic efficiency (Kyte, 1974) has raised doubts as to the validity of those and other 'rotating ball' type models (see Albers, 1967), since gross rotation of the Na^+-K^+-ATPase relative to the plane of the biomembrane would be unlikely if an antibody is bound to the enzyme. Furthermore, such a rotation of an integral membrane protein like the Na^+-K^+-ATPase would be energetically unfavourable within the context of current views on the structure of biomembranes (see Singer & Nicholson, 1972). An alternative model postulated that the ion transport process takes place by the passage of ions through the protein moiety of the enzyme (Skou, 1975). It was assumed that the passage of the ions was induced by a reversal in the affinity of the sodium and potassium sites for the respective ions. Such a model is compatible with an earlier allosteric model for membrane pumps (Jardetzky, 1966), and suggested that the Na^+-K^+-ATPase contains a gramicidin-like ionophore, for which some evidence has been reported (Shamoo, 1974; Shamoo & Meyers, 1974). This model has been questioned (Yager, 1977) since it would require some 35-42 precisely arranged ligands to be part of the structure of the pump, and that there be some restriction of diffusion between the bulk aqueous media and the cationic sites while the translocation takes place. Kyte (1975) has suggested the so-called 'rocker model' in which ion transport takes place by the alternations of the enzyme

between two major conformational states. The model suggests that there is only one cationic site at any one time, and postulates that the ions migrate across the membrane through channels between subunits on an oligomeric enzyme. Thus the minimal structure for the Na^+-K^+-ATPase demanded by this model is that of a dimeric enzyme exhibiting 'half-the-sites-reactivity'. This model is thus compatible with the previously described structural evidence and recently reported kinetic evidence supporting a dimeric Na^+-K^+-ATPase with 'half-the-sites-reactivity' (Grisham & Mildvan, 1975). However, this model has also been questioned (Yager, 1977) since it requires the 'rocking' of the enzyme to take place faster than the rehydration of the cation(s) as the cationic site is altered during the conformational 'rocking' changes.

Yager (1977) has proposed a novel mechanism for the Na^+-K^+-ATPase. This model postulates that the parts of the enzyme which span the membrane exist in an ordered helical form, and that the passage of ions through the helix is induced by $\alpha \rightarrow$ ¶ or ¶ $\rightarrow \alpha$ helical transitions over short sections of the helix ($\simeq$ two turns). The energetic push required to start the process would be supplied by the phosphorylation/dephosphorylation reactions, and the peptide oxygens of the distorted regions of the helix would replace the hydration shells of the respective ions. It was further proposed that once the translocation process was started, the ions would serve as catalysts for the α/¶ helical transitions. While there is some evidence that a significant part of the Na^+-K^+-ATPase is a highly ordered structure and probably 'buried' in the hydrophobic part of the biomembrane (Brazhnikov, Chetverin & Chirgazade, 1978), this model

and all the others so far presented can be subjected to two important criticisms. First, the models do not describe the enzyme such that it would account for the absolute discrimination between sodium and potassium ions. The results of some theoretical calculations have suggested that there may be a thermodynamic basis for such a discrimination only if the process involves a replacement of the primary hydration shell of the ions (Talekar, 1975), however, none of the models so far presented have overcome this problem. Secondly, there has not been a serious attempt to account for the stochiometry of the sodium pump (i.e. 3 moles Na^+ and 2 moles K^+ per mole of ATP) in any of the models so far proposed.

The involvement of the sodium pump (directly or indirectly) in a wide range of cellular functions (see Baker, 1972) has inevitably led to consideration of the factors involved in the fine control of this enzyme system. Given the firm association between the enzyme and membrane lipid and the current ideas pertinent to the probable regulation of membrane bound enzymes by membrane lipid (see review by Gazzoti & Peterson, 1977; Sandermann, 1978), a role for membrane lipid in the fine control of the Na^+-K^+-ATPase seems probable. Such ideas have been reinforced by the observed deviation of the temperature dependence of the enzyme from ideal Arrhenius temperature kinetics and the interpretation of these observations within the context of phase changes in biomembrane lipids (see Barnett & Parlazzoto, 1974). Such an interpretation is compatible with the reports that the temperature dependence of the Na^+-K^+-ATPase was responsive to changes in membrane lipids (Charnock, Cook, Almeida & To, 1973; Tanaka & Teruya, 1973). However, the

evidence supporting a role for membrane lipid in the fine control of this enzyme has been obtained, only after a severe disruption of the biomembranes by the use of phospholipases (Charnock, Cook, Almeida & To, 1973), detergents (Tanaka & Teruya, 1973) or enzymic lipid peroxidation (Sun, 1971). Since the effects of such experimental procedures are considerably greater than that likely in the *in-vivo* situation, the interpretation of such data within the context of lipid involvement in the fine control of the Na^+-K^+-ATPase can be questioned.

It has been suggested that the Na^+-K^+-ATPase is an enzyme subject to allosteric modulation (Robinson, 1972, 1973; Tobin, Banergee & Sen, 1970; Cavieres & Ellory, 1975). This may be of particular relevance to the consideration of the fine control of the Na^+-K^+-ATPase, especially since, as previously shown, there is evidence supporting an oligomeric structure for the Na^+-K^+-ATPase, and such a structure is the type which is susceptible to allosteric modulation of its kinetic properties. Given the weight of the evidence that the kinetic properties of the Na^+-K^+-ATPase is responsive to a variety of protein targeted modifiers (see Schwartz, Lindenmayer & Allen, 1975), the protein component of the enzyme as the prime factor in the fine control of the enzyme system cannot be discounted. The work presented in this thesis involves a study of some of the kinetic properties of the Na^+-K^+-ATPase, and their responsiveness to some lipid and protein targeted procedures with the aim of evaluating the role of its protein and lipid components in the fine control of this enzyme system.

The experimental approach to the objective outlined above was designed to test whether the fine control of the Na^+-K^+-ATPase

can be mediated via mild changes in the protein or lipid components of the enzyme. This required a careful choice of some of the kinetic parameters so as to monitor any such effects, and the choice of modulating procedures which would produce changes comparable to those probable in vivo. The main kinetic properties chosen for such studies were those of potassium activation, ouabain inhibition and temperature dependence. Temperature dependence was specially chosen for such studies since it was likely to give some information pertinent to the energetics of the rate limiting step(s) in the reaction mechanism, and on account of earlier evidence of its responsiveness to lipid changes. The other two parameters were considered since they were in effect the properties of the phosphorylated intermediate. Given that the reactions involved in the formation of the phosphorylated intermediate have been shown not to be rate limiting, the phosphorylated intermediate was considered to be the most likely target for any fine-control-modulatory changes. However, a survey of the literature revealed that there are considerable areas of uncertainty concerning the definition of the three kinetic parameters chosen. Consequently, an attempt was made to study these properties and to define the framework within which the relevant data will be interpreted.

The experimental modulatory procedures adopted were chosen since, in general, these were expected to exert their effects without gross inhibition of enzymic activity under optimal conditions. Both protein targeted and lipid targeted procedures were studied. The latter included both in-vivo lipid targeted modulators, and the in-vivo modulation of membrane lipids as a result of environmental temperature changes. The effects of these on the chosen kinetic

properties of the enzyme were studied, so as to enable an evaluation of the sensitivity of the enzyme to such changes.

CHAPTER 2

MATERIALS AND METHODS

General

(1) Tissue sources:

The following animals were used as sources of membranes containing Na^+-K^+-ATPase activity.

Rat (*Rattus norvegicus*) - remote wistar strain CFHB

Mouse (*Mus domesticus*)

Hamster (*Mesocricetus auratus*)

Guinea pig (*Cavia porcellus*)

Rabbit (*Oryctolagus cuniculus*)

Hedgehog (*Erinaceus europaeus*)

Pigeon (*Columba livia*)

Starling (*Sturnus vulgaris*)

Frog (*Rana temporaria*)

African clawed toad (*Xenopus laevis*)

Rainbow trout (*Salmo gairdneri*)

Carp (*Cyprinus carpio*)

Perch (*Perca fluviatalis*)

Notothenia (*Notothenia neglecta*) - an antarctic fish

Cockroach (*Periplaneta americana*)

Locust (*Locusta migratoria*)

(2) Reagents

All reagents were of analytical grade and purchased from the following sources:

Sigma Chemical Co.

Adenosine triphosphate (Sigma grade) disodium salt used for assay of Na^+-K^+-ATPase activity (see Appendix III).

Adenosine triphosphate (Grade 1) disodium salt used for washing membranes during detergent extraction.

L-Ascorbic acid

Bovine serum albumin - fraction V

Cholesterol - Sigma grade

Ethylmercuri-thiosalicylate (thimerosal) sodium salt

L-Histidine - free base

Imidazole - grade 1

β-mercaptoethanol - type I

ρ-nitrophenol

ρ-nitrophenol phosphate (Sigma 104 phosphatase substrate) - disodium salt.

L-α- phosphatidyl choline, palmitoyl

Tris(hydroxymethyl)-amino methane (tris) - Trizma base.

ρ-nitrophenyl hydrazine

Serva

Comassie brilliant blue G250

Concanavalin - A

Sphingosine

Supelco

Fatty acid methyl esters (standards for gas liquid chromatography)

Hexadecanal

Phospholipid standards (standards for thin layer chromatography)

Eastman Kodak

8-anilino, 1-napthalene sulphonic acid (sodium salt)

2,5-dimethyl benzene sulphonic acid.

Pye-Unicam

poly-ethylene glycol adipate

Applied Science Laboratories

Gaschrom Q

The Radiochemical Centre, Amersham

2-^{14}C-Acetic acid (sodium salt)

All other reagents were analytical grade or specially purified and supplied by the British Drug Houses.

Cirrasol-ALN-WF was a gift from I.C.I. Dyestuffs Division.

(3) Assay of sodium-potassium-adenosine triphosphatase activity (Na^+-K^+-ATPase)

The standard assay of Na^+-K^+-ATPase activity was done by the determination of the ouabain inhibitable enzymic release of inorganic phosphate from adenosine triphosphate at 37°C, a pH of 7.5 and at the following ligand concentrations:

Adenosine triphosphate (di sodium salt)	3mM
Magnesium chloride	3mM
Potassium chloride	20mM
Sodium chloride	130mM
Histidine	30mM

Prepared solutions:

(i) Adenosine triphosphate (di sodium salt) 4.4 x 3mM

The solution of the reagent was buffered at pH 7.5 at room temperature with Tris. It was stored in aliquots at -20°C until required.

(ii) Buffered ionic medium	pH 7.5 at 37°C
Histidine	2.2 x 30mM
Sodium chloride	2.2 x 124mM
Potassium chloride	2.2 x 20mM
Magnesium chloride	2.2 x 3mM
(iii) Ouabain	4.4 x 1mM

Inorganic phosphate was determined by the method described by Atkinson, Gatenby & Lowe (1973).

Prepared mixtures:

(i) Cirrasol ALN-WF solution 1% (w/v) in distilled water

(ii) Acid molybdate solution 1% (w/v) in 0.9M sulphuric acid.

The chromogenic solution was made by mixing equal volumes of acid molybdate and freshly prepared cirrasol solution.

Reaction mixtures (2.0 cm^3)

(i) Total ATPase mixture

1.0 cm^3 Buffered ionic medium

0.5 cm^3 Adenosine triphosphate

0.5 cm^3 distilled water

(ii) Ouabain insensitive ATPase mixture.

This was similar to that prepared for the total ATPase except that 0.5 cm^3 of ouabain was substituted for the distilled water.

0.2 cm^3 of an enzyme preparation was added to the reaction mixtures (thermoequilibriated at 37°C) so as to obtain a final volume of 2.2 cm^3. The reaction was quenched after a suitable run time by the addition of 4 cm^3 of the chromogenic solution. The mixture was allowed to stand at room temperature for 5 minutes and the extinction of the yellow colour formed determined at 390nm. The extinction at 390nm was interpreted in terms of released inorganic phosphates by calibrating the same with potassium di-hydrogen orthophosphate under the same ligand concentrations. The calibration graph was found to be linear for inorganic phosphate concentrations in the range 0 - 0.6 μmoles per cm^3 of final assay mixture (see figure 2.1). Na^+-K^+-ATPase activity was taken as the difference between the released inorganic phosphate in the total and ouabain insensitive ATPase mixtures.

(4) Assay of Protein:

The protein assay procedure adopted was adapted from that described by McGrath (1972) - see Appendix I.

Prepared solutions and reagents

(i) Acetate buffer with cyanide pH 4.6-4.7 at room temperature.

1.32M Sodium cyanide

1.54M Acetic acid

1×10^{-4}M Sodium cyanide

(ii) Ninhydrin solution - 0.5% (w/v) in 2-methoxyethanol

(iii) Barium hydroxide octahydrate - ground ≃ 100-120 mesh.

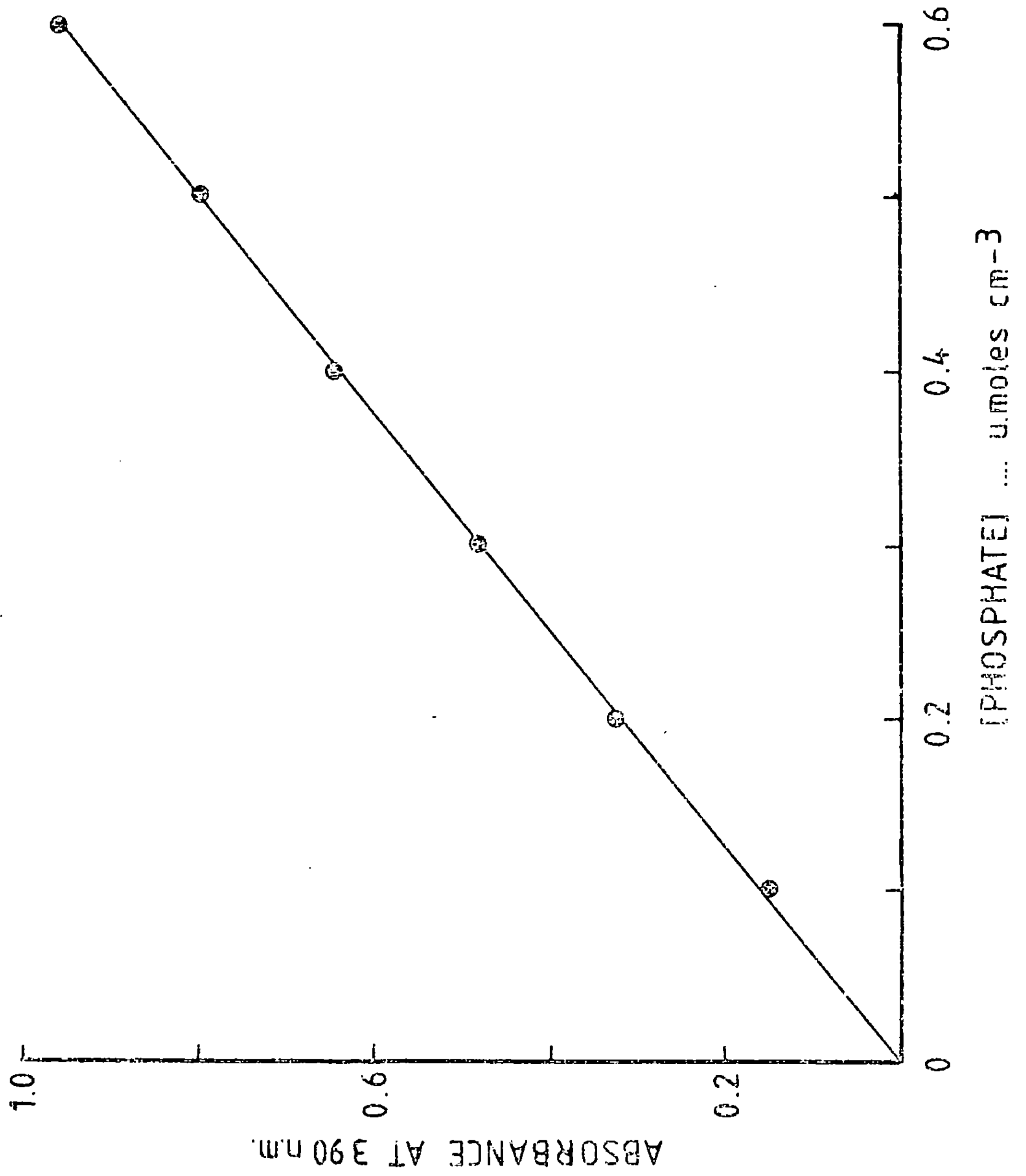


Fig. 2.1

INORGANIC PHOSPHATE CALIBRATION GRAPH

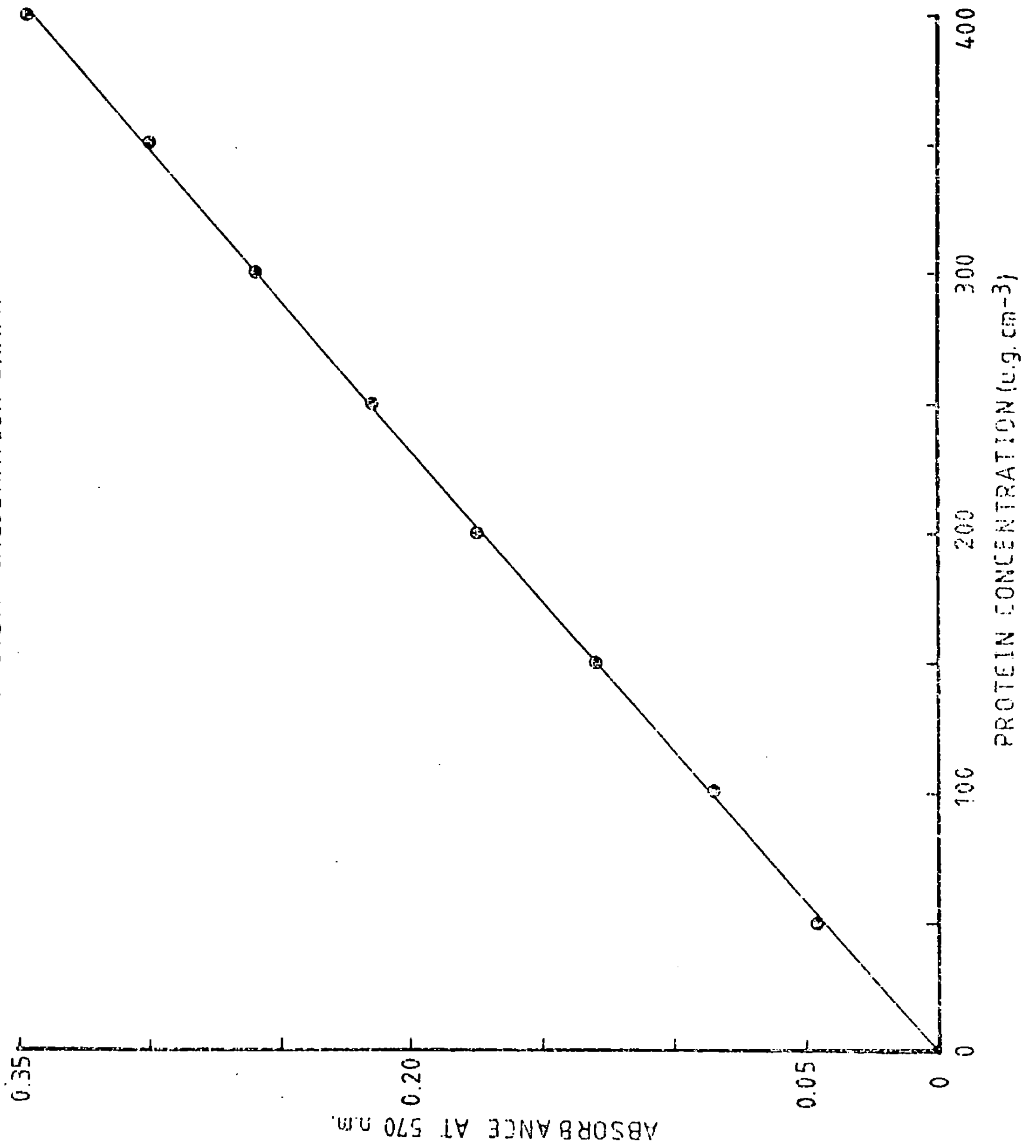
100mg samples of ground Barium hydroxide were weighed out into two clean, thin tall test tubes, and 0.2 cm^3 of the assay sample was added to each tube. One sample was steamed in a pressure cooker at 120°C (15 lb m^{-2}) for twenty minutes. After twenty minutes, the pressure was released and the steaming continued for a further thirty minutes. After the sample was cooled, 2 cm^3 of the acetate buffer was added to both samples, and thoroughly mixed so as to dissolve the Barium hydroxide. 3 cm^3 of ninhydrin solution was then added to both tubes which were thoroughly mixed and returned to the pressure cooker where they were steamed at 120°C for fifteen minutes. After the samples had cooled, they were then diluted to 25 cm^3 with 60:40 (v:v) 2-methoxy-ethanol:water. The extinction of the samples were then determined at 570nm. The difference in the absorbance at 570nm was attributed to the amino acids released by the alkaline hydrolysis of the protein in the sample. This was interpreted in terms of protein concentration by calibrating the same with Bovine serum albumin subjected to the above procedures. The calibration graph (see figure 2.2) was found to be linear in the protein concentration range 0-400 μg cm^{-3}. This adaptation of the assay procedure was found to tolerate low concentrations of Imidazole (20mM), E.D.T.A. (5mM) and E.G.T.A. (5mM). However, the assay sample was required to be free of any 'protein free' and latent amino groups.

(5) <u>Assay of potassium dependent ouabain sensitive para-nitro-phenol phosphatase activity (K^+-PNPase):</u>

The standard assay of K^+-PNPase activity was done by the determination of the ouabain inhibitable, enzymic release of para-nitrophenol from para-nitrophenol phosphate at 37°C, a pH of 7.5 and at the following ligand concentrations.

Fig. 2.2

PROTEIN CALIBRATION GRAPH



para-nitro-phenol phosphate	5mM
Magnesium chloride	5mM
Potassium chloride	20mM
Tris	50mM

Prepared solutions:

(i) Para-nitro-phenol-phosphate 4.4x5mM

The solutions of the reagent was buffered at pH 7.5 at room temperature with 10mM tris/HCl. It was stored in aliquots at $-20^{o}C$ until required.

(ii) Buffered ionic medium pH 7.5 at $37^{o}C$.

Tris	2.2 x 50mM
Magnesium chloride	2.2 x 5mM
Potassium chloride	2.2 x 20mM

(iii) Ouabain 4.4 x 1mM

Reaction mixtures (2.0 cm^3)

(i) Total K^+-PNPase mixture

1.0 cm^3 Buffered ionic medium

0.5 cm^3 para-nitro-phenol phosphate

0.5 cm^3 distilled water

(ii) Ouabain insensitive K^+-PNPase mixture

This was similar to that prepared for the total K^+-PNPase except that 0.5 cm^3 of ouabain was substituted for the 0.5 cm^3 of distilled water.

0.2 cm^3 of an enzyme preparation was added to the reaction mixtures (thermoequilibriated at $37^{o}C$) so as to obtain a final volume of 2.2 cm^3. The reaction was quenched after a suitable run time by the addition of a suitable volume of 1% sodium dodecylsulphate (usually 2 cm^3 or 4 cm^3), and the extinction of the yellow para-nitro-phenol determined at 410nm.

The extinction at 410nm was interpreted in terms of released p-nitrophenol by calibrating the same with authentic p-nitrophenol under the same ligand concentrations. The calibration graph was found to be linear for p-nitro-phenol concentrations in the range 0.15 μmoles cm^{-3} of final assay mixture (see figure 2.3). K^+-PNPase activity was taken as the difference between the p-nitro-phenol released in the total and ouabain insensitive K^+-PNPase mixtures.

(6) Preparation of synaptic membranes

(1) Rat brain synaptic membranes

All operations were carried out at 0-4°C. The animals were stunned and killed by cervical dislocation and their brains quickly excised out into ice cold extraction medium (320mM Sucrose, 30mM Imidazole, 2mM E.D.T.A. pH 7.2 at room temperature). The cerebral hemispheres were dissected free of blood vessels and underlying tissue, and then homogenised in ice cold extraction medium with a teflon lined glass homogeniser at a tissue concentration of 5-10%. The homogenate was then centrifuged at 900g (MSE Mistral 2L refrigerated centrifuge) for 10 minutes. The pellet was discarded, and the synaptosomal/mitochondrial fraction sedimented from the supernatant by centrifuging at 20,000g (MSE High Speed 18 refrigerated centrifuge) for 30 minutes. The synaptosomes were then lysed by osmotic shock, by being homogenised in a low ionic strength buffer (10mM Imidazole, 1mM E.D.T.A, pH 7.2 at room temperature) and the membranes and mitochondria sedimented from the homogenate by centrifuging at 20,000g for 30 minutes.

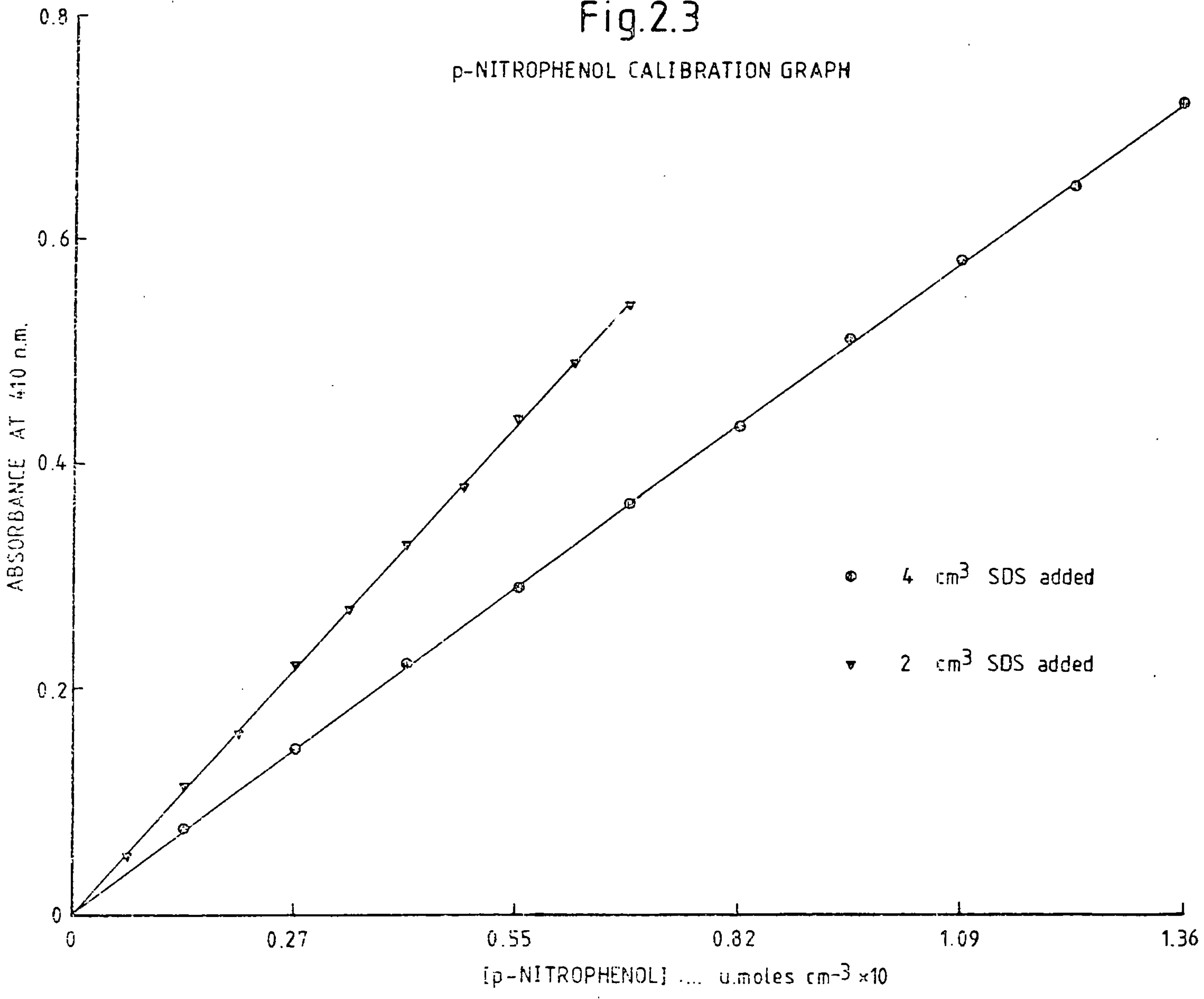


Fig.2.3
p-NITROPHENOL CALIBRATION GRAPH
ABSORBANCE AT 410 n.m.
0.8
0.6
0.4
0.2
0
0.27
0.55
0.82
1.09
1.36
[p-NITROPHENOL] .... u.moles cm-3 ×10
4 cm3 SDS added
2 cm3 SDS added

The fractionation of the synaptic membranes from the pellet then proceeded according to the quantity of material being prepared.

(a) Small scale preparation

The pellet was resuspended in the extraction medium and laid on a discontinuous sucrose density gradient (0.8M, 0.9M, 1.0M, 1.2M - buffered at pH 7.2 (room temperature) with 10mM Imidazole/HCl). The gradient was then centrifuged at 100,000g for 2 hours (MSE Prepspin 50 ultracentrifuge). The material floating at the interphase of the 1.0-1.2M sucrose layers was harvested, and the sucrose solution diluted with low ionic strength buffer to a sucrose concentration of less than 0.8M. The synaptic membranes were then sedimented from the solution by centrifuging at 100,000g for one hour. The pelleted membranes were resuspended in low ionic strength buffer to a convenient protein concentration ($\simeq$ 1 mg cm^{-3}) and stored on ice at 0-4°C until required.

(b) Large scale preparation

The pellet from the 20,000g spin was resuspended in 1.2M Sucrose, 10mM Imidazole (pH 7.2 at room temperature) and centrifuged at 100,000g for two hours. The supernatant was carefully decanted from the resulting pellet and the light membranous material floating on the surface. The supernatant was then diluted with half its volume of low ionic strength buffer, and the synaptic membranes sedimented by centrifuging at 100,000g for one hour. The pelleted membranes were resuspended in low ionic strength buffer to a protein concentration of 2-5 mg.cm^{-3}, and stored on ice at 0-4°C until required.

(2) Rainbow trout brain synaptic membranes

All operations were carried out at 0-4°C. The animals were killed by decapitation and their brains quickly dissected out into ice cold extraction medium. After dissecting the brain tissue free of blood vessels and connective tissues, the synaptosomal/mitochondrial fraction was prepared and lysed as described for the rat brain above. The membranous material was then sedimented by centrifuging at 20,000g for 30 minutes and the synaptic membranes were fractionated from the pellet by a procedure depending on the quantity of material being prepared.

(a) Small scale preparation

This was done using the same system of discontinuous sucrose density gradients described for the rat brain above. However, the material was harvested from the interphase of the 0.9-1.0M sucrose layers since the membranes floating at the 1.0-1.2M interphase were found to be heavily contaminated with mitochondria. The suspension harvested, was diluted to a sucrose concentration of less than 0.6M, and the membranes sedimented by centrifuging at 100,000g for 1 hour. The final pellet was resuspended in the low ionic strength buffer to a convenient protein concentration ($\approx$ 1 $mg.cm^{-3}$) and stored on ice at 0-4°C until needed.

(b) Large scale preparation

The large scale fractionation of these membranes was done by a procedure similar to that described for the rat brain above. However, the membranous fraction sedimented from the lysed synaptosomal/mitochondrial fraction was resuspended in 1.0M sucrose, 10mM Imidazole (pH 7.2 at room temperature). The membranes were

sedimented by centrifugation at 100,000g, and resuspended in low ionic strength buffer to a protein concentration of 2-5 $mg.cm^{-3}$ and stored on ice at 0-4°C until required.

NOTE: The preparation of brain synaptic membranes from sources other than the rat and the trout was done by one of the above mentioned procedures, as referred to in the text. Unless otherwise stated, large scale preparative procedures were only used for preparations from rats, mice, hamsters and rainbow trout.

CHAPTER 3

Ouabain Inhibition of the Na^+-K^+-ATPase

INTRODUCTION

The first reports of inhibition of coupled Na^+-K^+-transport by ouabain (Schatzmann, 1953) suggested that the cardiac glycoside acted at a site remote from the energy source. This was later confirmed after the discovery of the Na^+-K^+-ATPase (Skou, 1957), and the demonstration of a specific inhibition of its catalytic activity by the cardiac glycoside (Dunham & Glynn, 1961). The identification of the Na^+-K^+-ATPase as the target of the specific action of ouabain and other cardiac glycosides, has thus enabled the use of these reagents in the characterisation of Na^+-K^+-ATPase preparations.

The use of ouabain as a probe in mechanistic studies on the Na^+-K^+-ATPase has been very extensive. These studies have indicated that the optimal conditions for ouabain binding are the same as those for the formation of the phosphorylated intermediate in the overall reaction mechanism (Skou, Butler & Hansen, 1971); an observation which suggested that the action of the cardiac glycoside proceeded via the phospho-protein intermediate. This in turn suggested that the inhibition of the enzyme by ouabain would be uncompetitive with respect to ATP activation kinetics. This suggestion was later supported by experiment (Wolf & Peter, 1972). Other studies indicated that the kinetics of ouabain binding to the Na^+-K^+-ATPase are slow, and impeded by potassium ion (Akera, 1971), and in addition

the binding of ouabain to the enzyme was characterised by non-linear Scatchard plots (Taniguchi & Iida, 1972a;Hansen, 1976). On account of these observations, the ouabain inhibition of the Na^+-K^+-ATPase was expected to describe a complex concentration dependence. The early observations of this project were in accord with these expectations, a fact which made the comparison of different Na^+-K^+-ATPase preparations very difficult. The resolution of that problem required an attempt to rationalise the behaviour of the Na^+-K^+-ATPase in a description which would account for the observations. Such a description has been attempted in this study.

MATERIALS AND METHODS

(1) Preparation of membranes containing Na^+-K^+-ATPase activity

(a) Brain synaptic membranes from mammals.

These were prepared as described for rat brain (Chapter 2). The brains of rats, mice, hamsters and hedgehogs were used as sources of synaptic membranes.

(b) Brain synaptic membranes from non-mammalian vertebrates.

The brains of carp, rainbow trout, frogs, *Xenopus*, pigeons and starlings were used as sources of these membranes, which were all prepared as described for the rainbow trout in Chapter 2.

(c) Microsomal membranes from the cerebral ganglia of the locust and the cockroach.

All operations were carried out at $0^{o}C$ - $4^{o}C$.

The animals were anaesthetised with carbon dioxide, and the cerebral ganglia were quickly dissected out into an ice cold extraction medium (320mM sucrose, 30mM Imidazole, 2mM E.D.T.A, pH 7.2 at room temperature).

The tissue was then homogenised and centrifuged in extraction medium with a teflon lined glass homogeniser and centrifuged at 1000g (MSE 2L refrigerated centrifuge) for 10 minutes. The pellet was discarded and the supernatant centrifuged at 20,000g (MSE High Speed 18 refrigerated centrifuge) for 30 minutes. The pellet was discarded and the supernatant recentrifuged for 30 minutes at 20,000g. After carefully removing the supernatant from the pellet, the microsomes were sedimented from the supernatant by centrifuging at 100,000g for one hour (MSE Prepspin 50 ultra-centrifuge). The final pellet was then resuspended to a convenient protein concentration in a low ionic strength buffer (10mM Imidazole, 1mM E.D.T.A. pH 7.2 at room temperature), and stored on ice at 0° - $4^{o}C$ until required.

(2) Assay of Na^{+}-K^{+}-ATPase activity at non-saturating ouabain concentrations.

The assay conditions with respect to temperature, pH and concentration of essential ligands were as described for the standard assay conditions (Chapter 2). Since it had been reported that the equilibriation of ouabain with the enzyme is a slow process that is further impeded by potassium ion (Akera, 1971), the assay procedure was suitably modified to allow pre-equilibriation of the enzyme with the cardiac glycoside in a potassium free assay medium. Catalytic activity was subsequently started by the addition of potassium ion.

Prepared solutions

(i) Substrate - 4.4 x 3mM di-sodium ATP; pH 7.5 (Tris).
This was stored at $-20^{o}C$ when not in use.

(ii) Buffered ionic medium.
4.4 x 30mM Histidine; pH 7.5 at $37^{o}C$
4.4 x 124mM Sodium chloride
4.4 x 3mM Magnesium chloride

(iii) Ouabain

17 solutions were prepared containing ouabain at concentrations 3.4 x required concentrations, so as to give a final concentration range of $10^{-9}M - 10^{-3}M$.

(iv) Potassium Chloride/Ouabain

17 solutions were prepared containing 4.4 x 20mM potassium chloride and ouabain at the final concentration in the range $10^{-9}M - 10^{-3}M$. Each potassium chloride/ouabain solution was matched with a ouabain solution from (iii).

The equilibriation mixture (1.5 cm^3) was prepared thus

0.5 cm^3 Substrate

0.5 cm^3 buffered ionic medium

0.5 cm^3 Ouabain

The assay of Na^+-K^+-ATPase proceeded as follows:

0.2 cm^3 of an enzyme preparation was added to the pre-warmed equilibriation mixture and the equilibriation was allowed to proceed for 12 minutes. This preincubation time was used mainly for convenience since, from previous studies (Wallick & Schwartz, 1974), a preincubation period of 5-8 minutes was expected to be adequate for the equilibriation of the enzyme with ouabain at 37^oC, even at the lowest concentration used in this study. Na^+-K^+-ATPase activity was started by the addition of 0.5 cm^3 of a pre-warmed matched solution of potassium chloride/ouabain. The reaction was quenched after a suitable run-time and the liberated inorganic phosphate determined as previously described (Chapter 2.) The ouabain insensitive ATPase activity was determined by the enzymic release of inorganic phosphate under the same conditions

in the presence of 10^{-3}M ouabain. Other studies showed that the enzyme was stable under the pre-incubation and assay conditions (see Chapter 4).

(3) Assay of K^+-PNPase activity at non-saturating ouabain concentrations

The assay conditions with respect to temperature, pH and concentration of essential ligands were as previously described for the standard assay conditions in Chapter 2. The procedure was modified in a manner described for the Na^+-K^+-ATPase so as to circumvent the problems of slow equilibriation with ouabain and the effect of potassium ion on the kinetics of ouabain binding.

Prepared solutions.

(i) Substrate - 4.4 x 5mM p-nitrophenol phosphate; pH 7.5 (Tris/HCl).

(ii) Buffered magnesium solution
4.4 x 50mM Tris, pH 7.5 at 37°C
4.4 x 5mM Magnesium chloride

(iii) Ouabain. As described for the Na^+-K^+-ATPase.

(iv) Potassium chloride/Ouabain. As described for the Na^+-K^+-ATPase.

The equilibriation mixture (1.5 cm^3) was prepared thus
0.5 cm^3 Substrate
0.5 cm^3 Buffered magnesium solution
0.5 cm^3 Ouabain

The execution of the preincubation and assay was similar to that described for the Na^+-K^+-ATPase above. The preincubation period was 12 minutes and the K^+-PNPase activity was started by the addition of

0.5 cm^3 of potassium chloride/ouabain. The reaction was quenched after a suitable run time and the released p-nitrophenol determined as previously described (Chapter 2). The ouabain insensitive PNPase activity was determined by the enzymic release of p-nitrophenol under the same conditions and in the presence of 10^{-3}M ouabain.

RESULTS

The initial experiments were aimed at defining the dose response sensitivity of the rat brain Na^+-K^+-ATPase activity to ouabain. As shown in figure 3.1 (typical data set), the decay of enzymic activity with increasing ouabain concentration appeared to describe a biphasic curve of apparent pI_{50} 6.9. At the low concentrations of the cardiac glycoside (10^{-9} - 10^{-6}M), enzyme activity decayed with increasing concentration, to a 'plateau' at 65% ($\pm$ 3%) of maximal activity in the concentration range 10^{-6} - 10^{-5}M. The residual activity (30% V_{max}) was abolished at the higher concentrations of ouabain (10^{-5} - 10^{-3}M). Since ouabain inhibition of the Na^+-K^+-ATPase has been shown to be uncompetitive with respect to ATP (Wolf & Peter, 1972), the rate equation describing the dependence of enzyme activity on ouabain concentration (at saturating concentrations of all essential ligands) was expected to be of the form:

$$V_i = V_{max} \frac{1}{1 + (I/K_i)} \qquad (3a)$$

V_i . . . . observed rate

V_{max} . . . maximal rate

I . . . . Inhibition concentration

K_i . . . . Constant - concentration of inhibitor required to produce half maximal activity.

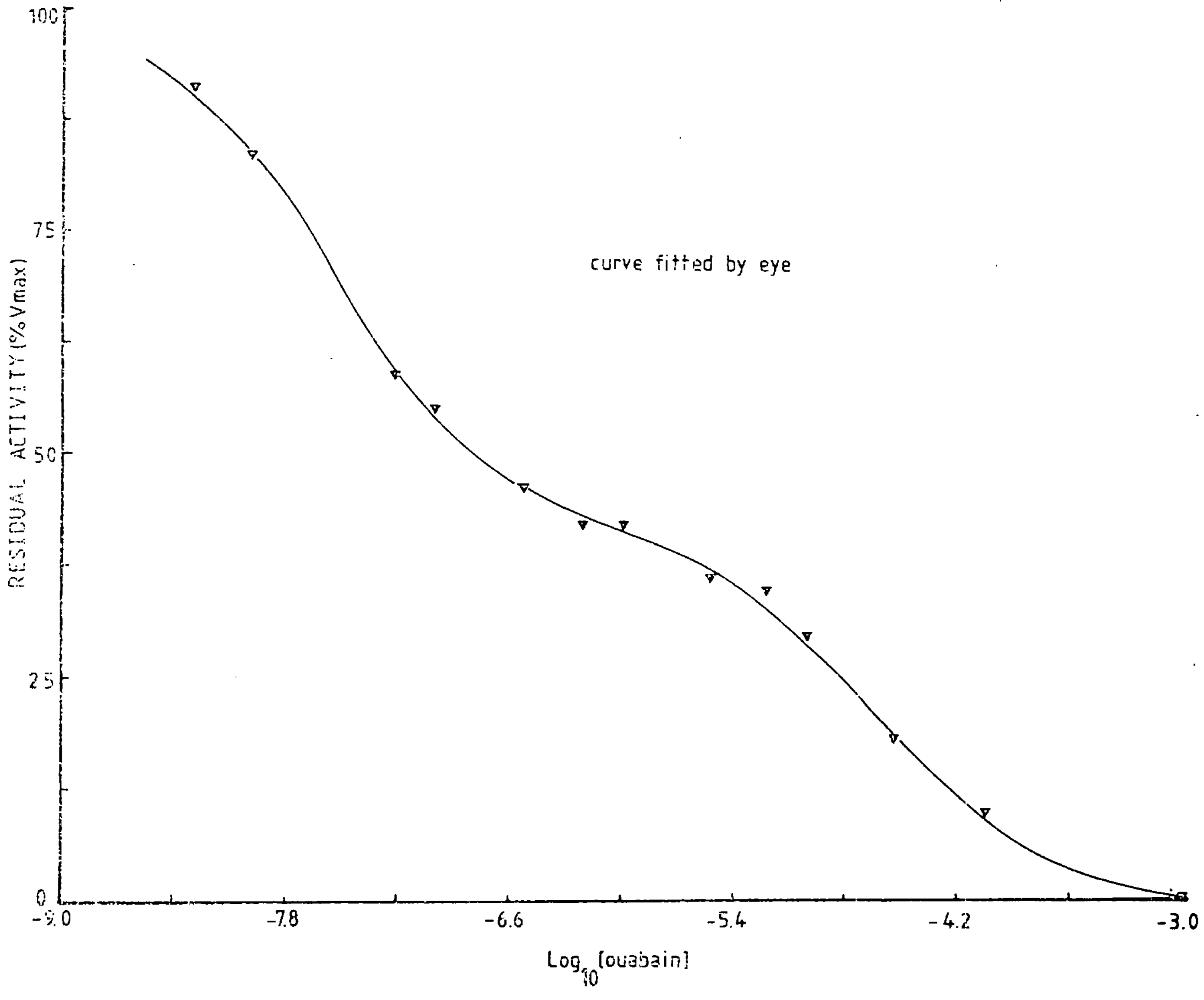


Fig. 3.1
OUABAIN INHIBITION OF RAT BRAIN Na+-K+-ATPase
curve fitted by eye
RESIDUAL ACTIVITY(%Vmax)
100
75
50
25
0
-9.0
-7.8
-6.6
-5.4
-4.2
-3.0
Log10 [ouabain]

The decay curves described by this equation (3a) are monophasic and the decay from 95% activity to 5% activity spans just over two orders of magnitude of inhibitor concentration. These curves converge at the extremities of inhibitor concentration and dilution, and do not intersect (see figure 3 .2). The decay curve described by the rat brain preparation deviated from the form predicted by the equation (3a), especially at thehigher concentrations of ouabain. Earlier reports (Tobin & Brody, 1972; Akera, Brody, So, Tobin & Baskin, 1974) have described Na^+-K^+-ATPase preparations obtained from rat tissues as being relatively insensitive to ouabain. Thus the initial experiments suggested that the reported 'ouabain insensitivity' of Na^+-K^+-ATPase preparations obtained from rat tissues may be the result of the apparent 'anomalous' behaviour at the higher ouabain concentrations. This consideration was approached experimentally by measuring the dose response sensitivity of Na^+-K^+-ATPase preparations from a variety of sources.

Figure 3 .3 shows the ouabain dose response curves described by the Na^+-K^+-ATPase preparations obtained from the four mammals used in this study. These curves all tend towards convergence at infinite dilution (10^{-9}M) and are all characterised by an obvious inflection which gives the appearance of a relative insensitivity to ouabain at concentrations near 10^{-6}M (10^{-7} - 10^{-5}M). The final decay towards zero activity at concentrations approaching 10^{-3}M ouabain. The deviation from the form expected of a simple uncompetitive inhibition system (as evidenced by the inflection in the curves) is most pronounced in the rat brain preparations and is considerably less obvious in the hedgehog preparations. This, however, is not reflected in the apparent pI_{50} values (see Table 3.1) which suggest that the mouse preparation (apparent pI_{50} 7.29) is the

Fig. 3.2

SIMPLE UNCOMPETITIVE INHIBITION CURVES

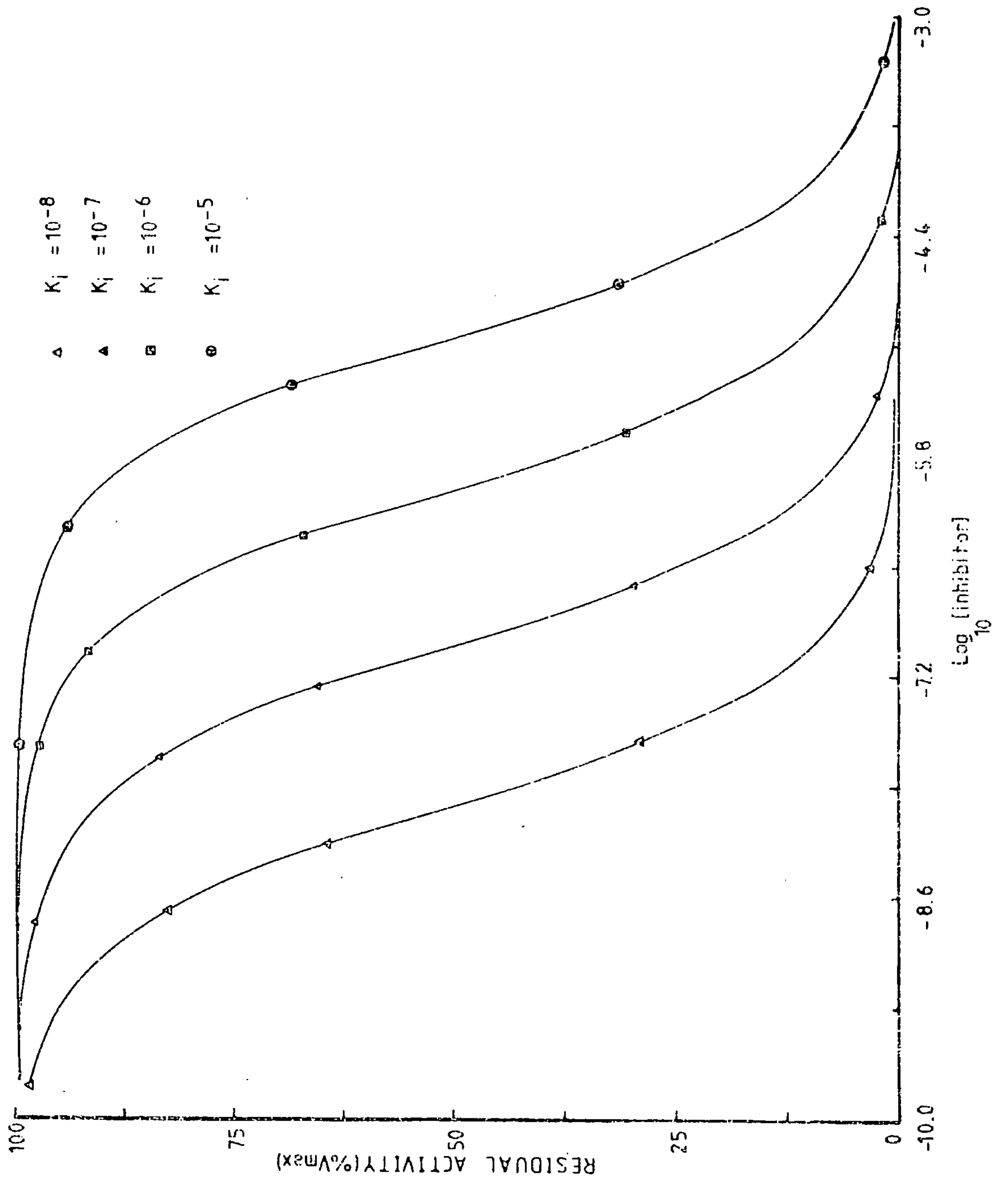


Fig. 3.3

OUABAIN INHIBITION OF THE Na^{+}-K^{+}-ase

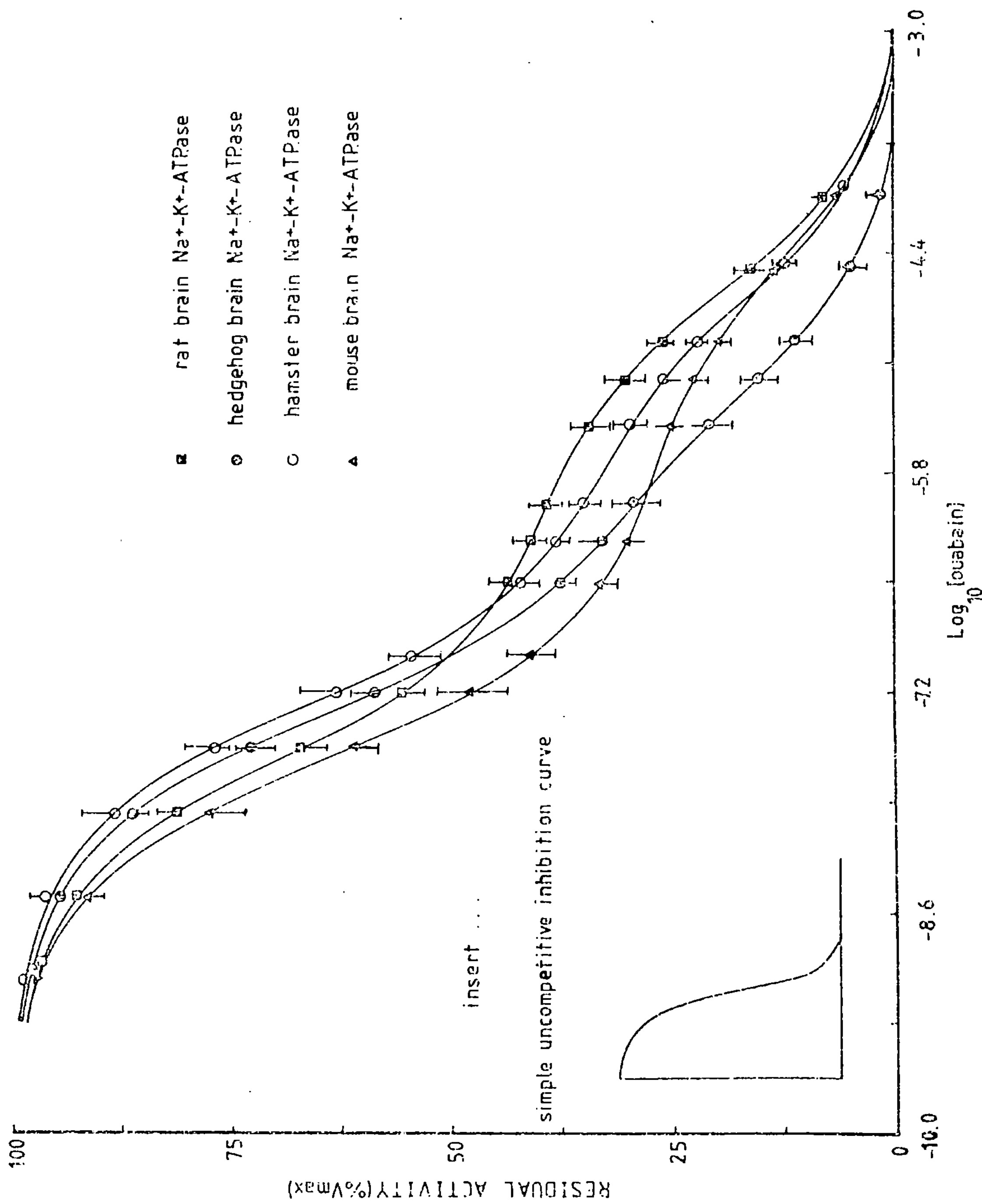


TABLE 3.1

Apparent pI_{50} values defining the ouabain inhibition of the Na^+-K^+-ATPase

Tissue Source	Apparent pI_{50}
Rat Brain	6.95
Hamster Brain	6.80
Mouse Brain	7.29
Hedgehog Brain	6.99
Frog Brain	7.34
Xenopus Brain	7.62
Carp Brain	7.79
Trout Brain	7.75
Starling Brain	7.58
Pigeon Brain	7.40
Locust 'Brain'	5.79
Cockroach 'Brain'	6.49

most ouabain sensitive of the mammalian preparations studied with the least ouabain sensitive of these being the hamster preparation (apparent pI_{50} 6.8).

The ouabain inhibition of the Na^+-K^+-ATPase from non-mammalian vertebrates appeared to describe monophasic decay curves (See figures 3.4 - 3.8). A closer inspection of these curves, showed that the enzyme activity range of 5% V_{max} - 95% V_{max} spanned nearly four orders of magnitude of ouabain concentration, as opposed to the two orders of magnitude expected of the simple uncompetitive inhibition system. In these cases, the curves described a sharp decay of activity to 10 - 20% V_{max} for ouabain concentrations up to 10^{-7}M. The residual 10% activity appeared to be relatively ouabain insensitive decaying finally to zero at ouabain concentrations near 10^{-4}M. In general, the Na^+-K^+-ATPase preparations from these non-mammalian vertebrates were more sensitive to ouabain than those from the mammals, with the decay of activity of the former occurring at lower ouabain concentrations. This was reflected in the larger values for the apparent pI_{50}'s (see Table 3.1) obtained for these preparations (apparent pI_{50} 7.3 - 7.8).

The ouabain inhibition of the Na^+-K^+-ATPase preparations from the two insects studied described simple monophasic decay curves which appeared to show little, or no, deviation from the form expected of simple uncompetitive inhibition (see figure 3.8). This was suggested by the observed decay from 95% V_{max} - 5% V_{max} over a ouabain concentration range of approximately two orders of magnitude. The apparent pI_{50} values obtained for these preparations (Table 3.1), indicated a relatively low sensitivity to ouabain with the locust

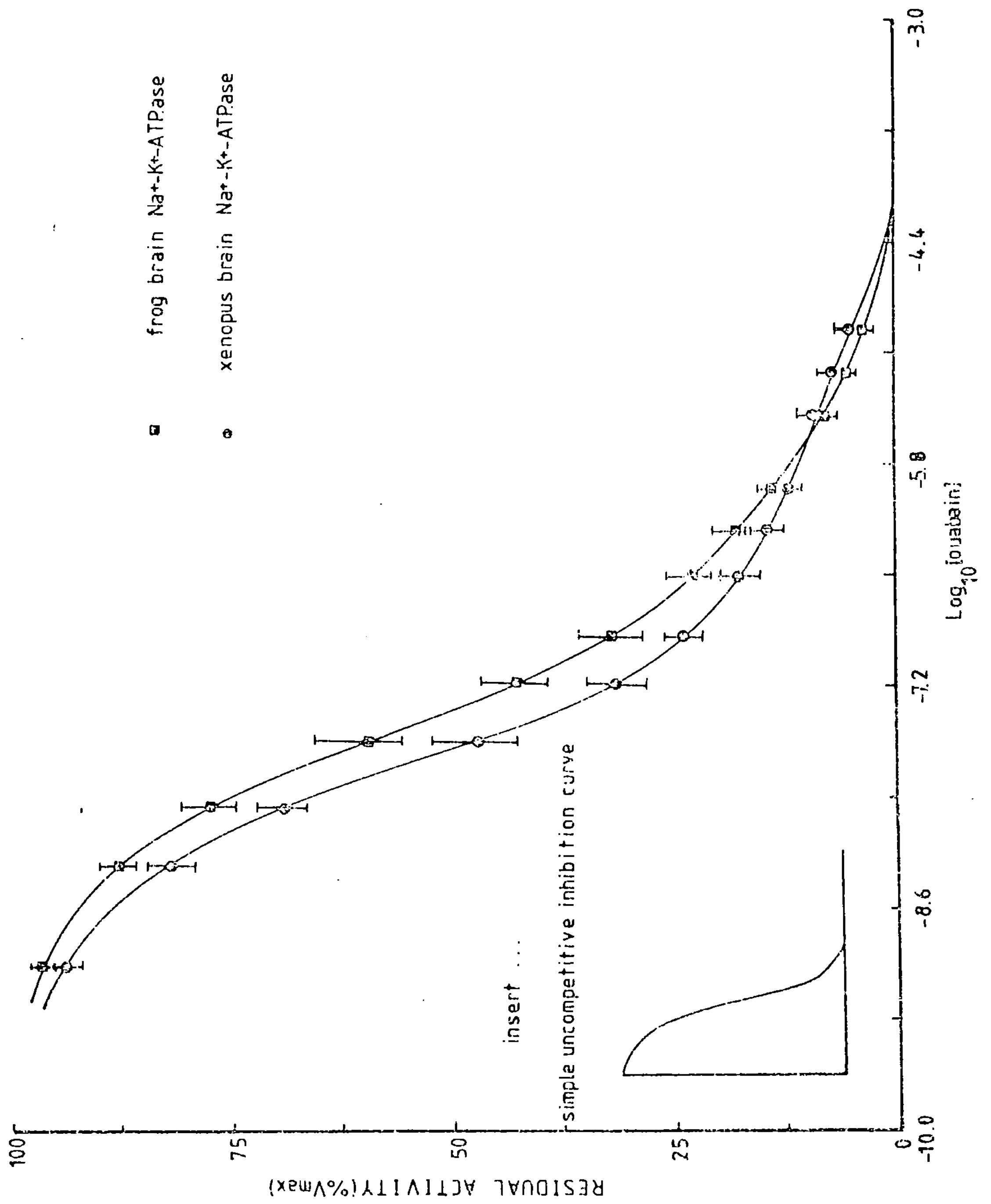
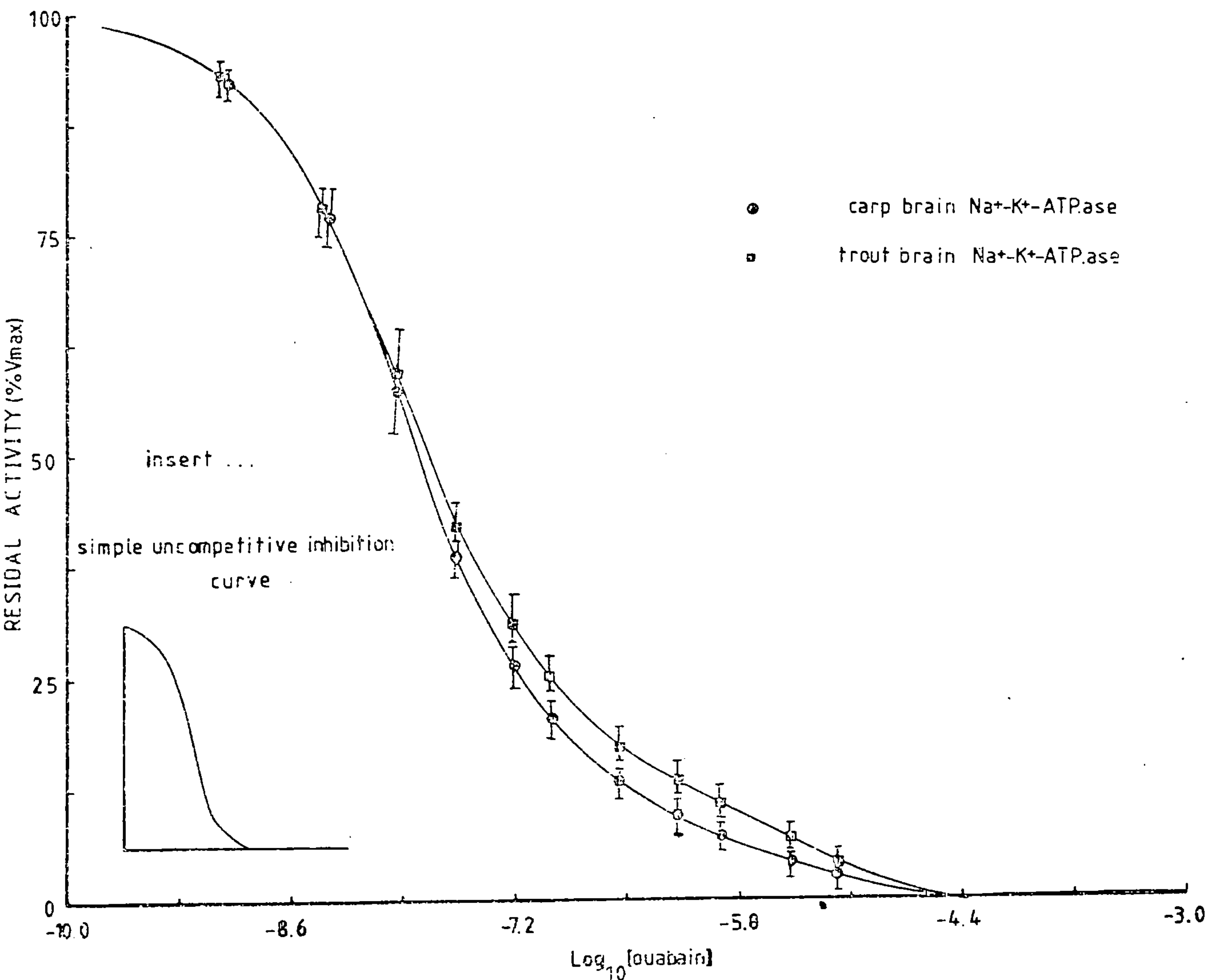


Fig.3.4
QUABAIN INHIBITION OF THE Na+-K+-ATP.ase
frog brain Na+-K+-ATPase
xenopus brain Na+-K+-ATPase
insert ...
simple uncompetitive inhibition curve
RESIDUAL ACTIVITY(%Vmax)
100
75
50
25
0
-10.0
-8.6
-7.2
-5.8
-4.4
-3.0
Log10 [ouabain]

Fig.3.5

OUABAIN INHIBITION OF THE Na^+-K^+-ATPase



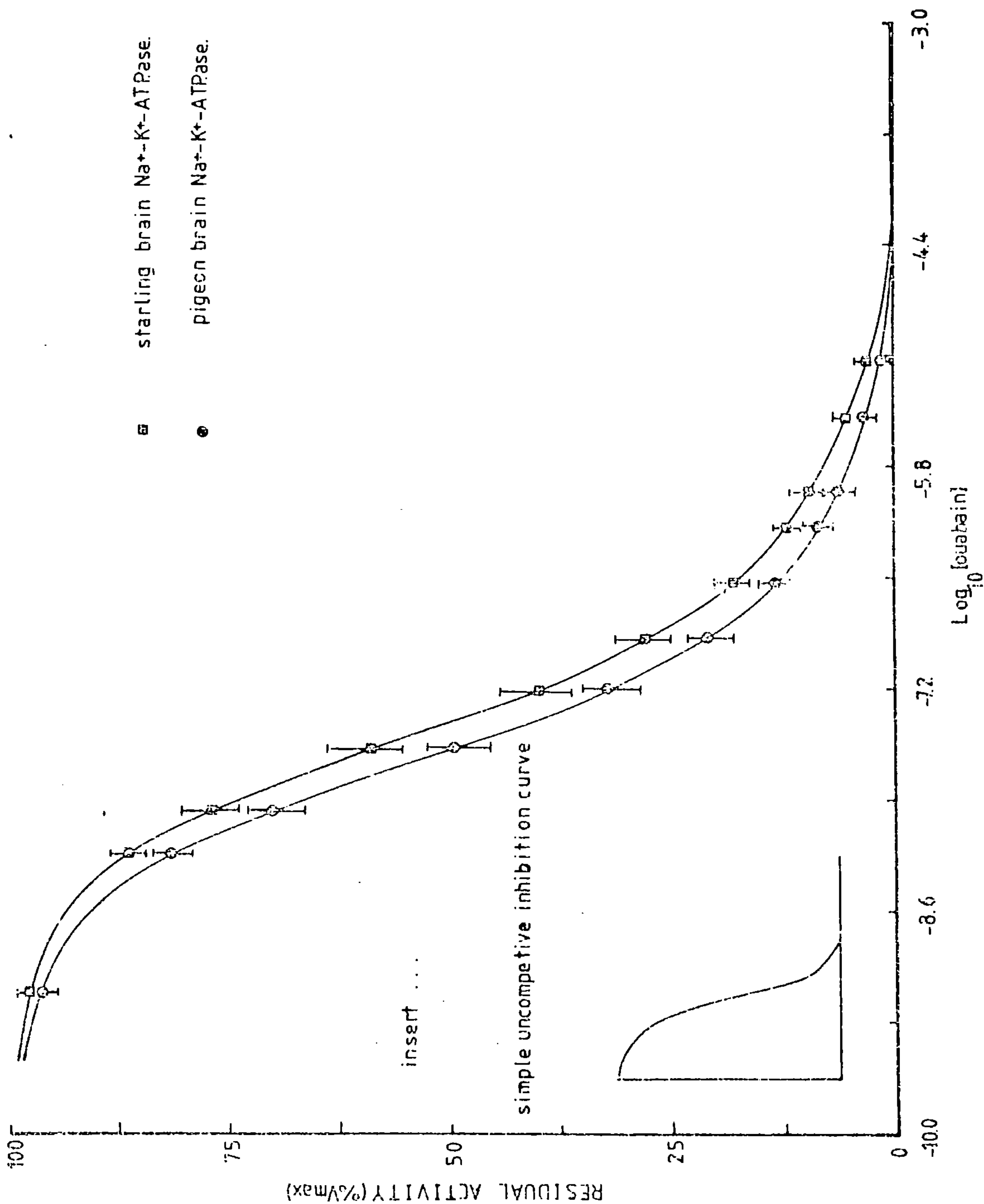


Fig. 3.6

OUABAIN INHIBITION OF THE Na+-K+-ATP.ase

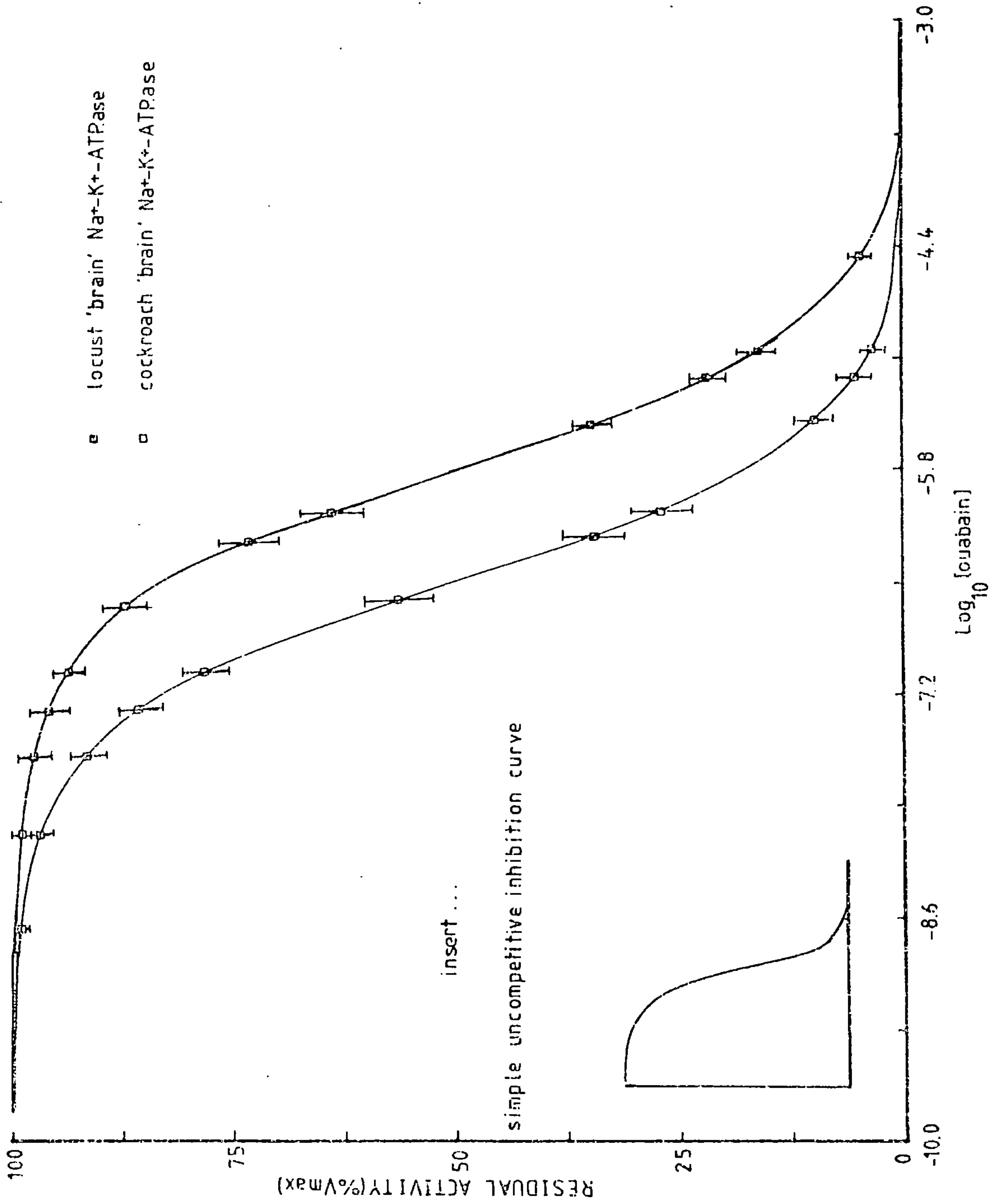


Fig. 3.7

OUABAIN INHIBITION OF THE Na+-K+-ATPase

Figure 3.8

Dose response kinetics as described by the two population and kinetic dimer models.

■ Decay pattern expected for the two population model of the following parameters:

K_i - 3.0×10^{-7}
β - 850
ϕ - 0.65
ψ - 0.45

□ Decay pattern expected for the co-operative kinetic dimer model of the following parameters:

K_i - 2.0×10^{-7}
β - 550
ϕ - 0.45

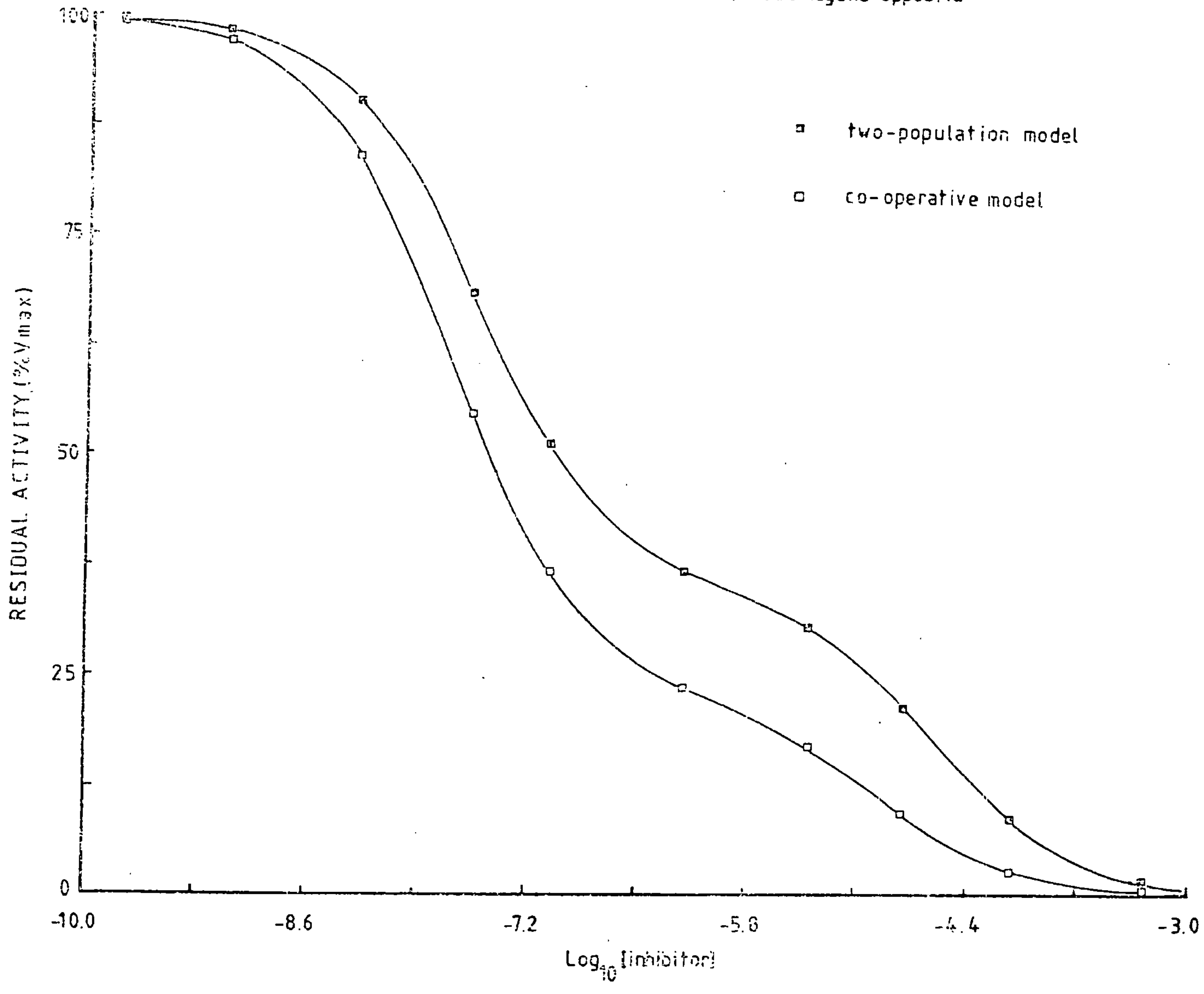


Fig. 3.8

SIMULATED DATA . . . see legend opposite

preparation (apparent pI_{50} 5.79) being the least ouabain sensitive of all the preparations studied.

The above measurements suggested that the apparent 'anomalous' ouabain dose response curves initially observed for the rat brain Na^+-K^+-ATPase preparation, while probably contributing to the reported ouabain sensitivity of these preparations, were not unique to Na^+-K^+-ATPase preparations from this source. Similar sets of observations have also been reported for the Na^+-K^+-ATPases from murine plasmocytoma cells (LeLievre, Charlemagne, Paraf, Jonkman-Bark & Zilberfarb, 1976; LeLievre, Charlemagne & Paraf, 1976; Zachowski, LeLievre, Aubry, Charlemagne & Paraf, 1977), Hela cells (Robbins & Baker, 1977) and pig heart (Schwartz, Lindenmayer & Allen, 1975). These measurements also suggested that the dose response sensitivity of the Na^+-K^+-ATPase is a very complex function of ouabain concentration. This had been previously suggested by reports of ouabain binding to the enzyme being described by non-linear Scatchard plots (Taniguchi & Iida, 1972a;Hansen, 1976) in which the observations were interpreted to be indicative of two types of ouabain binding sites on the Na^+-K^+-ATPase. The observed biphasic decay curves described by the mammalian preparations in this study were consistent with the above interpretation. However, the observation that the behaviour of Na^+-K^+-ATPase preparations ranged from an apparent agreement with simple uncompetitive inhibition, to the 'anomalous' form described by the rat brain preparation, suggested that the observations may all be resolved in a general description of which the behaviour described by preparations like the rat and the insects are near to the limiting extremes. These ideas were developed along two lines:

(a) The membrane preparations contained two independent significant populations of Na^+-K^+-ATPase enzymes.

(b) The functional Na^+-K^+-ATPase is a kinetic dimer with co-operativity between the subunits*.

The first of these has been suggested as possible reasons for non-ideal Scatchard binding of ouabain to the Na^+-K^+-ATPase (Taniguchi & Iida, 1972a;Hansen, 1976). This model describes a system in which the total enzyme assayed (Eo) is a mixture of two independent populations (A_o and B_o). The rate equation describing the conditions of saturating ligand concentrations would be given by the equations:

$$V_i = \alpha A + \theta B$$

$$V_{max} = \alpha A_o + \theta B_o$$

A + B . . . free forms of the enzymes

α . . . Rate constant for the species A

θ . . . Rate constant for the species B

V_{max} . . . Maximal activity

---

* The subunit in this discussion is defined as the minimum assemblage of protein species required for the hydrolysis of one molecule of ATP under saturation concentrations of essential ligands.

Since it must be assumed that the affinities for the cardiac glycoside are different, then the equilibrium between the free species (A and B) and the bound species (A_i and B_i) in the presence of a given concentration of the inhibitor (I) can be written

$$K_a = \frac{A.\ I}{A_i}$$

K_a . . . inhibitor equilibrium constant for A

$$K_b = \frac{B.\ I}{B_i}$$

K_b . . . inhibitor equilibrium constant for B

The essential conservation equations describing the above equilibria are given by

$$A_o = A + A_i$$

$$B_o = B + B_i$$

$$E_o = A_o + B_o$$

Given that the absolute rate constants α and θ are not experimentally accessible, a parameter ϕ can be defined such that $\phi = \theta/\alpha$.

If, in addition, Ψ represents the fraction of the species B in the sample (i.e. $\Psi = B_o/E_o$) then the observed rate (V_i) can be shown to be given by the equation:

$$V_i = V_{max} \frac{\dfrac{(1 - \Psi)}{(1 + I/K_a)} + \dfrac{\phi\Psi}{(1 + I/K_b)}}{(1 - \Psi) + \phi\Psi} \qquad (3b)$$

The alternative line describes the Na^+-K^+-ATPase as a dimeric species (E~E) with co-operativity between the subunits. The enzyme is

considered to be a symmetrical dimer such that catalytic activity of the free species (E~E) can be described in terms of a mean site rate constant (K_r). Thus at saturating concentrations of essential ligands, the maximal activity (V_{max}) can be given in terms of the total enzyme concentration (E_o)

i.e. $V_{max} = 2K_rE_o$

As described, there would be an equal probability of the inhibitor (ouabain) binding to any one site on the free enzyme. However, the binding of ouabain to any one site, abolishes the catalytic activity of that site and by co-operativity effects, alters the catalytic efficiency of the other site by a factor ϕ, and also alters the apparent ouabain equilibrium constant of the other site by a factor β. Thus the equilibria and conservation equations describing the system can be written thus:

$$K_i = \frac{E{\sim}E \cdot I}{E{\sim}E_i}$$

K_i . . . inhibitor affinity constant for the free dimer

$E{\sim}E_i$ . . species with one site blocked with inhibitor

I . . . concentration of inhibitor

$$\beta K_i = \frac{E{\sim}E_i \cdot I}{E_i{-}E_i}$$

$E_i{\sim}E_i$. species with both sites blocked with inhibitor

$$E_o = E{\sim}E + E_i{\sim}E_i + E_i{\sim}E$$

$$V_i = 2K_rE{\sim}E + \phi K_rE_i{\sim}E$$

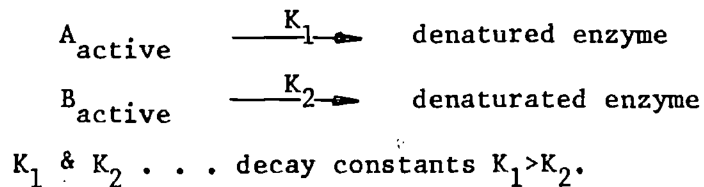
From these equations, an equation describing the observed rate as a function of the concentration of the inhibitor can be derived.

$$\text{i.e.} \quad V_i = \frac{V_{max}}{2}\left[\frac{2 + \phi\frac{I}{K_i}}{1 + (I/K_i) + (I^2/\beta K_i^2)}\right] \qquad (3c)$$

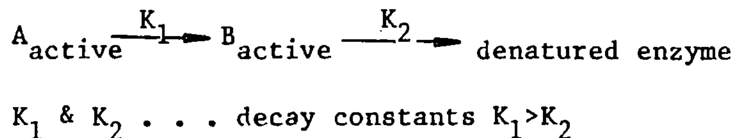
The dose response curves described by the equations (3b) and (3c) are both biphasic in the manner suggested by the experiments (see figure 3.8). This arises because both models postulate the existence of two catalytically active species at non-saturating concentrations of the inhibitor (ouabain). As a result, the models can not be distinguished by the type of dose-response measurements previously described. A possible approach to this problem was suggested by the results of the thermal inactivation of this enzyme (see Chapter 7).

A study of the kinetics of isothermal denaturation of the Na^+-K^+-ATPase (Chapter 7) revealed a complex decay process which could be described by any one of two schemes:

Scheme A



Scheme B



As described in Chapter 7, the scheme A is compatible with the description of the Na^+-K^+-ATPase defining two independent populations,

while the latter decay scheme is consistent with the description of the Na^+-K^+-ATPase as a dimeric enzyme. Since both schemes described the isothermal denaturation in terms of two species (A & B) with the species B being of greater thermal stability (i.e. $K_2<K_1$) it was, in principle, possible to prepare a sample in which all significant Na^+-K^+-ATPase activity was attributable to the more thermo-stable species. This was achieved by heating a normal preparation at a temperature and for a period of time such that the thermal inactivation process proceeded for ten half lives of the 'thermo-labile' species and two half lives of the 'thermo-stable' species (exact conditions described in Chapter 9). The preparation so obtained (heat treated preparation) could then be used for the characterisation of its dose response sensitivity to ouabain.

Figure 3.9 shows the ouabain dose response curves described by control and heat-treated preparations from rat brain and trout brain. The curves described by both heat treated preparations are monophasic and the decay from 95% V_{max} to 5% V_{max} spans two orders of magnitude of ouabain concentration. When compared with the control preparations, there was a well defined change in the shape of the decay curves and a significant change in their apparent ouabain sensitivities as estimated from the apparent pI_{50} values (Table 3.2). In both cases, there was a decrease in the apparent ouabain sensitivity of the heat treated preparations. However, the change in the apparent ouabain sensitivity after heat treatment was considerably greater in the case of the rat brain preparation.

Fig. 3.9

EFFECT OF 'HEAT-TREATMENT' ON THE
OUABAIN SENSITIVITY OF THE Na^{+}-K^{+}-ATPase

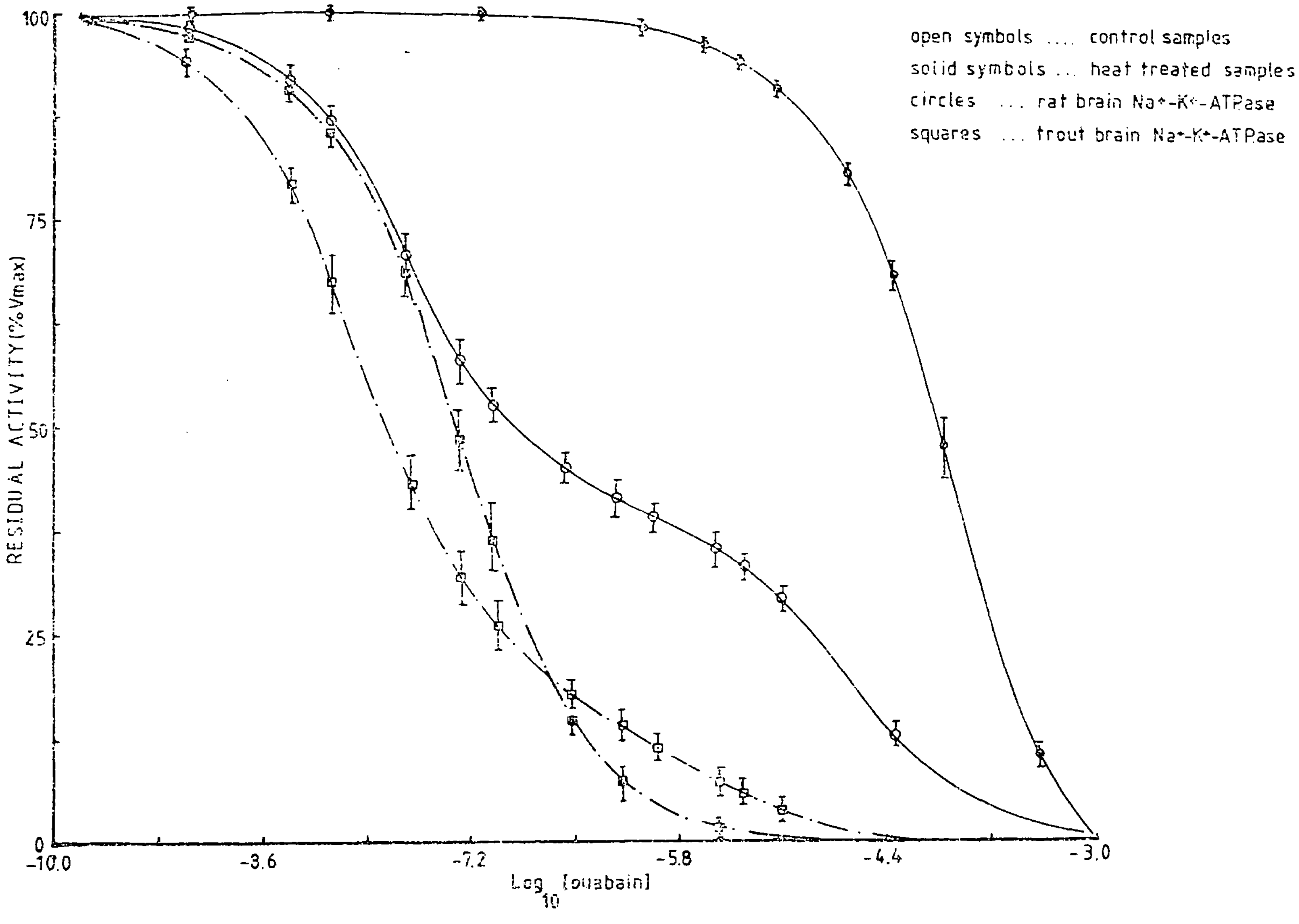


TABLE 3.2

Effect of heat treatment on the ouabain pI_{50} values of the Na^+-K^+-ATPase

Tissue Source	Apparent pI_{50} Values*	
	Control$^{\Delta}$	Heat Treated$^{\blacktriangle}$
Rat Brain	6.95 ± 0.003	4.04 ± 0.008
Trout Brain	7.75 ± 0.008	7.29 ± 0.007

* Values mean of 4 preparations $\pm$ 1 Standard deviation

Δ Values estimated by interpolation

▲ Values estimated by fitting an uncompetitive inhibition curve

The above observation, that the ouabain dose response sensitivities of the heat treated preparations described monophasic decay curves, that were definable in terms of simple uncompetitive inhibition, is consistent with both the thermal inactivation schemes previously described. Both schemes predicted that the heat treatment procedure employed should produce a sample in which all the significant Na^{+}-K^{+}-ATPase activity was attributable to a single one-site species, and for such a species, the description of a simple uncompetitive inhibition is inevitable. Since the thermal inactivation scheme A predicted that the properties of the heat treated preparation should be identical to those of one of the populations in the postulated mixture, it was possible to test the feasibility of this description by comparing the ouabain sensitive properties of the heat treated samples with those that would be defined if the system were treated as a mixture of two independent populations.

If the 'anomalous' dose response sensitivity of the Na^{+}-K^{+}-ATPase were taken to be the result of the presence of two independent populations of the enzyme in the membrane preparations, then the dose response curves described would be the summation of two uncompetitive inhibition dose response curves. Since the significant parts of such curves are described over two orders of magnitude of inhibitor concentrations, it follows that, as illustrated in figure 3.10, the composite curve can be resolved into its two component curves from which estimates of the apparent inhibitor equilibrium constants (and consequently their apparent pI_{50} values) can be obtained. Table 3.3 lists the apparent pI_{50} values for the heat treated rat brain and trout brain preparations, and those values obtained by resolving the curves

Fig. 3.10

RESOLUTION OF A BIPHASIC DECAY CURVE INTO TWO MONO-PHASIC DECAY CURVES

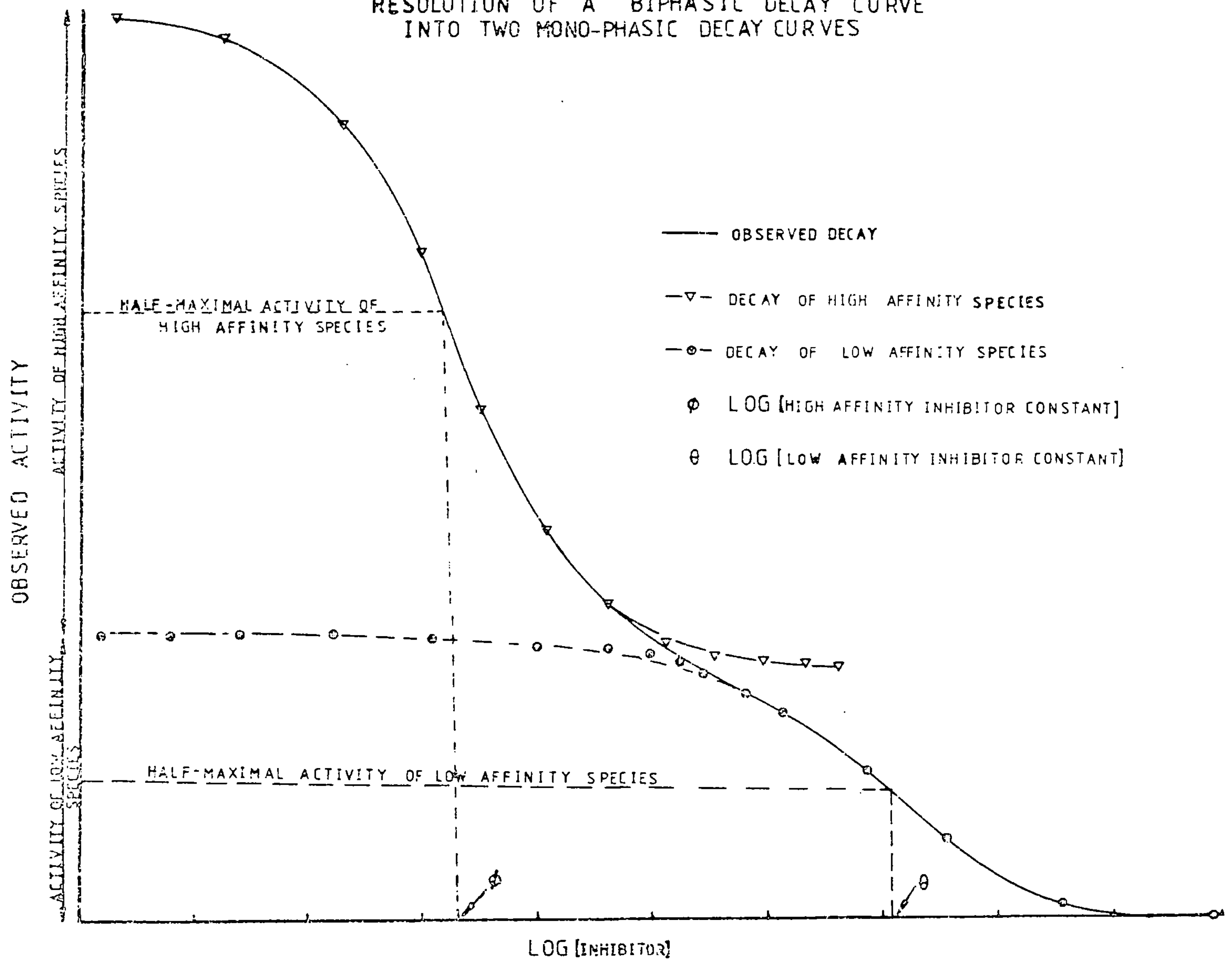


TABLE 3.3

Comparison of ouabain pI_{50} values with those calculated for the two populations of Na^+-K^+-ATPase enzymes

Tissue source	Apparent pI_{50} values*		
	Heat treated Δ membrane	High affinity ▲ population	Low affinity ▲ population
Rat Brain	4.04±0.008	7.70±0.01	4.96±0.03
Trout Brain	7.29±0.007	8.06±0.02	5.88±0.04

* Values mean of 3 preparations ± 1 standard deviation

Δ Values estimated by fitting an uncompetitive inhibition curve.

▲ Values estimated by interpolation on the uncompetitive inhibition curves resolved from the control curves.

described by the relevant control preparations, in the manner illustrated in figure 3.10. In both cases, the apparent pI_{50} values for the heat treated preparations were significantly different from those obtained by the resolution of the control curves into two uncompetitive inhibition curves. The heat treated rat brain preparation was found to be less ouabain sensitive (pI_{50} 4.04) than any of the postulated populations (pI_{50} values 7.7 and 4.96). In the case of the trout, the ouabain sensitivity of the heat treated preparation (pI_{50} 7.33) was greater than that of the 'low affinity' population (pI_{50} 5.89), but significantly less than that of the 'high affinity' population (pI_{50} 8.06).

The failure to match the ouabain sensitive properties of the heat treated preparation with any of those that would be described for a mixture of two Na^+-K^+-ATPase enzymes, argued against the presence of such a mixture. Although such a mixture has been proposed to account for the 'anomalous' ouabain binding to the Na^+-K^+-ATPase (Taniguchi & Iida, 1972a;Hansen, 1976), recent reports have presented evidence in favour of a dimeric structure for the Na^+-K^+-ATPase (Kyte, 1975; Grisham & Mildvan, 1975; Liang & Winter, 1977). The evidence presented in these reports, favoured the functional Na^+-K^+-ATPase as being made up of two subunits each of which is an assembly of one large polypeptide (M.W.≃ 95,000-100,000) and one sialo-glycoprotein (M.W.≃ 45,000-55,000). Since it has been demonstrated that the large polypeptide contains the ATP phosphorylation site (Kyte, 1971a) and the ouabain binding site (Ruoho & Kyte, 1974), the structural dimer described in these reports, is compatible with the kinetic dimer described in the model (b) above. Consequently this description was implemented as the framework for interpreting the ouabain dose response sensitivity of the Na^+-K^+-ATPase.

Equation (3c) describes the dose response kinetics in terms of the four defining parameters V_{max}, ϕ, β and K_i. Under conditions of saturation concentrations of essential ligands, V_{max} defines the maximal activity (i.e. observed activity in the absence of inhibitor), K_i defines the concentration of inhibitor needed to half-saturate the 'first' site on the dimer, ϕ defines the extent to which the catalytic efficiency of the second site is altered (relative to the mean site activity defining V_{max} conditions) by ouabain binding to the first site, and β defines the extent to which inhibitor binding to the first site alters the affinity of the second site for the inhibitor, such that βK_i represents the concentration of inhibitor needed to half saturate the second site on the dimer. This equation can be treated as a numerical problem involving a dependent variable (V_i), an independent variable (I) and four adjustable parameters, and as such it can be solved numerically by a computer assisted non-linear least squares minimisation procedure (see Appendix II). Table 3.4 lists the values of the defining parameters returned for the various preparations (except those obtained from the insects) by a computer programme employing such a procedure. These results show very small differences among the various preparations with respect to the K_i parameter (ouabain concentration needed for half saturation of the first site). All the values returned for this parameter were in the range 1×10^{-8} - 6×10^{-8}M. The values returned for the mammalian preparations (2.3×10^{-8} - 6×10^{-8}M) were generally higher than those returned for the preparations from the non mammalian vertebrates (1×10^{-8} - 3.5×10^{-8}M). The values returned for the ϕ parameter (factor by which the catalytic efficiency of the second site is altered by ouabain binding to the first site), are all less than unity, with those for the mammalian

TABLE 3.4

List of parameters defining the ouabain inhibition of the Na^+-K^+-ATPase from different sources.

Tissue Source	n	▲Defining Parameters		
		ϕ	Ki	β
Rat Brain	4	0.799 ± 0.031	$2.32 \times 10^{-8} \pm 3.5 \times 10^{-9}$	866 ± 25
Hamster Brain	4	0.675 ± 0.027	$5.25 \times 10^{-8} \pm 1.0 \times 10^{-9}$	399 ± 12
Mouse Brain	4	0.555 ± 0.061	$2.30 \times 10^{-8} \pm 9.8 \times 10^{-10}$	1154 ± 170
Hedgehog Brain	2	0.619 ± 0.026	$4.11 \times 10^{-8} \pm 8.0 \times 10^{-10}$	153 ± 16
Frog Brain	2	0.355 ± 0.022	$2.99 \times 10^{-8} \pm 1.4 \times 10^{-9}$	90 ± 11
Xenopus Brain	2	0.293 ± 0.021	$1.71 \times 10^{-8} \pm 8.3 \times 10^{-10}$	282 ± 40
Carp Brain	2	0.207 ± 0.019	$1.33 \times 10^{-8} \pm 9.5 \times 10^{-10}$	163 ± 23
Trout Brain	4	0.336 ± 0.08	$1.19 \times 10^{-8} \pm 6.0 \times 10^{-10}$	157 ± 43
Starling Brain	3	0.117 ± 0.05	$2.34 \times 10^{-8} \pm 4.3 \times 10^{-9}$	181 ± 51
Pigeon Brain	2	0.173 ± 0.02	$3.31 \times 10^{-8} \pm 9.9 \times 10^{-10}$	101 ± 14

▲ Values mean of n preparations $\pm$ 1 standard deviation

preparations (0.5 - 0.8) being greater than those returned for the non-mammalian vertebrates (0.1 - 0.4). These values describe a system in which the binding of ouabain to the first site, results in a reduction of the catalytic efficiency of the second site. In all cases the values for the β parameter (factor by which the apparent ouabain equilibrium constant of the second site is altered by ouabain binding to the first site) were very much greater than unity, with the values returned for the mammalian preparations (150-1200) being generally greater than those returned for the non mammalian vertebrates (80 - 300). The values described a system in which the binding of ouabain to one of the sites on the dimer, resulted in a reduction in the ouabain sensitivity of the other site.

The data obtained from the insect preparations was not optimised by the computer assisted procedure used. These preparations appeared to be better described by simple uncompetitive inhibition curves. In principle these observations can be accommodated within the model described by assuming that one or both of the co-operativity parameters (ϕ and β) is very small. However, direct experimental support for such a supposition was precluded by the necessity for having to assay very low levels of enzyme activity, and by the unavailability of the insect preparations in the quantities needed for thermal inactivation studies. A possible approach to this problem was suggested by the observation that the ouabain dose response sensitivities of the Na^+-K^+-ATPase and K^+-PNPase from heat treated rat brain preparations were not significantly different from each other (see Chapter 9). Given that the cardiac glycoside interacts with the phosphorylated intermediate, and since the properties of that

intermediate must be independent of the method if its formation, the above observation is consistent with the presence of a single one-site ouabain sensitive species in the heat treated preparation. Thus, if the insect preparations are genuinely described by a simple uncompetitive inhibition system, no difference would be expected between the dose response sensitivity curves of the Na^+-K^+-ATPase and K^+-PNPase activities of such preparations. Figure 3 .11 shows the ouabain dose response curves for the Na^+-K^+-ATPase and K^+-PNPase from the locust 'brain' preparation. This describes the K^+-PNPase curve as being a monophasic decay curve, and like the Na^+-K^+-ATPase it can be in terms of simple uncompetitive inhibition since the significant part of the curve (95% V_{max} - 5% V_{max}) spans two orders of magnitude of ouabain concentration. However, the apparent ouabain sensitivity of the Na^+-K^+-ATPase (apparent pI_{50} 5.79) was significantly greater than that of the K^+-PNPase (apparent pI_{50} 4.76). This result whilst not directly supporting the supposition that the ouabain dose response sensitivities of the insect preparations were definable as limiting cases of the model implemented, suggested that their observed consistency with simple uncompetitive inhibition kinetics, was probably better defined as the limiting extreme of a more complex system.

DISCUSSION

The results of this study suggest a description of the ouabain inhibition of the Na^+-K^+-ATPase in which the dose response sensitivity is a complex function of ouabain concentration. Such a description is supported by the observation that most of the preparations studied

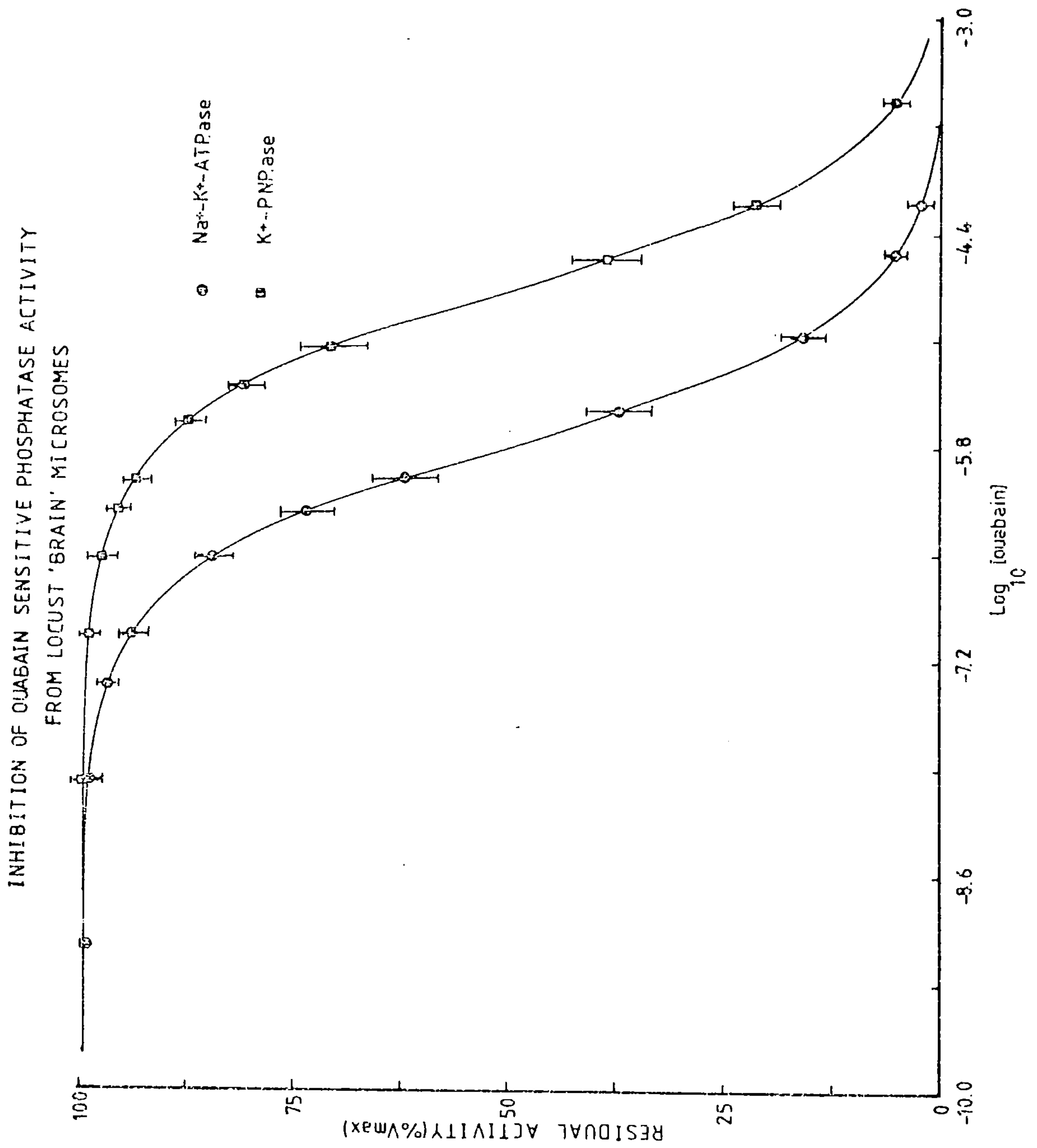


Fig.3.11

INHIBITION OF OUABAIN SENSITIVE PHOSPHATASE ACTIVITY FROM LOCUST 'BRAIN' MICROSOMES

are characterised by a relative insensitivity to ouabain at concentrations near 10^{-6}M. In the cases where the dose response sensitivities are definable in terms of simple uncompetitive inhibition kinetics (e.g. the insect preparations), the comparison of the Na^{+}-K^{+}-ATPase and K^{+}-PNPase activities with respect to their ouabain sensitivities suggest that the apparent consistency with the simple system is probably better described as the limiting case of a more complex system, since the apparent ouabain sensitivity varies with the phosphorylating ligand used. Therefore the results of this study compare favourably with the reported non-linear Scatchard plots described by ouabain binding to the Na^{+}-K^{+}-ATPase (Taniguchi & Iida, 1972a;Hansen, 1976), and the complex system suggested by the observed variation of the ouabain sensitivity with the nature of the phosphorylating ligand used (Chapter 10; Bond & Post, 1971; Israel & Titus, 1967; Yoshida, Izumi & Nagai, 1966).

The apparent ouabain sensitivity of the various preparations as would be estimated from the pI_{50} values, was found to be greater than those values of similar preparations previously reported (Grasso, 1967; Bonting, 1966; Tobin & Brody, 1972). However, this was expected since, with few exceptions, most of the pI_{50} values reported in the literature were not determined by a similar pre-incubation assay procedure designed to circumvent the problems of slow equilibriation with ouabain and the effects of potassium ion. Previous reports (Akera, 1971; Tobin & Brody, 1972) have shown that in the absence of such a preincubation step, the apparent pI_{50} values were found to be smaller, and in addition the values so obtained were dependent upon the assay time (Allen & Schwartz, 1970). However, in cases where comparable values are available (Akera, 1971; Tobin & Brody, 1972), the values reported compared favourably with those

found in this study. In spite of this agreement, the complexity of the concentration dependence of the ouabain inhibition of the enzyme suggests that some caution must be exercised in the use of the pI_{50} values as an index of the ouabain sensitivity of the Na^+-K^+-ATPase.

The model adopted for the description of the ouabain inhibition of the enzyme, describes a kinetic dimer with co-operativity between the subunits. Whilst the available evidence does not support this model to the exclusion of alternative descriptions, the model adopted is consistent with the recently reported structural evidence (Kyte, 1975; Grishan & Mildvan, 1975; Liang & Winter, 1977), describing the functional enzyme as an $\alpha_2\beta_2$ dimer. Once this description is assumed, the co-operativity parameters (ϕ and β) calculated describe a system of negative co-operativity between the subunits, affecting both catalytic efficiency and ouabain binding affinity. This is suggested by the observation that the ϕ values calculated were all less than unity, indicating a reduced catalytic efficiency at the second site by ouabain binding to the first site. Negative co-operativity with respect to ouabain binding affinity is supported by the values returned for the β parameter (all >>1). Since small differences are found between the K_i values returned for the various preparations, the results suggested that the major differences in the apparent sensitivity to ouabain were almost entirely the result of co-operative effects. Thus the relatively 'ouabain insensitive' preparations (e.g. rat brain) were characterised by ϕ values which approach unity (0.8 for rat), and large values for β (866 for the rat), when compared with

the smaller co-operativity parameters for the more 'ouabain sensitive' preparations (e.g. for trout brain ϕ = 0.34 and β = 157). Recent mechanistic models (Stein, Lieb, Karlish & Eilam, 1973; Kyte, 1975), have described the Na^{+}-K^{+}-ATPase as a dimeric enzyme with 'half-the-sites-reactivity'. Given that 'half-the-sites-reactivity' is the limiting case of negative co-operativity*, and given that the results provide evidence for large co-operative effects (e.g. for starling brain ϕ = 0.12) the mechanistic models reported and the model developed in this study are comparable.

The data obtained in this study argues strongly against a simple description of the concentration dependence of the ouabain dose response sensitivity of the Na^{+}-K^{+}-ATPase. The 'anomalous' behaviour found can be accommodated within a model describing the enzyme as a kinetic dimer with negative co-operativity between the subunits. Although the experimental evidence does not support this model to the exclusion of all others, the model described appears to accommodate most of the currently held views of the structure and mechanism of the Na^{+}-K^{+}-ATPase, and as a result, this model has been used for the interpretation of the ouabain inhibition of the Na^{+}-K^{+}-ATPase.

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* In the model implemented, 'half-the-sites-reactivity' would be defined by small values of ϕ. In practice, half-the-sites-reactivity is kinetically indistinguishable from negative co-operativity for ϕ values less than 0.1.

CHAPTER 4

The temperature dependence of the Na^+-K^+-ATPase

INTRODUCTION

A study of the temperature dependence of the activity of an enzyme forms an integral part in its characterisation and consequently is an important step in the elucidation of an enzyme's catalytic mechanism. Such temperature studies are usually resolved according to the rate theory of ARRHENIUS (Arrhenius, 1889). Within this description, the rate of a reaction is related to temperature by the equation:

$$\text{Rate} = Ae^{-\frac{u}{RT}}$$

A	Proportionality constant
u	Apparent activation energy
R	Gas constant
T	Absolute temperature

Thus a graph of $\log_e$(Rate) plotted against $^1/T$ should be linear with a slope $-u/_R$, and a value for 'u' can be experimentally obtained. Ideally, such a treatment should only be applied to simple, one step reactions, in which case, the measured Arrhenius 'u' would approximate to the activation energy of the reaction. This approach, however, has also been applied to the complex, multi-step reaction mechanisms, that are typical of enzyme catalysed reactions. In such cases, the apparent Arrhenius 'u' value obtained, can be taken only as a rough measure of the energy difference between the initial state of the system, and the 'transition state' of the rate limiting step of the overall reaction mechanism.

The early attempts at the characterisation of the temperature kinetics of the Na^+-K^+-ATPase (Gruener & Avi-Dor, 1966), were done on rat brain preparations. Those results suggested that the temperature

kinetics of the Na^{+}-K^{+}-ATPase could not be described by an Arrhenius straight line. This suggestion was later supported by observations from goldfish preparations (Smith, 1967), though the deviation from ideal Arrhenius behaviour was less pronounced. On reflection, such observations may have been expected since non-linear Arrhenius plots had already been observed in other biological systems (Kistiakovusky & Lumry, 1949; Levey, Sharon & Koshland, 1959), and more pertinently, low temperature inactivation of potassium linked sodium transport had been previously reported (Whittam & Davies, 1953; Page, Grove & Storm, 1964). . Those early observations have since been confirmed in other laboratories using a range of different tissue sources, preparative techniques, assay conditions and methods. It is now generally accepted that the temperature dependence of the Na^{+}-K^{+}-ATPase activity, cannot be described by simple Arrhenius temperature kinetics.

Inevitably, the deviation of the temperature kinetics of the Na^{+}-K^{+}-ATPase, and other enzyme systems, from the form predicted by the Arrhenius equation, has made the interpretation of such data problematical. This uncertainty has been augmented by the absence of agreement on a comprehensive principle, a set of principles, within which the observed behaviour could be defined. Proposals meant to account for the non-ideal Arrhenius behaviour observed in many biological systems are varied. They include:

(a) A temperature induced change from one rate limiting reaction to another (Crozier, 1924).

(b) The temperature dependence of thermodynamic interconversions between high and low temperature stable states (Kavanau, 1950).

(c) The occurrence of thermal phase transitions and, or conformational changes, which directly or indirectly modify the catalytic properties of

the enzyme (Massey, Curti & Ganther, 1966; Kumamoto, Raison & Lyons, 1971; Wynn-Williams, 1976).

(d) The effects of temperature on the substrate binding affinity of the enzyme (Silvius, Read & McElhaney, 1978).

These and other proposals, have been the subject of some extensive theoretical calculations (Han, 1972), from which it was concluded that non-ideal Arrhenius kinetics can be the result of a variety of kinetic and thermodynamic factors. This, along with the accumulation of evidence in the literature, that there may be different factors governing the non-linearity of the Arrhenius plots of different enzyme systems (Massey et al, 1966; Lyons & Raison, 1970a, b; Charnock, Cook & Opit, 1971) have suggested that one, all embracing, principle within which all cases of non-ideal Arrhenius temperature kinetics can be defined, is unlikely. Consequently it may be necessary to define the temperature kinetics of a given enzyme system within the theoretical framework which seems most applicable to it.

Apart from defining a non-linear Arrhenius graph, any mechanism attempting to define the temperature kinetics of the Na^+-K^+-ATPase should account for the sharp decline in the observed catalytic activity at temperatures near 4^oC (Charnock, Cook & Casey, 1971), as well as the smaller deviation from ideal Arrhenius behaviour reported for fish preparations (Smith, 1967), when compared with mammalian preparations (Gruener & Avi-Dor, 1966; Charnock, Cook & Opit, 1971). Attempts at so doing have defined two approaches to this problem:

(a) A description within which the Arrhenius graph of the Na^+-K^+-ATPase would appear as a catenary curve (Neufeld & Levey, 1970; Russel & Peach, 1974).

(b) A description within which the Arrhenius graph of the Na^+-K^+-ATPase would be interpreted as two Arrhenius straight lines (Charnock, Cook & Opit, 1971).

Since, in principle, non-ideal Arrhenius temperature kinetics of the form observed in the Na^+-K^+-ATPase system, can be accounted for by any one of a variety of proposals, an evaluation of the applicability of these and other suggestions (Han, 1972), to the particular case of the Na^+-K^+-ATPase should be helpful. Such has been the object of this study, with the eventual aim of implementing the mechanistic framework which appears to be the most applicable.

MATERIALS AND METHODS

(a) Preparation of external synaptic membranes. These were prepared from the brains of rats, mice, hamsters and rainbow trout as has been previously described in Chapter 3.

(b) Assay of Na^+-K^+-ATPase activity. This enzyme was assayed as previously described for the standard assay conditions (see Chapter 2). In addition, it was necessary to restrict the temperature dependent change in the pH of the buffer system (Histidine/HCl) to $\pm$ 0.05 pH units from the set value. This was achieved by the preparation of four otherwise identical solutions that were set at pH 7.5 at different temperatures. The temperature ranges chosen were:

Assays at 4^oC - 11^oC	pH 7.5 at 7^oC
Assays at 12^oC - 21^oC	pH 7.5 at 15^oC
Assays at 22^oC - 29^oC	pH 7.5 at 25^oC
Assays at 30^oC - 38^oC	pH 7.5 at 35^oC

Preliminary experiments showed that the buffer systems maintained their pH within $\pm$ 0.05 pH units of the set value under the experimental conditions.

RESULTS AND DISCUSSION

The temperature dependence of the Na^+-K^+-ATPase from the brain synaptic membranes of rats, mice, hamsters and rainbow trout are shown in Arrhenius form in figures 4.1 - 4.4. (typical data sets). Although no attempt was made to define the observations by any curve, line or set of lines, it is clear that the data sets are consistent with the observations of several workers in that they do not define Arrhenius straight lines. As mentioned previously, non-ideal Arrhenius behaviour can be the result of a variety of causative factors. These include:

(a) Kinetic factors related to the enthalpy of formation of the enzyme-substrate complex.

(b) Thermodynamic factors associated with the interconversions between different stable states of the enzyme.

(c) Thermal phase transitions which directly or indirectly affect catalytic efficiency.

(d) Thermal inactivation processes.

The theoretical basis of the above will be examined within the context of the Na^+-K^+-ATPase, with the aim of establishing which provides the most likely explanation for the behaviour patterns seen in figures 4.1 - 4.4.

(i) Kinetic considerations related to the heat of dissociation of the enzyme-substrate complex.

The kinetic factors that can give rise to non-ideal Arrhenius behaviour have been discussed in general terms (Han, 1972; Silvius *et al* 1978), and with special reference to the Na^+-K^+-ATPase (Russel & Peach, 1974). The effect arises directly as a result of the application of Arrhenius kinetics to the complex reaction mechanisms typical of enzyme

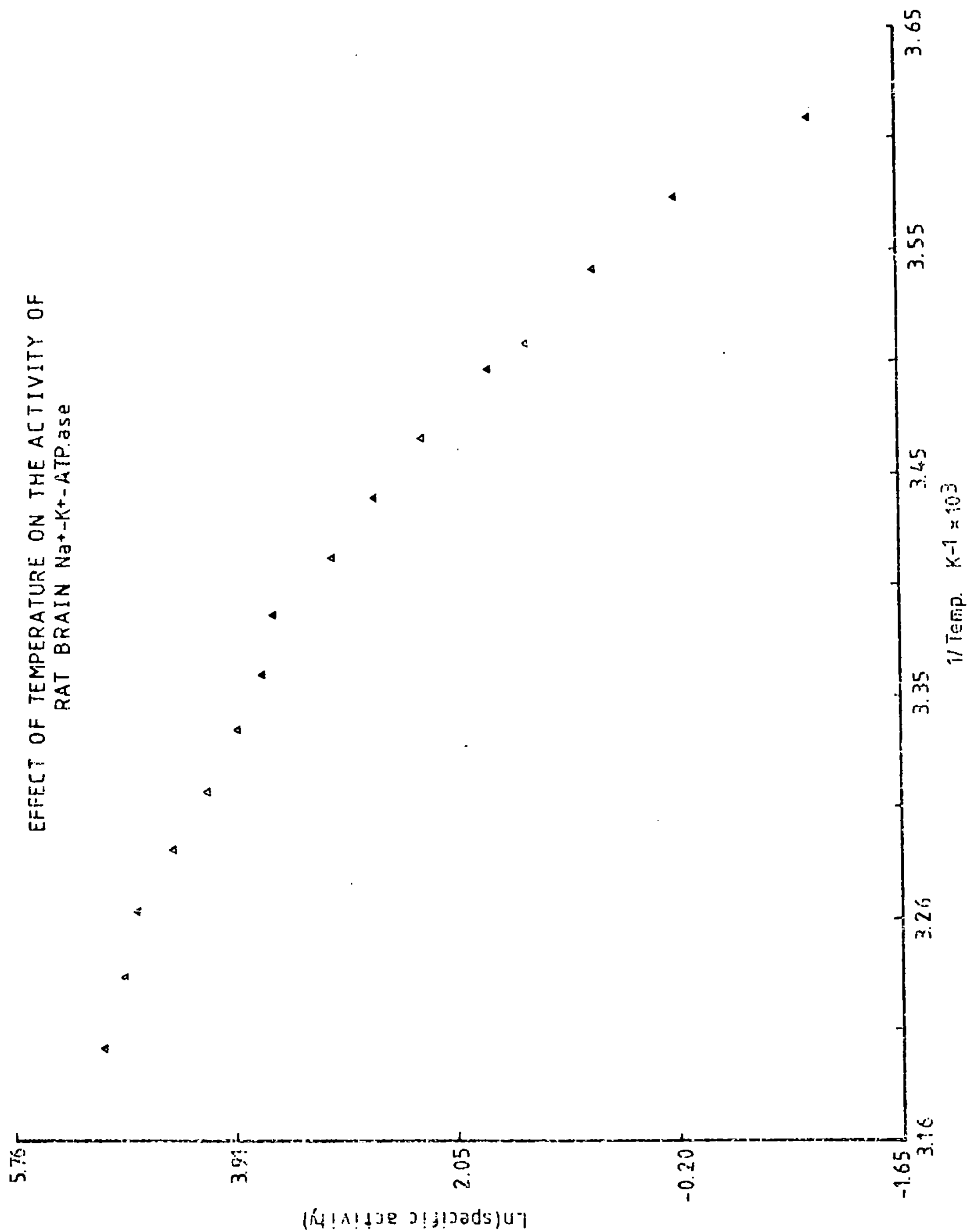


Fig. 4.1

EFFECT OF TEMPERATURE ON THE ACTIVITY OF RAT BRAIN Na^+-K^+-ATP.ase

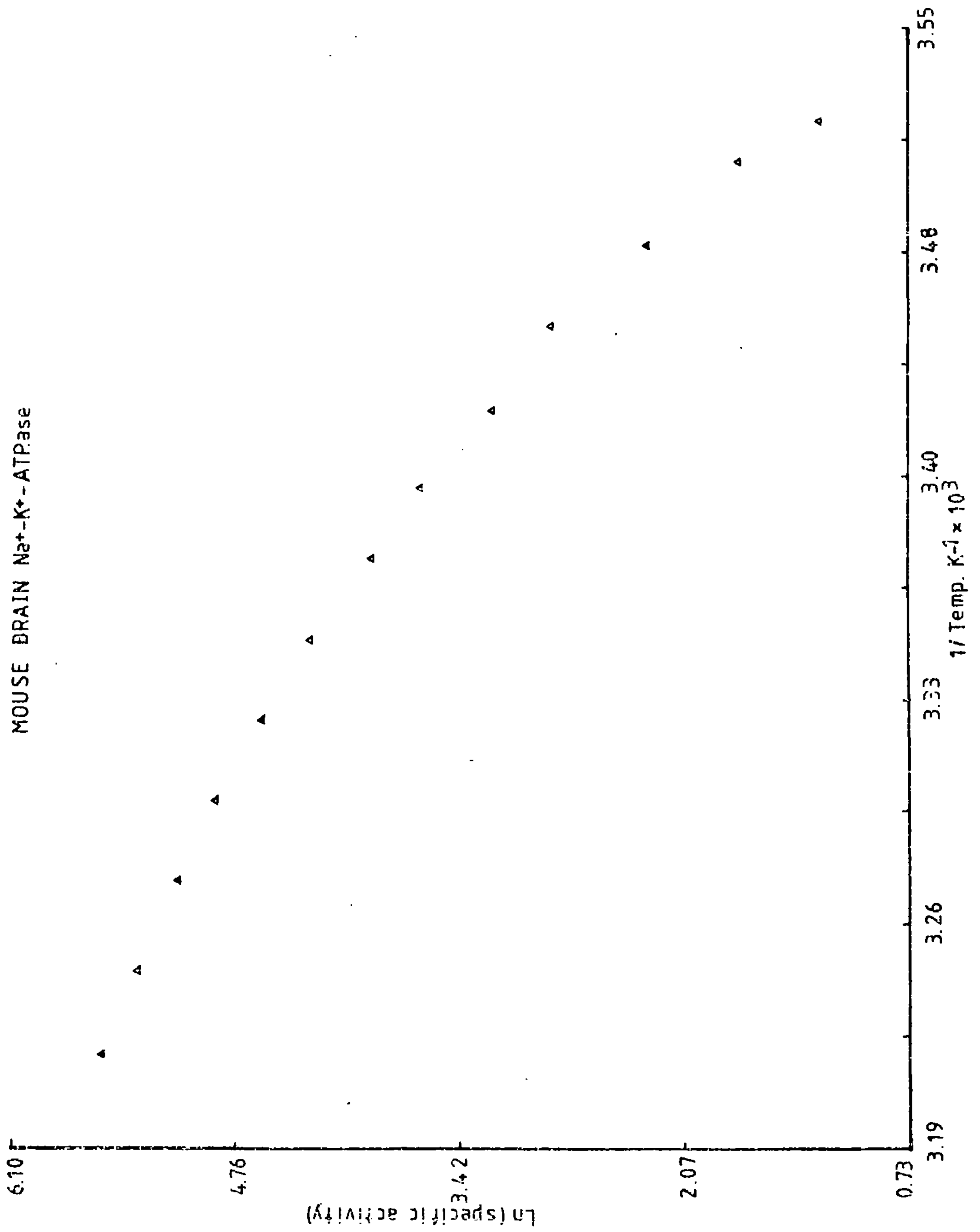


Fig. 4.2

EFFECT OF TEMPERATURE ON THE ACTIVITY OF MOUSE BRAIN Na^+-K^+-ATPase

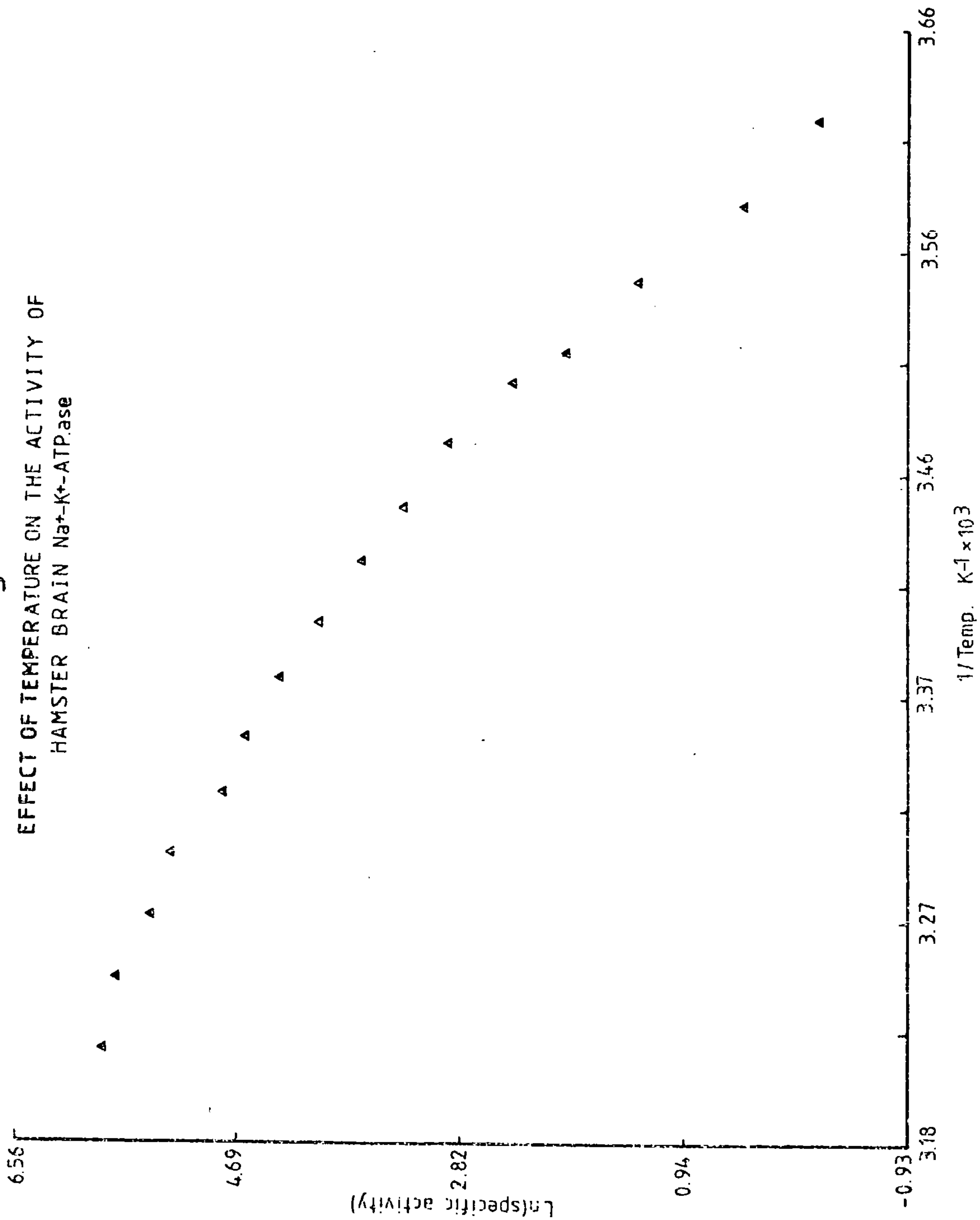
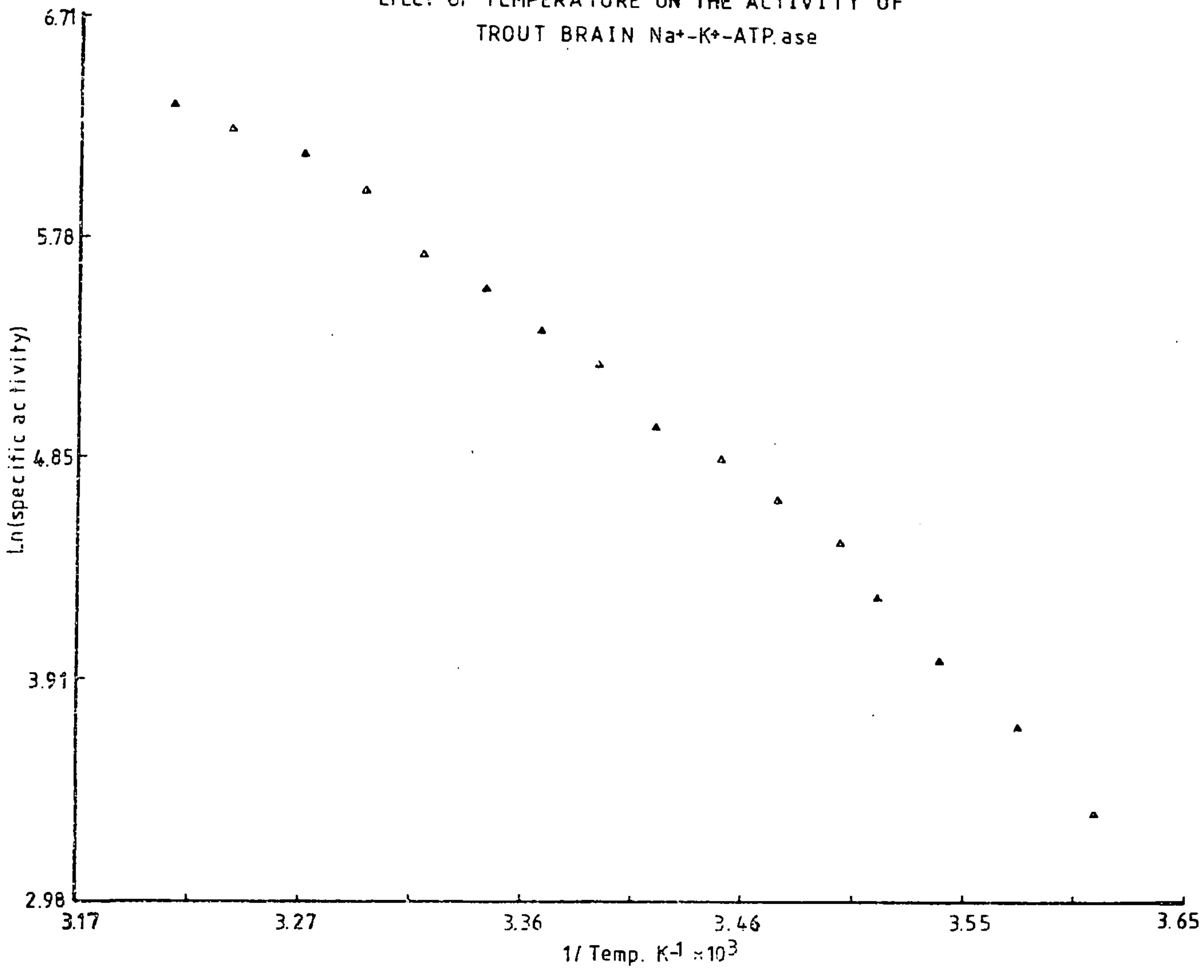


Fig. 4.3

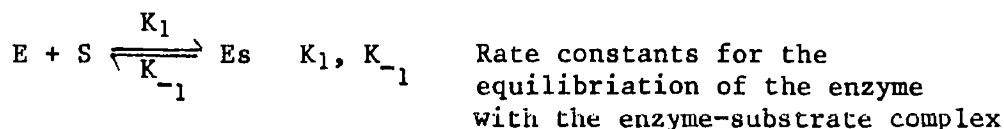
EFFECT OF TEMPERATURE ON THE ACTIVITY OF HAMSTER BRAIN Na^{+}-K^{+}-ATP.ase

Fig. 4.4

EFECT OF TEMPERATURE ON THE ACTIVITY OF TROUT BRAIN Na^{+}-K^{+}-ATP.ase



catalysed reactions (Gibson, 1953). This effect can be quantified for a simple Michaelis-Menten mechanism, within which the enzyme (E) enters into a rapid equilibrium with its substrate (S), to form an enzyme-substrate complex (Es), followed by a rate limiting transformation of this complex along the pathway resulting in product formation. The essential rate equations are:



K_r ... Rate constant for the formation of the products.

For such a system operating under steady state conditions, a Michaelis-Menten constant (K_m) can be defined thus:

$$K_m = \frac{K_{-1} + Kr}{K_1} = \frac{E \quad S}{Es}$$

If it is assumed that the sum of all the enzyme molecules in the system (Eo) exists either in the free state (E) or as the enzyme-substrate complex (Es),

i.e. $Eo = E + Es$

then the reaction rate equation (Rate = KrEs) can be described in terms of the total enzyme present in the system thus:

$$\text{Rate} = \frac{Kr \quad Eo}{1 + \frac{K_m}{S}}$$

Hence, an enzyme concentration independent parameter, specific activity

$(V = \frac{Rate}{Eo})$ can be defined by

$$V = \frac{Kr}{(1+\frac{K_m}{S})} \qquad (4a)$$

Since Kr is a rate constant, its temperature dependence can be defined by the Arrhenius equation, thus after substitution for Kr the equation (4a) becomes:

$$V = \frac{Ae^{-\frac{u}{RT}}}{(1+\frac{K_m}{S})} \qquad (4b)$$

Thus, equation (4b) can be expressed in the form accessible to an Arrhenius graph by taking logarithms of its constituent terms.

i.e. $$\log_e V = \log_e A - \frac{u}{RT} - \log_e (1 + \frac{Km}{S}) \qquad (4c)$$

From the equation (4c), it is obvious that a straight line would describe the resultant plot only if the ratio $\frac{Km}{S}$ does not vary with temperature. By definition the Michaelis-Menten constant (Km) is given by:

$$Km = \frac{K_{-1} + Kr}{K_1}$$

Since it is assumed (as part of the mechanism) that the rate constant Kr is much smaller than either K_1 or K_{-1}, then a reasonable approximation for Km is the dissociation constant of the enzyme-substrate complex. This being a thermodynamic parameter, can be defined in terms of a Gibbs free energy (ΔG)

i.e. $$\Delta G = -RT \log_e Km$$

Since the free energy can be expressed in terms of its enthalpy (ΔH) and entropy (ΔS) components, the constant Km can be given by the equation:

$$Km = e^{\frac{\Delta S}{R}} \times e^{-\frac{\Delta H}{RT}}$$

Thus the equation (4c) can be rewritten in terms of its full temperature dependence thus:

$$\log_e V = \log_e A - \frac{u}{RT} - \log_e \left(1 + \frac{e^{\frac{\Delta S}{R}} \; e^{-\frac{\Delta H}{RT}}}{S}\right) \quad (4d)$$

The equation (4d) predicts that, for experiments at constant substrate concentration, the Arrhenius plot would be non-linear and defined by a catenary curve. The slope of the curve at any temperature can be obtained by differentiating the equation (4d) with respect to the reciprocal of the absolute temperature. The expression obtained defines the slope of the line as:

$$\text{Slope} = -\frac{u}{R} + \frac{\Delta H}{R}\left(\frac{1}{1 + \frac{S}{Km}}\right)$$

Thus the apparent Arrhenius 'u' (U_{app}) at any temperature would be given by the equation:

$$U_{app} = U - \Delta H\left(\frac{1}{1 + \frac{S}{Km}}\right) \quad (4e)$$

The equation (4e) predicts that:

(a) The curvature of the Arrhenius plot obtained would depend on the magnitude of the enthalpy of formation of the enzyme substrate complex, and the ratio of substrate concentration with respect to the Michaelis-Menten constant at any given temperature.

and

(b) The curve should approach a straight line when the substrate

concentration is in large excess over the Km, in which case the apparent Arrhenius 'u' (U_{app}) approaches the activation energy (u) of the reaction. At the other extreme, when the substrate concentration is much smaller than Km, the curve should also approach linearity of apparent activation energy (U_{app}) would be given by

$$U_{app} = u - \Delta H$$

The above considerations are too simple for an unqualified application to the Na^+-K^+-ATPase. However, the fact that the substrate (or ligand) activation kinetics of this enzyme do at least approximate to saturation kinetics (Bakkeren & Bonting, 1968; Robinson, 1974a; Gache, Rossi & Lazdunski, 1977), suggests that the application of the above principles, though not strictly correct, should be sufficiently accurate to enable the prediction of at least the order of magnitude of the deviation from ideal Arrhenius behaviour, that is attributable to such considerations in the case of the Na^+-K^+-ATPase. The calculation of the magnitude of such effects requires an estimate of the enthalpy of formation of the enzyme-substrate complex as well as an estimate of the change in the substrate concentration to Km ratio for all the essential ligands over the temperature range investigated. The former is accessible via a study of the temperature dependence of the activation kinetics of the enzyme with respect to its essential ligands. This aspect of the characterisation of the Na^+-K^+-ATPase has not been extensively studied, but, of those ligands studied, the activation kinetics with respect to A.T.P. has been found to be the most temperature sensitive (Neufeld & Levy, 1970). These authors reported an apparent Km for A.T.P. at 43°C of 2×10^{-4}M, while at 1°C it was 5×10^{-6}M. From these figures, a value for the enthalpy of formation of the enzyme -ATP complex (63.23 $K.J.mol^{-1}$) was calculated using the van't Hoff isotherm. Since, of all the ligands, the binding of ATP was reported to be the most temperature sensitive, it follows

that the value calculated for ATP would be greater than that of any other ligand. Thus allowing for errors in the estimation and possible species variations, the value of 90 $K.J.mol^{-1}$ was taken as the maximum that could be expected for the enthalpy of binding of the Na^{+}-K^{+}-ATPase to any of its essential ligands.

The assumed maximum value for the heat of substrate binding, was used to calculate the maximum change in apparent Arrhenius 'u' between 37°C and 1°C, using literature values for the apparent Km for the various ligands, and the concentration of the ligands used for assay (see Chapter 2). Table 4.1 lists the results of the calculations. This shows that in spite of the overestimation involved in the assumption of 90 $K.J.mol^{-1}$ for the ΔH value, the maximum change in the apparent Arrhenius 'u' between 1°C and 37°C predictable by the above mechanism, is around 19 $K.J.mol^{-1}$. On comparing these values with those actually observed (Table 4.2), it becomes obvious that they are too small to account for the observed values, and that it is very unlikely that this mechanism describes the observed temperature kinetics of the Na^{+}-K^{+}-ATPase.

(ii) <u>The effects of phase changes</u>

Phase changes are considered to be of some relevance to the Na^{+}-K^{+}-ATPase, and other membrane bound enzymes, on account of the close association of the enzyme with the phospholipid constituent of the membrane, in which it sits. Sharp thermal transitions have been detected in model membranes made from pure phospholipids by a variety of physical techniques (Ladbroke & Chapman, 1969; Hubbell & McConnell, 1969; Veatch & Styrer, 1977), and these are believed to be

TABLE 4.1

Changes in apparent Arrhenius 'u' attributable to ligand concentration effects

Ligand	Assay Concentration (M)	●Apparent Km (M)		(S)/Apparent Km		Enthalpy term in apparent Arrhenius 'u'		Change in apparent 'u' between 37°C - 1°C
		37°C	▲1°C	37°C	1°C	37°C	1°C	
Mg^{++}	$3x10^{-3}$	Δ$8x10^{-4}$	$8.14x10^{-6}$	3.75	369	18.95 K.J.	0.24 K.J.	18.71 K.J.
A.T.P.	$3x10^{-3}$	†$2x10^{-4}$	$2.03x10^{-6}$	15	1478	5.63 K.J.	0.06 K.J.	5.57 K.J.
Na^{+}	$1.3x10^{-1}$	*$6x10^{-3}$	$6.1x10^{-5}$	21.67	2131	3.97 K.J.	0.04 K.J.	3.93 K.J.
K^{+}	$2.0x10^{-2}$	+$9x10^{-4}$	$9.16x10^{-6}$	22.22	2183	3.88 K.J.	0.04 K.J.	3.84 K.J.

Δ Robinson (1974a)

† Neufeld and Levey (1970)

* Bakkeren and Bonting (1968)

+ Robinson (1974b)

▲ Calculated for an Enthalpy of Substrate Binding of 90 $K.J.Mol^{-1}$.

● Values quoted for saturating concentrations of all other ligands

TABLE 4.2

Variations in apparent Arrhenius 'u' with temperature

Tissue Source	$^{\Delta}$Apparent Arrhenius'u' (K.J.Mol^{-1})		ΔU_{app} K.J.Mol^{-1}
	$30^{o}C$ - $37^{o}C$	$4^{o}C$ - $11^{o}C$	
Rat brain	61.87±4.99	208.54±12.49	≃157
Mouse brain	67.51±2.43	213.26±15.54	≃146
Hamster brain	62.68±4.90	197.41±12.97	≃135
Rainbow trout brain	31.65±3.41	74.49±2.95	≃43

Δ

Mean of 4 preparations ± 1 standard deviation

the result of a phase change in the lipid bilayers. Given that thermal events, attributed to lipid phase changes, have been detected in biomembranes (Rienert & Steim, 1970; Chapman & Urbina, 1971; Eletr & Inesi, 1972), and that non-ideal Arrhenius behaviour is more frequently observed in membrane bound enzymes, as compared with soluble enzymes, some workers have suggested a possible role for lipid phase changes in the temperature kinetics of the Na^{+}-K^{+}-ATPase (Charnock, Cook & Opit, 1971; Barnett & Parlazzotto, 1974; Boldyrev, Ruuge, Smirnova & Tabak, 1977), and other membrane bound enzymes (Kumamoto, Raison & Lyons, 1971; Wynn-Williams, 1976).

The thermodynamic principle underlying the phase change effect is that the chemical potential of a pure system is independent of the amount of that system under consideration. Thus its chemical potential can be defined by the equation:

$$U = U_o - RT\log_e \Theta$$

U.... chemical potential of the system

U_o .. standard chemical potential of the system

R ... gas constant

T ... absolute temperature

Θ ... constant independent of the magnitude of the system but unique to it.

Consequently, an equilibrium between two such systems is defined by the equality of their chemical potentials. Since a phase change is effectively the conversion of one pure system to another, and given that the equations defining the chemical potentials of such states contain only one system independent variable, temperature, it follows that an equilibrium would exist between the two states on either side of a phase change, only at some critical temperature at which their chemical potentials are equal. Consequently, at all temperatures except this

critical temperature, there would be a complete separation into macroscopic domains, with the state of lower energy existing to the exclusion of the other. Furthermore, the factors governing the change from one phase to another, are external to any one phase, and as a result, abrupt changes in the temperature dependent properties of the system would take place at the phase transition temperature, and neither the change in properties, nor the temperature at which the change takes place would be governed by the law (or laws) describing the temperature dependence of the properties under observation.

The interpretation of biological data within the framework provided by phase-transition theory, must be tempered by the knowledge that biological systems are far removed from the pure systems to which the above discussion applies. In the case of membrane bound enzymes in general, and the Na^{+}-K^{+}-ATPase in particular, where the proposed phase transitions are believed to be lipid dependent, the fact that the biomembrane is a very complex mixture of lipids and proteins must be considered. The effect of lipid heterogeneity, and the presence of protein in biomembranes on the thermal properties of such membranes have been studied (Pagano, Cherry & Chapman, 1973; De-Kruyff, Demel, Slotboom, Van Deenen & Rosenthal, 1973; Fienstein, Fernandez & Sha'Afi, 1975), and the results suggested that the sharp thermal transitions, characteristic of pure lipid bilayers, are unlikely in real biomembranes where much broader transitions are expected.

In spite of such considerations, the phase change effect has been suggested as the dominant factor contributing to the non-ideal Arrhenius temperature kinetics observed in many enzyme catalysed reactions (Kumamoto _et al_ 1971), and has since been used as a framework for the

interpretation of the temperature kinetics of the Na^+-K^+-ATPase (Charnock, Cook & Opit, 1971; Barnett & Parlazzotto, 1974). Within this description, the enzyme is assumed to exist in a system within which the phase change takes place. In each phase, the temperature dependence of its catalytic activity is defined by the Arrhenius equation, and at some critical temperature (when the two phases are in equilibrium), there is an abrupt change in the defining Arrhenius parameters. As described, the data would describe the two Arrhenius straight lines with a marked-discontinuity at the so called critical temperature, at which the lines may or may not intersect. In most cases where this mechanism has been implemented, the data have been interpreted as two Arrhenius straight lines intersecting at the 'critical' temperature have also been reported (Lyons & Raison, 1970a, b; Grinna, 1975).

Since the system under study, the Na^+-K^+-ATPase, is not ideal (as defined by the phase change effect), the data obtained from this system should reflect the heterogeneity of the biomembrane lipids and the presence of protein in its structure, by a perturbation of the ideal description near the phase transition temperature. Thus the best that could be expected of the Na^+-K^+-ATPase, is a tendency for the data to fit an Arrhenius relationship at temperatures remote from the transition temperature, with some curvature of the lines as the experimental temperature approaches the phase transition temperature. One added complication is that the effective temperature range within which it is feasible to measure the catalytic activity of the Na^+-K^+-ATPase is limited. This is because the enzyme activity quickly

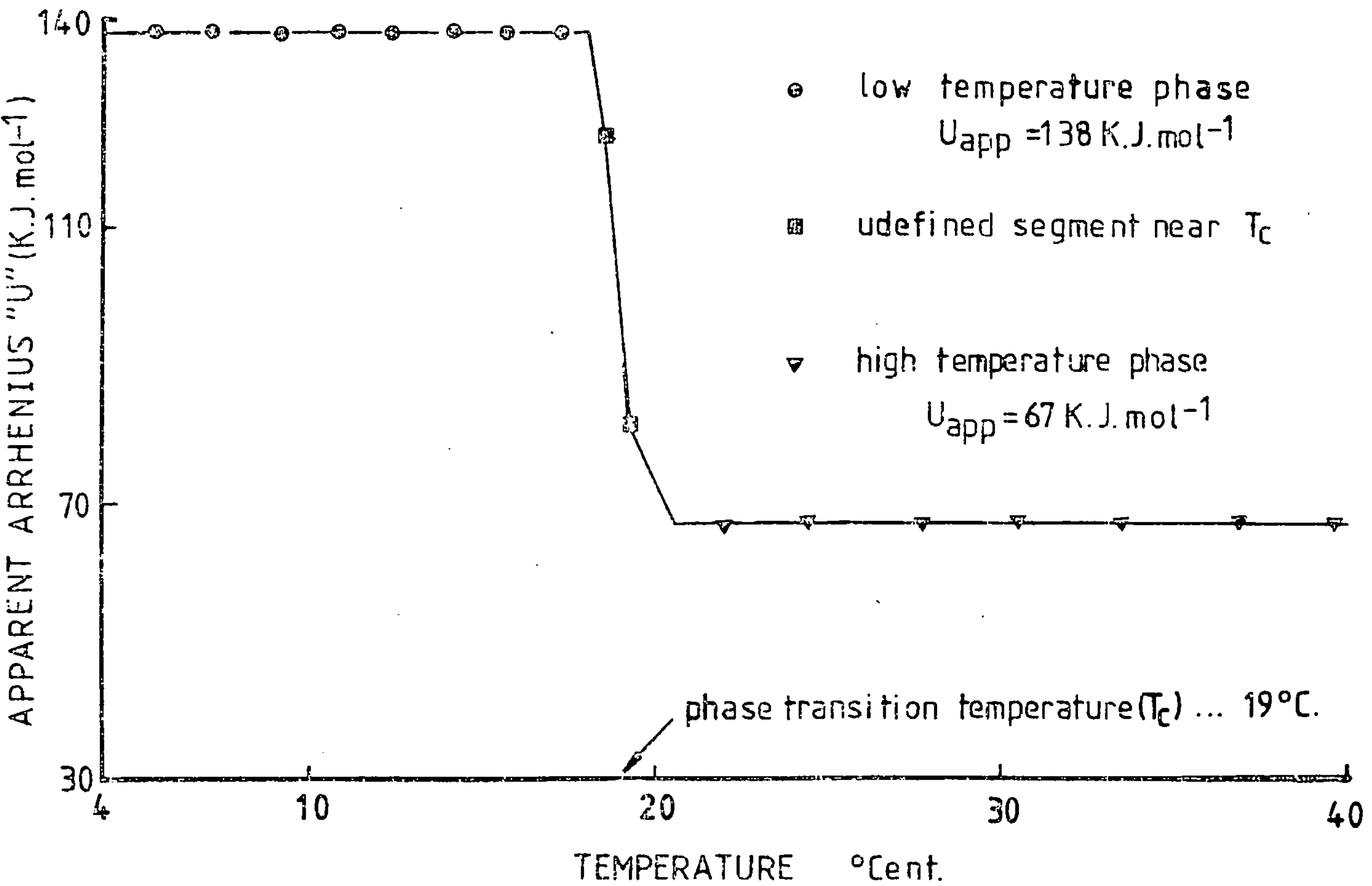
approaches zero at temperatures near $4^{o}C$ (Charnock, Cook & Casey, 1971), and thermal denaturation becomes significant at temperatures above $41^{o}C$ (see Chapter 7). These limitations, along with the inevitable errors in measurement, must restrict the degree of compliance of the study system with the behaviour predicted by the phase change effect. In an effort to rationalise these problems, a computer assisted procedure based on a published resolution method (Bogartz, 1968), has been used (Charnock, Cook & Opit, 1971). By this method, the authors were able to resolve the data into two Arrhenius straight line segments, and to obtain the defining parameters which were then compared by a students 't' test. The objectivity provided by this procedure gives a useful aid to the interpretation of such data. However, it is based on the assumption that the temperature kinetics of the Na^{+}-K^{+}-ATPase are defined by the phase change effect, and thus does not evaluate the applicability of this process to the enzyme under study.

If the-non ideal Arrhenius behaviour is best described by the phase change effect, then a graph of apparent Arrhenius 'u' against temperature should give a plot of the form shown in figure 4.5. This shows two linear segments parallel to the temperature axis, separated by an undefined section in the region of the transition temperature, where the apparent Arrhenius changes rapidly with temperature. The data obtained from the rat brain and trout brain preparations have been subjected to this test. Calculation of apparent Arrhenius 'u' values (U_{app}) for each data point shows that when these separate values for U_{app} are plotted against temperature, a curved line provides the best fit (see figure 4.6). At 'high' temperatures, the observed

Fig. 4.5

BEHAVIOUR PREDICTED BY PHASE CHANGE EFFECTS

simulated data .... two linear segments intersecting at T_c = 19°cent.



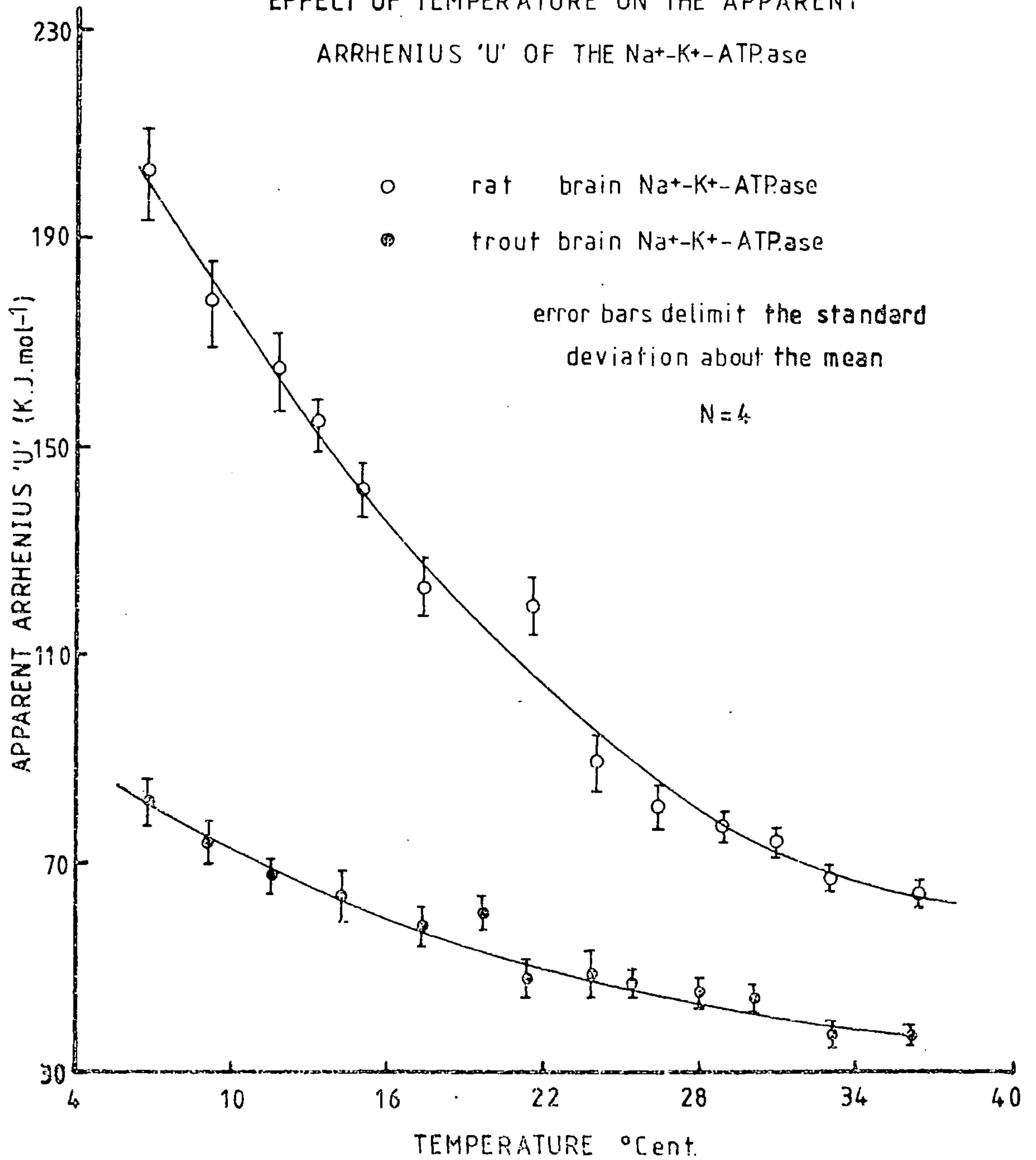


Fig.4.6
EFFECT OF TEMPERATURE ON THE APPARENT
ARRHENIUS 'U' OF THE Na+-K+-ATPase
rat brain Na+-K+-ATPase
trout brain Na+-K+-ATPase
error bars delimit the standard
deviation about the mean
N=4
APPARENT ARRHENIUS 'U' (K.J.mol-1)
230
190
150
110
70
30
4
10
16
22
28
34
40
TEMPERATURE °Cent.

U_{app} tends towards the form predicted, but at the lower temperatures there is a marked increase in the observed U_{app} as the temperature is lowered. These observations can be interpreted by assuming that either

(a) The phase change effect does not describe the temperature kinetics of the Na^{+}-K^{+}-ATPase, and that an alternative description should be sought.

or

(b) The perturbations caused by the heterogeneity of the biomembrane lipids, and the variety of proteins bound to them produce a transition that is broad enough to cover the entire temperature range accessible for measurements.

If the latter assumption is true, then it would be impossible to define the Arrhenius parameters of any phase, since by the principle of separation into macroscopic domains, such parameters would be accessible only when the phase change is complete. Thus, while these observations do not rule out the phase- change effect as a plausible mechanism for defining the temperature kinetics of the Na^{+}-K^{+}-ATPase, they suggest that the procedure of resolving the data from this enzyme into two Arrhenius straight lines, cannot be expected to return meaningful parameters.

(iii) The effect of thermal denaturation

Since the measurement of the catalytic activity of an enzyme assumes that the molecule is stable under the assay conditions, the possibility that the observed non-ideal Arrhenius temperature kinetics of the Na^{+}-K^{+}-ATPase may in part be the result of denaturation under the assay conditions, must be considered. That this may play a role depends

on the fact that the conformational states necessary for the biological activity of proteins represent a kinetic stability as opposed to a thermodynamic stability, and that those states are high energy states, as evidenced by the ease with which active proteins are denatured by changes in temperature, pH, ionic strength etc. Thus, a decay of the enzyme from its biologically active state is to be expected, and the measured activity would in consequence be affected by the rate at which the decay proceeds under the assay conditions.

The rate of decay of biological activity, under any given conditions, is usually described by a pseudo first order decay process (Joly, 1965). Thus at any time (t), the number of biologically active molecules (Et) would be given by the equation:

$$Et = E_o e^{-K_d t}$$

E_o.. Initial number of active molecules.

K_d.. pseudo first order decay constant

If it is assumed, that the assayed enzyme activity is proportional to the number of biologically active molecules present, then at any time the activity (W_t) would be given by

$$W_t = K_r E_o e^{-K_d t}$$

K_r.. Catalytic rate constant

Thus an enzyme concentration independent specific activity ($V_t = W_t/E_o$) can be defined by the equation

$$V_t = K_r e^{-K_d t} \qquad (4f)$$

Since the parameters K_r and K_d are both rate constants, they are both expandable by the Arrhenius equation from which their temperature dependence can be expressed.

$$\text{i.e.}\qquad K_r = A_r e^{-\frac{U_r}{RT}}$$

$$K_d = A_d e^{-\frac{U_d}{RT}}$$

Hence the equation (4f) can be expanded so as to show the temperature dependence of V_t, and then expressed in the form accessible to an Arrhenius plot

$$\text{i.e.}\ \log_e V_t = \log_e A_r - \frac{U_r}{RT} - tA_d e^{-\frac{U_d}{RT}} \qquad (4g)$$

The equation (4g) describes the observed specific activity as being time dependent, and predicts a non-linear Arrhenius plot for such a system. The Arrhenius plot would describe a catenary curve, the curvature of which is dependent on time, temperature and the magnitude of the energy term (U_d) defining the kinetics of the denaturation process. The slope of the Arrhenius plot is defined by the differential of the equation (4g) with respect to the reciprocal of the absolute temperature.

$$\text{i.e. Slope} = -\frac{U_r}{R} + \frac{U_d}{R} \cdot K_d t$$

Since K_d is a pseudo first order rate constant, it can be expressed in terms of a half life (ϕ).

i.e. Slope $= -\frac{U_r}{R} + \frac{U_d}{R} \quad \frac{t}{\phi} \log_e 2 \qquad \phi = \frac{\log_e 2}{K_d}$

The apparent Arrhenius 'u' (U_{app}) can thus be given by the expression

$$U_{app} = U_r - U_d \ \frac{t}{\phi} \log_e 2 \qquad (4h)$$

The apparent Arrhenius 'u' as described by the equation (4h) is dependent on time and temperature, but it should be essentially independent of these if the assay time (t) is small relative to the half life (ϕ) of the denaturation process. Given that a finite time is required for the assay of the enzyme activity, the independence of U_{app} from time and temperature would most likely be observed at the low temperatures where the denaturation rate constants should be small. At the higher temperatures, the Arrhenius plot should deviate from linearity, by an extent which, for any finite time (t) depends on the magnitude of the activation energy of denaturation.

An assessment of the relevance of these considerations to the Na^+-K^+-ATPase requires an evaluation of the kinetic stability of this enzyme under the conditions of assaying its catalytic activity. Such is accessible from a study of the kinetics of thermal denaturation of the enzyme, from which estimates of its half life, the temperature coefficient of its half life, and the activation energy of denaturation can be obtained. A study of the kinetics of thermal denaturation of this enzyme (see Chapter 7) revealed that:

(a) The kinetics of thermal denaturation of the Na^+-K^+-ATPase could not be described by a single first order decay process, and consequently estimates of its half life, and the temperature dependence of its half life were difficult to obtain.

(b) The temperature range accessible for accurate measurements of the rate of denaturation was small (approximately $6^{o}C$). This suggested that the activation energy of denaturation (U_d) was very large.

These observations severely restricted the experimental methods of quantifying the effects attributable to thermal denaturation processes. The best that was practical was to evaluate whether the rate of thermal denaturation at the higher temperatures used (approx. $37^{o}C$ - where the predicted variations should be greatest), was sufficient to account for the observations. This was pursued along two lines of experimentation.

First, a determination of the time dependence of the catalytic activity of the enzyme was made at $37^{o}C$. This involved a monitoring of the ouabain inhibitable enzymic hydrolysis of ATP as a function of time. The results (fig.4 .7, typical data set) described curvilinear plots. Initially the release of inorganic phosphate was linear in the range 0-800 n.moles, but beyond that the rate of inorganic phosphate release progressively declined. In further experiments, the concentration of the enzyme preparation assayed was varied. The results of these (fig.4 .8, typical data set) indicated that the deviation from linearity was independent of time since it was only apparent after 800 n.moles of inorganic phosphate were released, irrespective of the time taken for this to occur. These results were not consistent with there being significant thermal denaturation. More likely, they resulted from substrate depletion or product inhibition. Since the amount of substrate hydrolysed at the

Fig. 4.7

P_i release by rat brain Na^+-K^+-ATPase vs. time

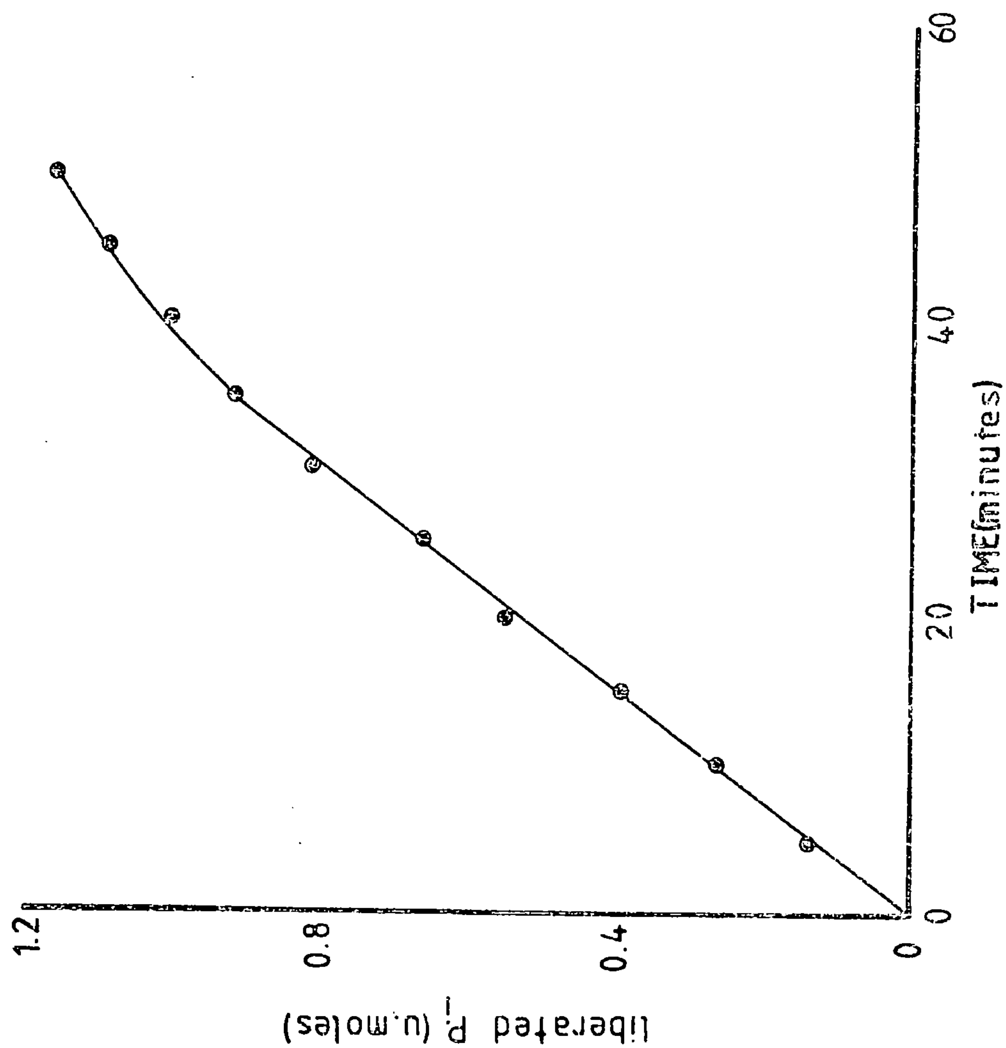
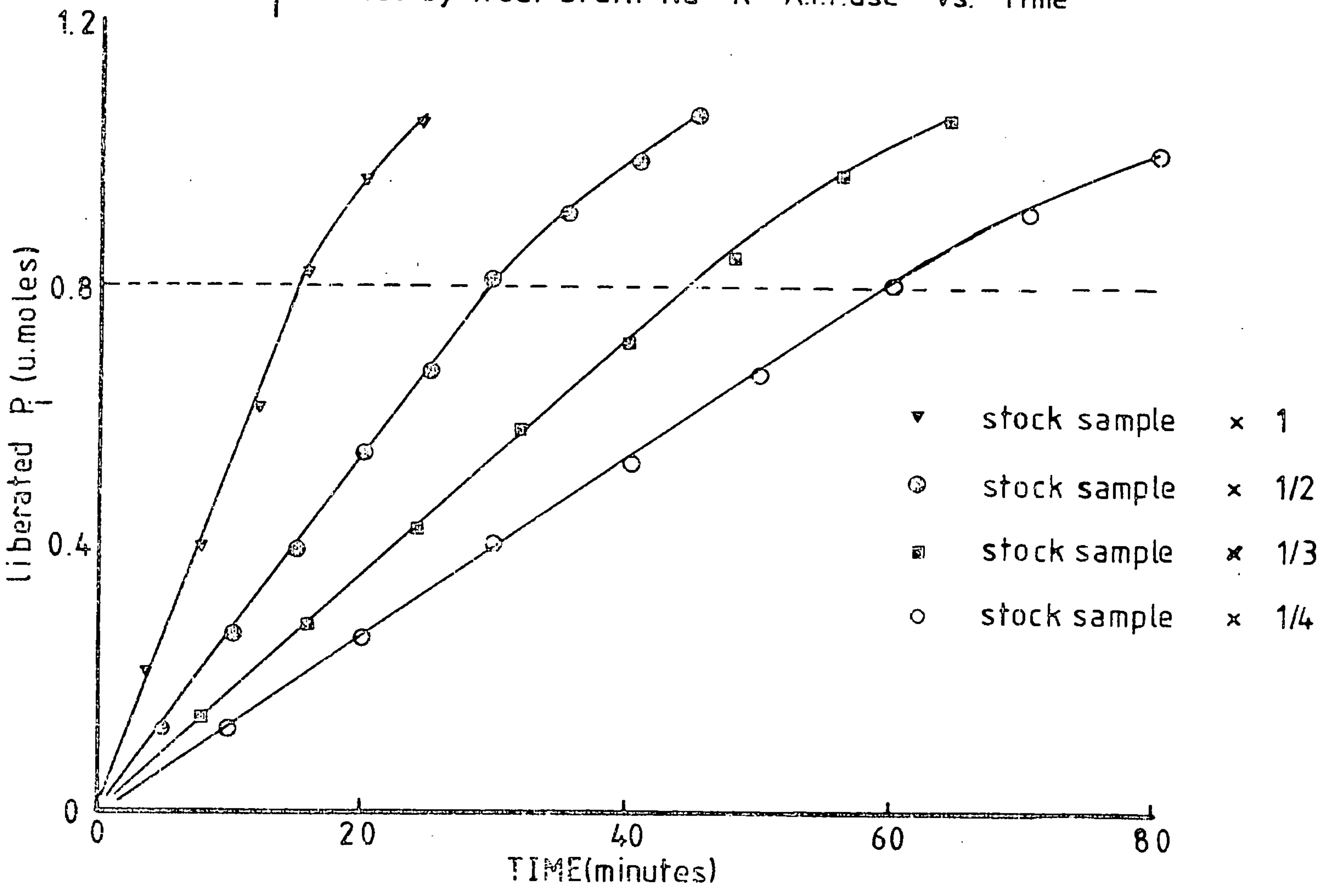


Fig. 4.8

P_i release by trout brain Na^+-K^+-A.T.Pase vs. time



stage the non-linearity became apparent (i.e. 800 n.moles) was only a small fraction of the total amount added (6600 n.moles), product inhibition seemd to be the more likely cause. Such is consistent with the reports of inorganic phosphate being an inhibitor of the Na^+-K^+-ATPase (Baskin & Leslie, 1968; Robinson, Hashner & Martin, 1978).

In the second set of experiments, the thermal stability of the enzyme in the absence of any catalysis was investigated. One approach involved an incubation of the enzyme in a low ionic strength buffer at pH 7.2, over a temperature range extending beyond the upper temperature limits used for the assay of its catalytic activity. This formed part of a broader based study of the thermal stability of Na^+-K^+-ATPase preparations from several sources (see Chapter 7). The results indicated that, for incubation periods of fifteen minutes, there was no significant loss of activity at temperatures below 39°C (fig. 7.1), and were consistent with the previously reported stability of Na^+-K^+-ATPase preparations at 37°C (Baskin & Leslie, 1968). In the other approach, the thermal stability of the enzyme under the conditions of high ionic strength needed for the assay of its activity was investigated. Thus the activity of the enzyme was determined by the standard assay procedure and by the preincubation assay procedure described in Chapter 3. The latter procedure involved the preincubation of the enzyme in a potassium free assay medium for fifteen minutes at 37°C, before starting the reaction by the addition of potassium ion. The results (Table 4.3) showed no significant difference in the measured activities, the observations being independent of the assay time as well as the preincubation period at 37°C.

TABLE 4.3

Effect of assay procedure on the observed specific activity of the Na^+-K^+-ATPase (μ-moles Pi.mg/protein^{-1}hr^{-1})

Source	[A] Na^+-K^+-ATPase activity at 37°C	
	Assay Method I	Assay Method II
Rat Brain	85.7 ± 9.3	83.3 ± 10.2
Trout brain	187.6 ± 15.9	189.2 ± 18.1
Hamster brain	109.3 ± 11.0	105.8 ± 14.2
Mouse brain	98.4 ± 7.3	98.1 ± 8.5

[A] Results mean of 4 preparations ± 1 standard deviation

Assay Method I - Standard Assay Procedure

Assay Method II - Preincubation Assay Procedure

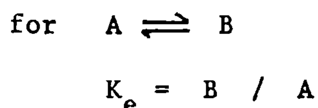
The results of these testing experiments supported the view that the temperature range within which the temperature kinetics of the Na^{+}-K^{+}-ATPase was studied was too low for there to be significant thermal denaturation of the enzyme. Consequently, thermal denaturation was not considered to be a significant contributory factor to the observed non-ideal Arrhenius temperature kinetics of the Na^{+}-K^{+}-ATPase.

(iv) The effect of thermodynamic interconversions between different stable states of the enzyme.

The idea that the non-ideal Arrhenius behaviour observed in many biological systems, may be the result of thermodynamic interconversions between different stable states, was first considered (Kistiakowsky & Lumry, 1949), after these authors demonstrated that the mechanism describing a change from one rate limiting reaction to another (Crozier, 1924), required differences in activation energy too large to be biologically feasible. Previously, there had been suggestions that a thermodynamic equilibrium between active and inactive forms of certain enzymes may exist in-vivo (Johnson, Eyring & Williams, 1942). This idea was later shown to be a plausible mechanism for the interpretation of the observed non-ideal Arrhenius temperature kinetics of many enzyme catalysed reactions (Kavanau, 1950). The mechanism has been used to describe the behaviour of alkaline phosphatase and peroxidase (Maier, Tappel & Volman, 1955), and has been supported by a demonstration of a thermodynamic equilibrium between different states of D-amino acid oxidase, within the temperature range normally accessible for kinetic measurements (Massey, Curti & Ganther, 1966).

This mechanism assumes that an equilibrium exists between a

high temperature stable state (A), and a low temperature stable state (B). This equilibrium is definable by an equilibrium constant (K_e) such that



$$\text{for} \quad A \rightleftharpoons B$$

$$K_e = B \;/\; A$$

The non-ideal Arrhenius behaviour would then be described in terms of the effect of temperature on the magnitude of the equilibrium constant (K_e). Once these assumptions are made, two variations must be considered:

(1) The observed catalytic activity is the sum of significant contributions from both stable states.

(2) Only one stable state contributes significantly to the observed activity.

<u>An equilibrium between two active species</u>

This mechanism describes the observed rate (V_i) as the sum of the activities of the species A and B.

$$\text{i.e. } V_i = K_a \; A \; + \; K_b \; B \qquad (4i)$$

K_a .. Catalytic rate constant of species A.

K_b .. Catalytic rate constant of species B.

The total amount of enzyme present (Eo) is given by the conservation equation

$$Eo = A + B \qquad (4j)$$

This conservation (4j) and the rate equation (4i) can be rewritten in terms of one of the stable species (e.g. species A) and the equilibrium

constant (K_e)

i.e. $E_o = A(1 + K_e)$ (4k)

$V_i = (K_a + K_bK_e)A$ (4l)

From these equations (4k) and (4l), an enzyme concentration independent specific activity (i.e. specific activity = V = V_i E_o) can be defined thus

$$V = \frac{K_a + K_bK_e}{1 + K_e} \quad (4m)$$

The parameters K_a and K_b are rate constants definable by the Arrhenius equation, while K_e an equilibrium constant is definable in terms of an enthalpy change (ΔH) and an entropy change (ΔS)

i.e. $K_a = A_a e^{-\frac{U_a}{RT}}$

$K_b = A_b e^{-\frac{U_b}{RT}}$

$K_e = e^{\frac{\Delta S}{R}} \cdot e^{-\frac{\Delta H}{RT}}$

Thus the rate equation describing the temperature dependence of V is given by

$$V = \frac{A_a e^{-\frac{U_a}{RT}} + A_b e^{-\frac{U_b}{RT}} \; e^{\frac{\Delta S}{R}} \; e^{-\frac{\Delta H}{RT}}}{1 + e^{\frac{\Delta S}{R}} \; e^{-\frac{\Delta H}{RT}}}$$

The slope of the Arrhenius plot ($\frac{d}{d(1/T)}$ $\log_e V$) is thus given by the equation

$$\text{Slope} = -\frac{U_a}{R}\left(\frac{K_a}{K_a+K_bK_e}\right) - \frac{U_b + \Delta H}{R}\left(\frac{K_bK_e}{K_a+K_bK_e}\right) + \frac{\Delta H}{R}\left(\frac{Ke}{1 + Ke}\right) \quad (4o)$$

The apparent Arrhenius 'u' can thus be given by

$$U_{app} = U_a\left(\frac{K_a}{K_a+K_bK_e}\right) + (U_b + \Delta H)\left(\frac{K_bK_e}{1 + K_bK_e}\right) - \Delta H\left(\frac{Ke}{1 + Ke}\right) \quad (4p)$$

The complexity of the equation (4p) describing the apparent Arrhenius 'u' makes a prediction of its behaviour over the entire temperature range difficult. However, its behaviour can be predicted for the limiting conditions of high and low temperature. By definition, the equilibrium constant (Ke) is defined by

$$Ke = \frac{\text{Concentration of low temperature stable species B}}{\text{Concentration of high temperature stable species A}}$$

i.e. Ke decreases with increasing temperatures.

Thus, at 'low' temperatures (Ke large, $\frac{K_a}{(K_a+K_bK_e)} \approx 0$, $\frac{K_bK_e}{K_a+K_bK_e} \approx \frac{Ke}{(1 + Ke)} \approx 1$),

the apparent Arrhenius 'u' approaches that of the low temperature stable state,

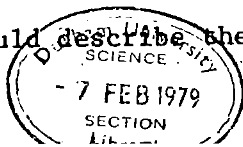
i.e. $U_{app} \approx U_b$ (low temperatures)

Similarly, at 'high' temperatures (Ke small, $\frac{K_a}{K_a+K_bK_e} \approx 1$, $\frac{K_bK_e}{K_a+K_bK_e} \approx \frac{Ke}{1 + Ke} \approx 0$),

the apparent Arrhenius 'u' approaches that of the high temperature stable state

i.e. $U_{app} \approx U_a$

Consequently, the observed Arrhenius plot should describe linear segments of slopes $-\frac{U_a}{R}$ and $\frac{U_b}{R}$ at the high and low temperature limits respectively, and a smooth curve should describe the transition between the linear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segments. As described, the defining parameters of limiting linear segments, are expected to be independent of each other, and the enthalpy term (ΔH) which describes the width of the transition between the linear segments. As a result, values for U_a and U_b would be accessible only by making measurements in the relevant temperature ranges.

The equation (4p) predicts that, for any enzyme system described by this mechanism, a graph of apparent Arrhenius 'u' plotted against temperature, should be described by horizontal segments at the extremities of temperature, with a smooth curve joining these segments. As was previously demonstrated, figure 4 .6) the Na^+-K^+-ATPase preparations studied, showed a tendency towards the predicted behaviour at high temperatures, while at low temperatures there was a steady increase in the apparent Arrhenius 'up' as the temperature is lowered. This can be accommodated within the model if it is assumed that the conversion to the low temperature stable state, is not completed within the temperature range of the experiments. Such an assumption is incompatible with the reported failure to demonstrate significant catalytic activity by mammalian Na^+-K^+-ATPase preparations at temperatures near 4^oC (Charnock, Cook & Casey, 1971; Gruener & Avi-Dor, 1966). Furthermore, this model requires that the low temperature state of the Na^+-K^+-ATPase be described by an apparent activation energy of at least 100 $K.J.Mol^{-1}$ (see fig. 4 .6), values which outside the range expected of biological processes (30-85 K.J. mol^{-1}). Thus the experimental observations while not ruling out the plausibility of this mechanism, with respect to the Na^+-K^+-ATPase, suggest it to be unlikely, and that if the behaviour of this enzyme is defined by this model, meaningful parameters are inaccessible.

An equilibrium between an active and an inactive species

A description of the temperature kinetics of the Na^+-K^+-ATPase within this model requires the consideration of two possibilities:

(a) The low temperature state is catalytically active.

(b) The high temperature state is catalytically active.

The former describes a system of reversible thermal inactivation at the higher temperatures, in which case the observed rate is proportional to the concentration of the low temperature stable species (B).

$$\text{i.e. Rate} = K_b \quad B \tag{4q}$$

The equation (4q) can be rewritten in terms of an enzyme concentration independent specific activity (V), and the previously defined equilibrium constant (Ke)

$$\text{i.e. } V = \frac{K_b K_e}{(1 + Ke)} \tag{4r}$$

After redefining equation (4r) in terms of the temperature dependence of K_b and Ke, it can be shown that the slope of the Arrhenius plot would be described by the equation

$$\text{Slope} = -\frac{U_b}{R} - \frac{\Delta H}{R} \quad (1 - \frac{K_e}{(1+K_e)}) \tag{4s}$$

The equation (4t) describes a non linear Arrhenius plot of apparent Arrhenius 'u' (U_{app}) given by the equation.

$$U_{app} = U_b + \Delta H \left(\frac{1}{1+Ke}\right) \tag{4t}$$

Thus at low temperatures (Ke large, $\frac{1}{1 + Ke} \simeq 0$), the apparent Arrhenius 'u' should approach the activation energy of the low temperature stable state, while at high temperatures (Ke small, $\frac{1}{1 + Ke} \simeq 1$), the specific

activity should approach zero (see equation (4r)) and the apparent Arrhenius 'u' (U_{app}) should approach the limiting value of (U_b + ΔH). Since, by the definition of the equilibrium constant, the ΔH must be negative, the apparent Arrhenius 'u' is thus expected to decrease with increasing temperature.

The behaviour predicted by this model does not match the observations made on the Na^+-K^+-ATPase preparations studied in several important respects. First, the observed activity decays to zero at low temperatures, an observation which can be accommodated within the model only by the assumption that the catalytically active species is described by an unacceptably large apparent activation energy (>100 K.J.Mol^{-1}). Secondly, the apparent Arrhenius 'u' should be independent of temperature at low temperatures. As previously shown in figure 4 .6, this is not observed. Finally, the predicted time independent decay of activity to zero at high temperatures is not observed in practice. Whilst there is a decay of activity at high temperatures, such is time dependent in a manner characteristic of irreversible thermal denaturation, and as previously shown (fig. 7.1), this occurs at temperatures outside the temperature range of the kinetic measurements. Since the behaviour predicted by this model was found to be at variance with the experimental observations, it was not considered plausible for the description of the temperature kinetics of the Na^+-K^+-ATPase.

The alternative possibility, describes an equilibrium between an active high temperature state and an inactive low temperature stable state. Thus the observed rate, is proportional to the concentration of the high temperature stable species (A).

$$\text{i.e. Rate} = K_a \ A \qquad (4u)$$

On rewriting the equation (4u) in terms of the previously defined equilibrium constant (Ke), the enzyme concentration independent

specific activity (V) will be given by the equation

$$V = \frac{K_a}{(1 + Ke)} \qquad (4v)$$

After expanding K_a and Ke in terms of their temperature dependence, it can be shown that the slope of the Arrhenius plot would be given by the equation

$$\text{Slope} = -\frac{U_a}{R} + \frac{\Delta H}{R}\left(\frac{Ke}{1 + Ke}\right) \qquad (4w)$$

Thus at low temperatures (Ke large, $\frac{1}{1 + Ke} \simeq 0$, $\frac{Ke}{1 + Ke} \simeq 1$), the specific activity should approach zero (see equation (4v)), and the slope of the Arrhenius plot should approach the limiting value of $-\left(\frac{U_a - \Delta H}{R}\right)$ (equation (4w)), with an apparent Arrhenius 'u' of $(U_a - \Delta H)$. At 'high' temperatures (Ke small, $\frac{1}{1 + Ke} \simeq 1$, $\frac{Ke}{1 + Ke} \simeq 0$), the apparent Arrhenius 'u' is expected to approach the value U_a (equation (4w)). Since, by the definition of Ke, the ΔH must be negative, the apparent Arrhenius 'u' should increase from a limiting high temperature value (U_a) to a limiting low temperature value (U_a + abs (ΔH), as the temperature is decreased.

On comparing the behaviour predicted by this description with the experimental observations on the Na^+-K^+-ATPase preparations, considerable areas of agreement are found. The apparent Arrhenius 'u' when plotted as a function of temperature (see figure 4.6), increases from a high temperature asymptotic value as the temperature decreases. However, the approach to a low temperature asymptotic value was not observed. This apparent discrepancy can be rationalised by noting that the low temperature limiting conditions are expected to occur as the specific activity approaches zero. Thus experimental

verification requires the measurement of activity levels that may be below the sensitivity limits of the assay procedure. The predicted decay of activity to zero at low temperatures (equation (4v)) is consistent with the observations of this study as well as the reported failure to demonstrate significant catalytic activity at temperatures near $4^{o}C$ (Charnock, Cook & Casey, 1971). The extent of agreement between the predicted behaviour and the experimental observations suggested this mechanism as a plausible framework for the description of the temperature kinetics of the Na^{+}-K^{+}-ATPase.

CONCLUSIONS

Of the mechanisms discussed above, there are three which predicted effects that were approached by the observations on the Na^{+}-K^{+}-ATPase.

(a) A phase change affecting the catalytic properties of the enzyme.

(b) A thermodynamic equilibrium between catalytically active high and low temperature stable states of the enzyme.

(c) A thermodynamic equilibrium between a catalytically active high temperature stable state and an inactive low temperature stable state.

The description provided by the mechanisms (a) and (b) above, show the lesser agreement with the experimental observations, in that they predict Arrhenius plots with linear segments at the temperature extremes which are not observed. Furthermore, they do not predict the observed tendency towards zero activity at low temperatures, without the assumption that the apparent activation energy describing the low temperature forms of the enzyme are considerably greater than that normally expected of biological systems. Some of this disagreement may be

rationalised by assuming that the temperature range over which the phase change or equilibrium conversion takes place is too broad to be fitted into the range accessible to kinetic measurements on this enzyme. Such would imply that the accessible measurements would only describe the undefined interphase between the high and low temperature forms and that meaningful defining parameters would be inaccessible.

In contrast, the description provided by the mechanism (c) defines the behaviour of the Na^{+}-K^{+}-ATPase in a manner that agrees with most of the experimental observations. The model predicted the observed low temperature decay to zero activity, as well as the tendency towards a linear Arrhenius plot at the higher temperature. It also predicted a similar tendency at the lower temperatures, but experimental verification was precluded by the necessity of having to make measurements at activity levels near to or possibly beyond the resolution limits of the procedures used for assay of the Na^{+}-K^{+}-ATPase. In spite of this, the defining parameters should be accessible provided that the experimental measurements define the approach to the limiting conditions and the phase between them. Since the data suggested that the experimental observations covered that range, the mechanism (c) was adopted as the framework for the interpretation of the temperature kinetics of the Na^{+}-K^{+}-ATPase.

The rate equation taken to describe the temperature kinetics of the Na^{+}-K^{+}-ATPase is given by

$$V = \frac{A\, e^{-\frac{U}{RT}}}{1 + e^{\frac{\Delta S}{R}}\, e^{-\frac{\Delta H}{RT}}}$$

V... observed specific activity

A... constant as defined by Arrhenius 'A'

U ... apparent activation energy of the active species.

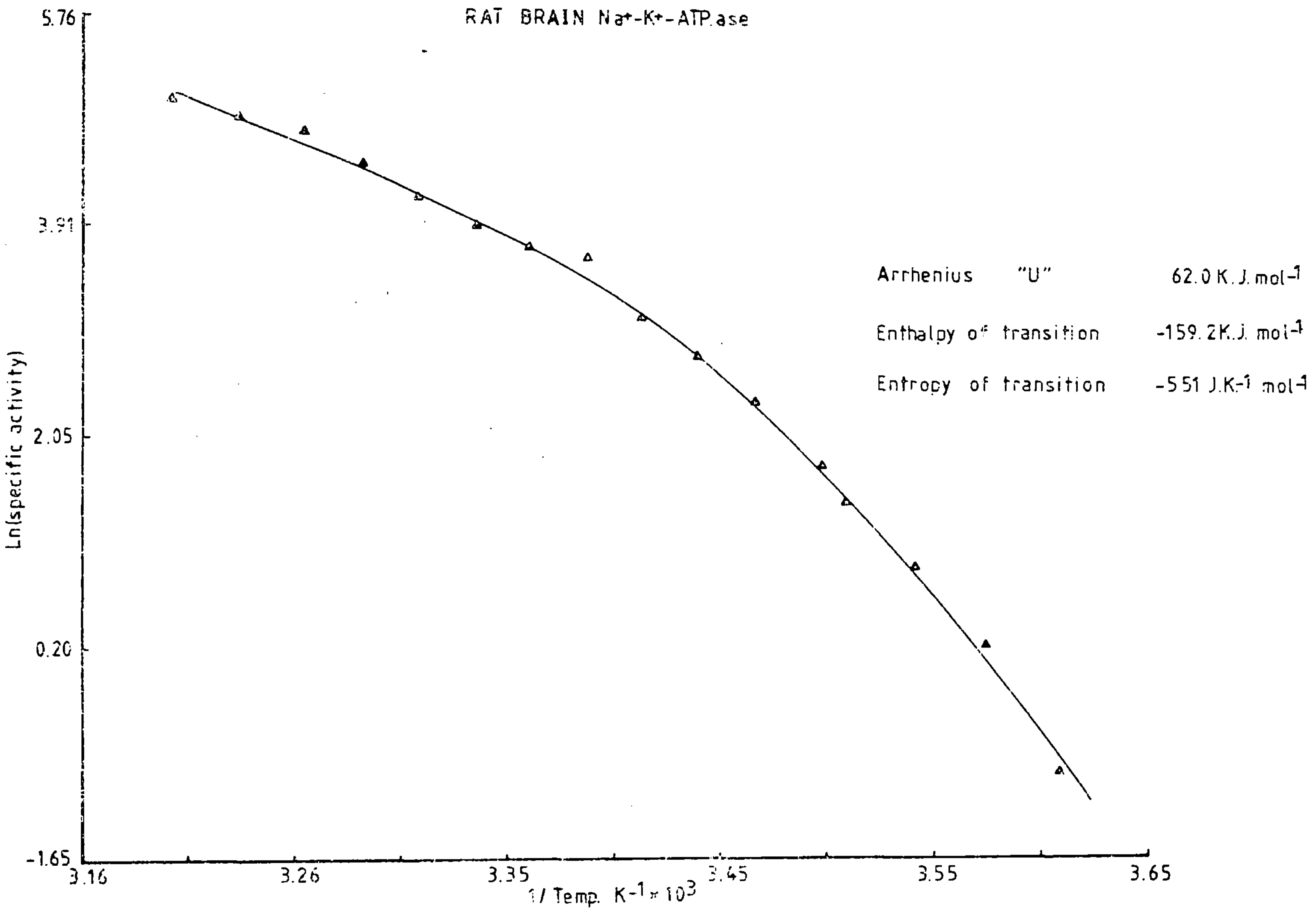
ΔS ... standard entropy change describing the change from the high temperature state to the low temperature state

ΔH ... standard enthalpy change describing the change from the high temperature state to the low temperature state.

This equation can be treated as a numerical problem in which the dependent variable (V) is a function of the independent variable (T) and the four adjustable parameters (A, U, ΔH, ΔS), and as such can be solved numerically by a computer assisted non linear least squares minimisation procedure. Figures 4.9 - 4.12) are typical sets of data to which non_ideal Arrhenius curves, described by the above equation, have been fitted, by a computer program using such a procedure. The Arrhenius plots were all curvilinear, and the data points were all within the five percent error range of the best fit theoretical points. The values for the defining parameters are listed in Table 4.4. The apparent Arrhenius 'u' values defining the high temperature state were somewhat lower than those reported elsewhere (Charnock, Cook & Opit, 1971; Charnock, Cook, Almeida & To, 1973; Boldyrev, Ruuge, Smirnova & Tabak, 1977). However this was expected since the apparent Arrhenius 'u' values returned by this method, were calculated from the limiting high temperature asymptotes, and not by interpolation within the range of the experimental measurements. The values returned for the thermodynamic parameters ΔH and ΔS were large (see Table 4.4). In the absence of reports of other attempts at interpreting the behaviour of the Na^+-K^+-ATPase in this way, comparable

Fig. 4.9

NON-LINEAR ARRHENIUS TEMPERATURE KINETICS OF RAT BRAIN Na^{+}-K^{+}-ATPase



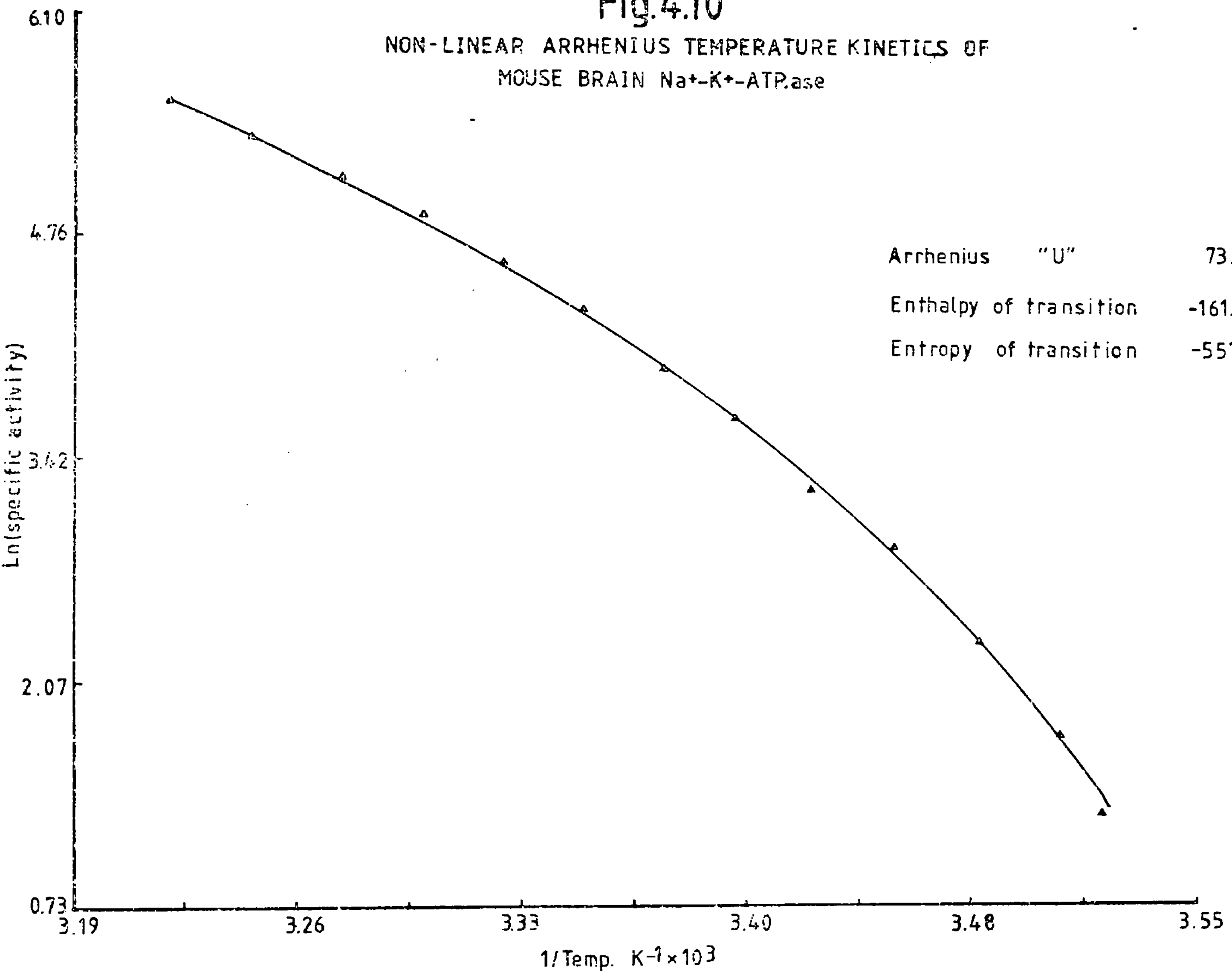


Fig.4.10

NON-LINEAR ARRHENIUS TEMPERATURE KINETICS OF MOUSE BRAIN Na+-K+-ATPase

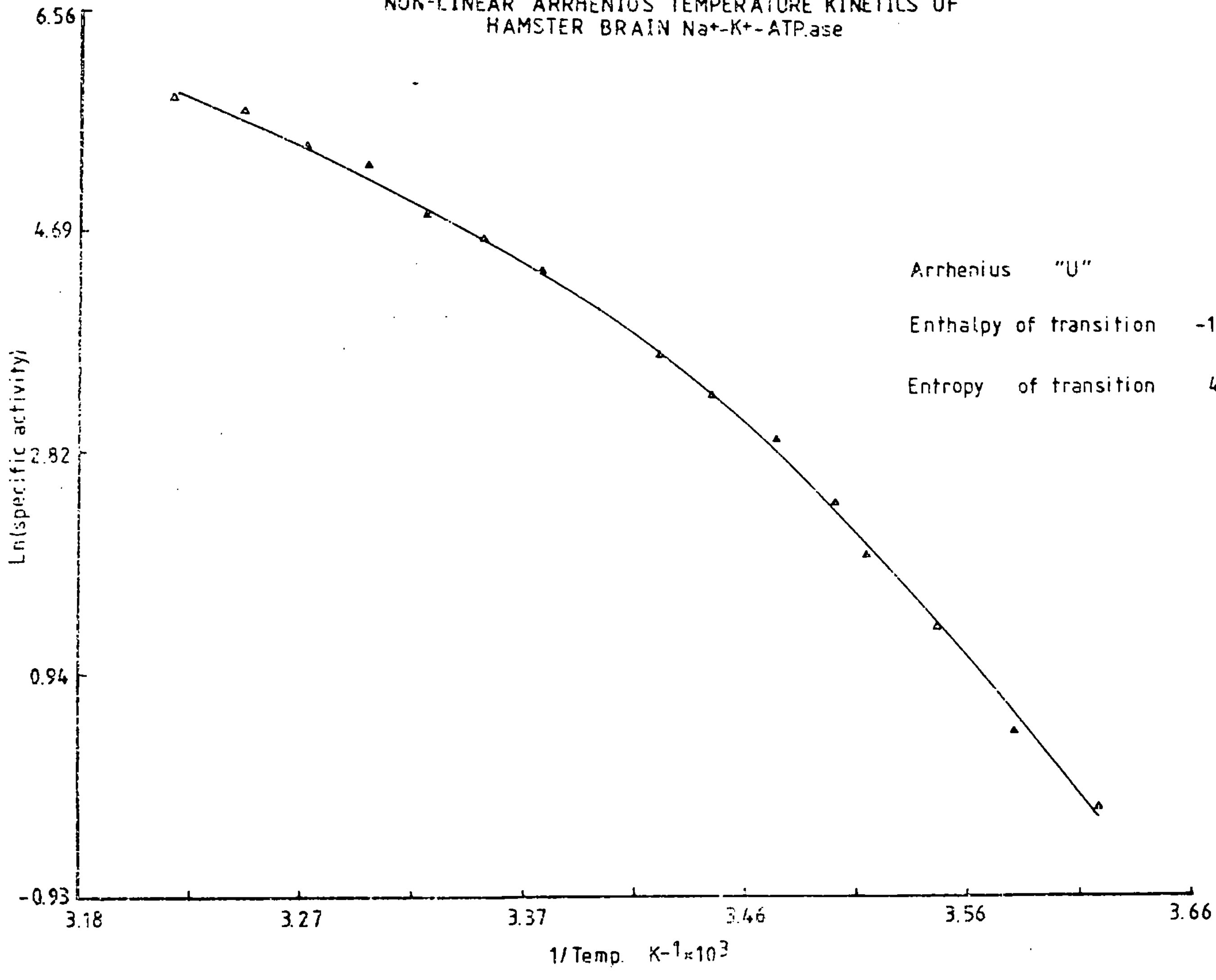


Fig.4.11
NON-LINEAR ARRHENIUS TEMPERATURE KINETICS OF HAMSTER BRAIN Na+-K+-ATP.ase
Arrhenius "U" 67.9 K.J.mol-1
Enthalpy of transition -141.1 K.J.mol-1
Entropy of transition 486 J.K-1 mol-1
Ln(specific activity)
6.56
4.69
2.82
0.94
-0.93
3.18
3.27
3.37
3.46
3.56
3.66
1/Temp. K-1×10^3

Fig. 4.12

NON-LINEAR ARRHENIUS TEMPERATURE KINETICS OF TROUT BRAIN Na^+-K^+-ATPase

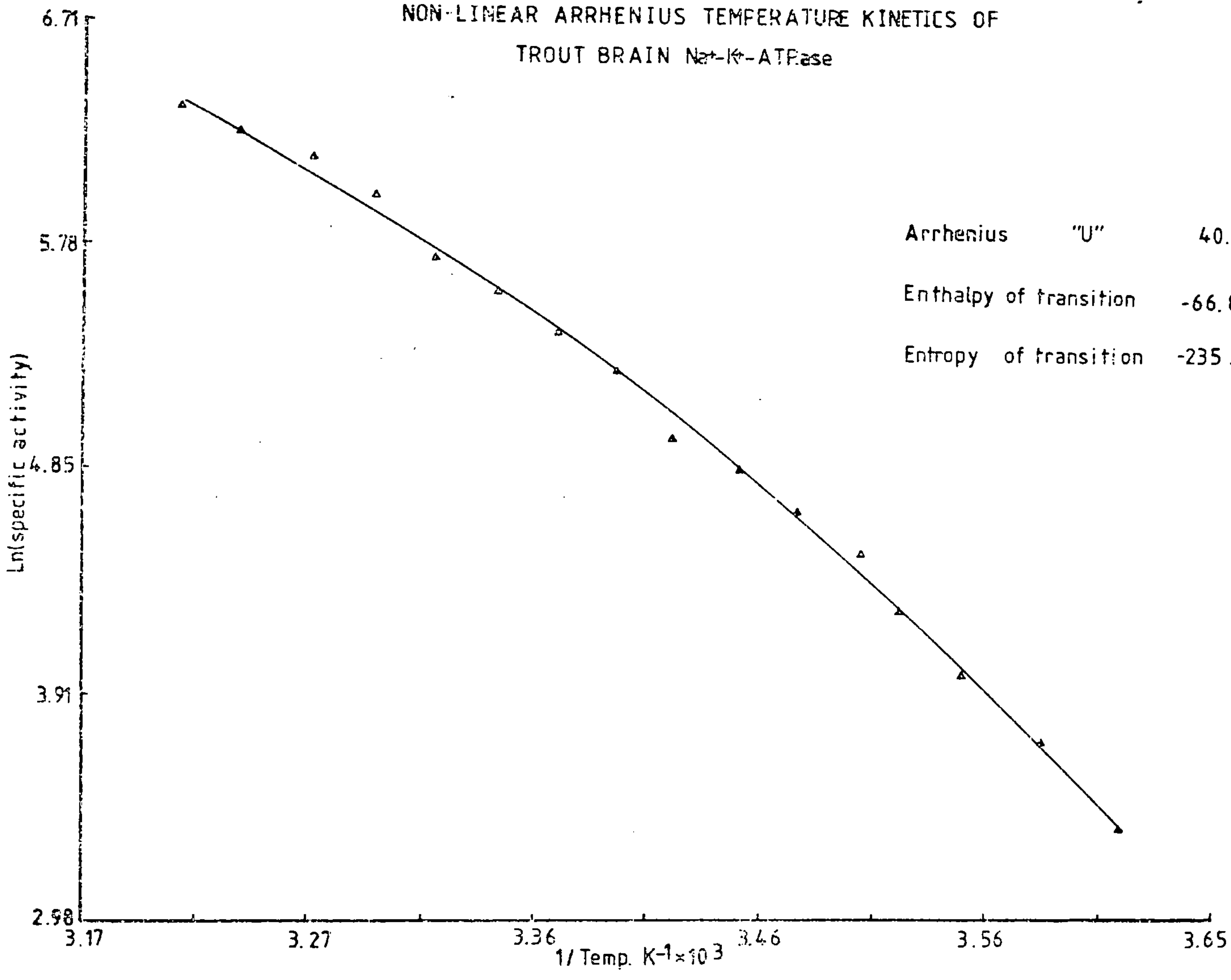


TABLE 4.4

Non Linear Arrhenius parameters describing the temperature kinetics of Na^{+}-K^{+}-ATPase

Source	Arrhenius 'u' High Temperature $K.J.Mol^{-1}$	Enthalpy of Transition $K.J.Mol^{-1}$	Entropy of Transition $J.K.Mol^{-1}$
Rat brain	67.7 ± 4.4	-170.7 ± 9.0	-593 ± 32
Mouse brain	67.02 ± 7.3	-160.2 ± 10.4	-551 ± 38
Hamster brain	69.92 ± 4.4	-150.09 ± 10.1	-519 ± 36
Rainbow trout	41.53 ± 4.0	- 74.31 ± 6.2	-263 ± 21

Values - mean of 4 preparations ± 1 standard deviation

values are unavailable. However in a similar treatment of the data from the soluble enzymes peroxidase and alkaline phosphatase (Maier _et al_ 1955), the values reported were in the range 79-107 $K.J.Mol^{-1}$ for ΔH and 310-410 $J.K.^{-1}Mol^{-1}$ for ΔS. Those values are considerably smaller than those calculated for the Na^{+}-K^{+}-ATPase, and have been attributed to the energy conversions involved in the making and breaking of hydrogen bonds (Maier _et al_ 1955). Although such energy conversions are expected in the case of the Na^{+}-K^{+}-ATPase, the magnitude of the parameters calculated suggested another factor. This along with the knowledge that the Na^{+}-K^{+}-ATPase sits in a highly ordered biomembrane, prompts the suggestion of a possible contribution from the protein-lipid and lipid-lipid interactions, in the membrane fragments containing the enzyme. Given that there is experimental evidence for a greater structural rigidity of mammalian biomembranes in comparison with those of the gold fish (Cossins, 1977), such a suggestion would be compatible with the observation that the values returned for the fish preparations were consistently lower than those of the mammalian preparations. Furthermore, the negative values returned for the ΔH and ΔS for the change from the high temperature to low temperature state, were in the same direction as those of thermal events detected in model membranes (Ladbroke & Chapman, 1969), and biomembranes (Rienert & Steim, 1970). If such is the case, the most probable physical event would be the postulated order-disorder structural change in the lipid bilayers (Nagle, 1973; Firpo, Durpin, Albinet, Bois, Casalta & Baret, 1978). The calorimetric estimation of the ΔH values for such events were in the range 25-45 $K.J.Mol^{-1}$ (Chapman, Peel, Kingston & Lilley, 1977). These values were too small to account for the large ΔH values returned from the kinetic measurements. A similar discrepancy has also been reported for the observed (calorimetric), and calculated (Van't Hoff isotherm) ΔH values for the biomembrane order-disorder changes

(Trauble, 1971). This discrepancy has been rationalised by the assumption that the biomembrane is organised in co-operative units of 20-30 lipid molecules (Rothman, 1973; Yellin & Levin, 1977). If such is the case, then the magnitude of the energy changes associated with the lipid-lipid and protein-lipid interactions may be large enough to account for the large values calculated from the kinetic measurements on the Na^+-K^+-ATPase.

As described the significant determinants of the behaviour of the Na^+-K^+-ATPase, as regards its temperature kinetics, are the apparent activation energy of the active state, and the width of the conversion to the inactive state (as defined by ΔH). Thus within this context, the difference in the behaviour of the fish preparation and the mammalian preparation, are defined in terms of a smaller activation energy and a broader thermal transition to the low temperature state, defining the fish preparation. Furthermore, the smaller entropy characterising the fish preparation, suggests that the degree of reorganisation characterising the change to the low temperature stable state is considerably smaller in the case of the fish. Thus the mechanism described enables a reasonable interpretation of the behaviour of the Na^+-K^+-ATPase. However, it requires that there be a thermally induced change of state that is characterised by large enthalpy and entropy changes, to which no physical process has yet been assigned. Hence a full understanding of the temperature kinetics of the Na^+-K^+-ATPase must await unambiguous studies of the thermal changes which occur in this enzyme system.

CHAPTER 5

Potassium activation of the Na^{+}-K^{+}-ATPase

INTRODUCTION

The mechanism of the Na^{+}-K^{+}-ATPase is generally envisaged to involve a sodium dependent phosphorylation of the enzyme followed by a potassium dependent discharge of the phosphorylated intermediate so formed. Under 'ideal' phosphorylating conditions, the formation of the phospho-protein intermediate takes place quickly and quantitatively at $0^{o}C$ (Fahn, Koval & Albers, 1968; Bond, Bader & Post, 1971), while under similar temperature conditions, the activity of the enzyme proceeds very slowly (Charnock, Cook & Casey, 1971). This suggested that the rate limiting step in the overall reaction mechanism occurs somewhere in the steps involved in the dephosphorylation of the enzyme and the regeneration of the 'free' enzyme. Thus, the activation of the enzyme by potassium ion may be relevant in the study of the steps near to the rate limiting step in the reaction mechanism.

The effect of increasing potassium on the activity of the Na^{+}-K^{+}-ATPase (at saturating concentrations of all other ligands), is usually described in terms of a sigmoid curve (Robinson, 1967; Lindenmayer, Schwartz & Thompson, 1974). Some workers have described the potassium activation kinetics of this enzyme in terms of a Michaelis-Menten rectangular hyperbola (Bakkeren & Bonting, 1968). The latter work however was carried out at relatively high concentration range (>0.4mM). It has consequently been shown that the response of the sodium pump deviates from an apparent compliance with Michaelis-Menten

kinetics only at low potassium concentrations (Sjodin & Beauge, 1968). Thus the discrepancy between these data can be explained if the potassium activation kinetics of the Na^+-K^+-ATPase is sigmoidal but approximates to a rectangular hyperbola at 'high' potassium concentrations.

Although the sigmoidal nature of the potassium activation kinetics is generally accepted, there is, as yet, no agreed framework for the interpretation of the behaviour. The simplest and most widely used method (Robinson, 1967, 1969, 1970; Gache, Rossi & Lazdunski, 1977) is based on the Hill equation (Hill, 1910). Alternative descriptions (Lindenmayer, Schwartz & Thompson, 1974; Fukushima & Tonomura, 1975) define the sigmoidal kinetics of the Na^+-K^+-ATPase in terms of multiple potassium sites. In both cases computer assisted numerical methods have been used to obtain accurate fits of experimental data to sigmoidal curves. However, the empirical nature of these approaches allows only a numerical definition of the fitted curves. Clearly, a description of the Na^+-K^+-ATPase such that the sigmoidal potassium activation kinetics follows naturally, could enable a mechanistic interpretation of the data. In this study, such descriptions will be examined with the eventual aim of implementing the one which accommodates most of the experimental observations.

MATERIAL AND METHODS

(1) Preparation of brain synaptic membranes.

These were prepared from the brains of rats and rainbow trout as previously described in Chapter 2.

(2) Assay of Na^+-K^+-ATPase at non-saturing potassium concentrations.

The assay conditions with respect to temperature, pH and concentrations of essential ligands (except potassium) were as previously described in Chapter 2.

Prepared solutions

(a) Disodium A.T.P. 4.4 x 3mM . . pH 7.5 (Tris)

(b) Buffered ionic medium pH 7.5 at 37°C (Tris/HCl)

4.4 x 124mM Sodium Chloride

4.4 x 3mM Magnesium Chloride

4.4 x 30mM Histidine

(c) Ouabain 4.4 x 1mM

(d) Potassium Chloride 4.4 x final concentration

18 solutions were prepared so as to obtain potassium chloride solutions of final concentrations in the range 1.15×10^{-4}M - 1.5×10^{-2}M with a geometric separation of potassium concentrations.

Reaction mixtures

(i) Total ATPase activity

0.5 cm^{-3} ATP

0.5 cm^{-3} buffered ionic medium

0.5 cm^{3} distilled water

0.5 cm^{3} potassium chloride

(ii) Ouabain insensitive ATPase activity

This was essentially the same as that for the total ATPase except that 0.5 cm^{3} of ouabain was substituted for the distilled water.

The reaction mixtures were thermoequilibriated at 37°C for a suitable time (usually 20-30 minutes), and the reaction started by the addition of 0.2 cm^{3} of an enzyme preparation. The reaction was quenched after a suitable run time and liberated inorganic phosphate determined as previously described (Chapter 2). The Na^{+}-K^{+}-ATPase activity was taken as the difference between the inorganic phosphate released in the total ATPase mixture and that in the ouabain insensitive ATPase mixture.

RESULTS AND DISCUSSION

The potassium activation kinetics of the Na^{+}-K^{+}-ATPase from the rat brain and trout brain preparations are shown in figures 5.1 and 5.2 (typical of 4 preparations). In these diagrams, the line drawn is a Michaelis-Menten rectangular hyperbola fitted by a computer assisted least squares minimisation procedure (see Appendix II) In both cases, the observed data points appear to fit the rectangular hyperbola drawn at the higher potassium concentrations, but deviate downwards from the hyperbola at the low potassium concentrations ($<10^{-3}$M). This is further emphasised in the Lineweaver-Burk transformations of the data (see inserts in figures 5.1 and 5.2), in which, the data points for potassium concentrations less than 10^{-3}M, all deviate upwards from the Lineweaver-Burk line calculated from the best fit Michaelis-Menten hyperbola. These observations are thus similar to those previously reported (Robinson, 1967), and clearly indicate that the potassium activation of the Na^{+}-K^{+}-ATPase does not satisfy Michaelis-Menten kinetics and may better be described by a sigmoid curve.

The fitting of a sigmoid curve to this type of data is usually done according to the Hill equation (Robinson, 1967, 1969, 1970; Gache, Rossi & Lazdunski, 1977). This is an empirical relationship in which the observed activity (v) is given by the equation

$$v = \frac{V_{max} S^{n}}{K_{0.5}^{n} + S^{n}}$$

V_{max} ... Maximal activity

$K_{0.5}$ ... Substrate concentration required for half maximal activity

S ... Substrate concentration

n ... Hill exponent

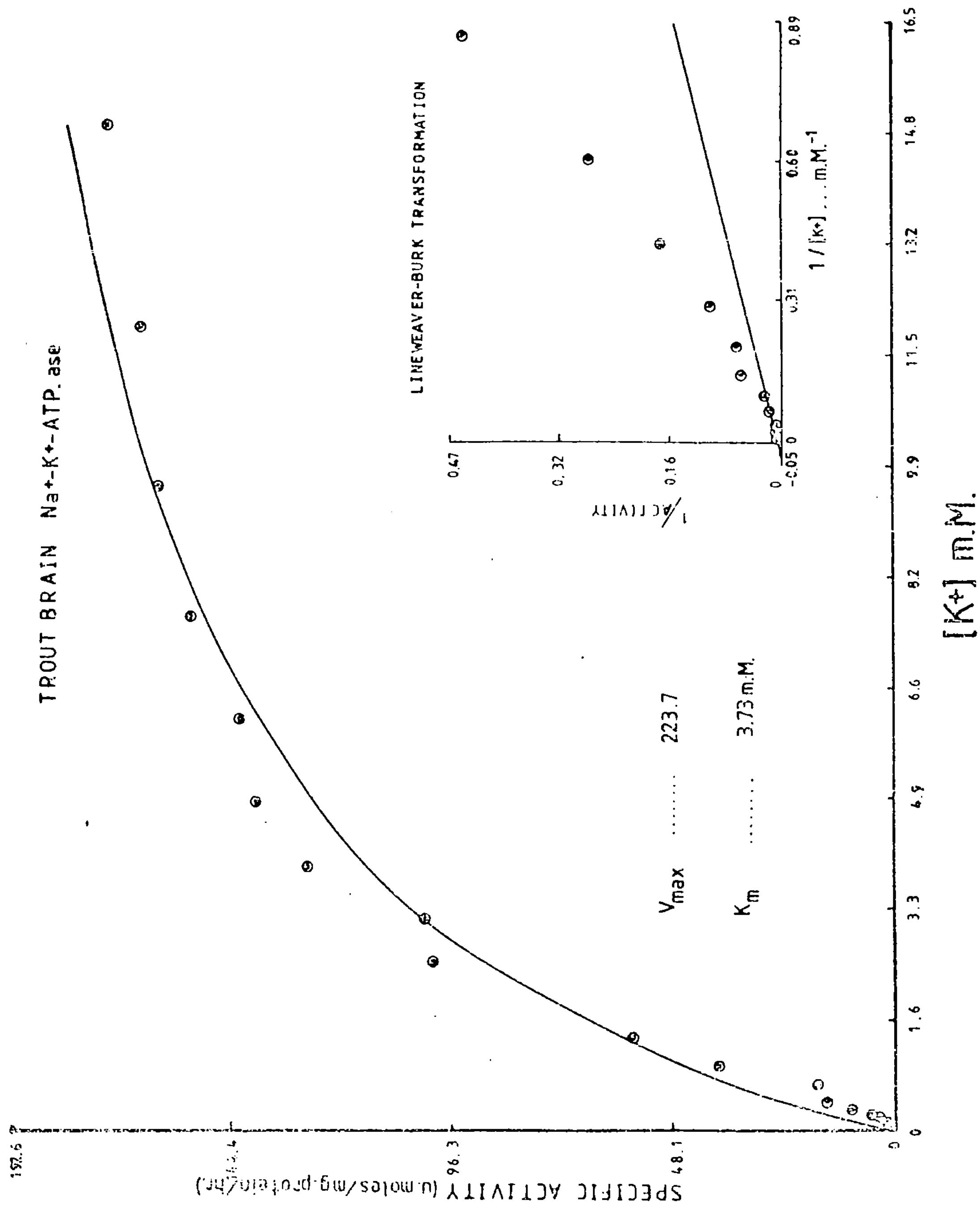


Fig. 5.1
TROUT BRAIN Na+-K+-ATP.ase
LINEWEAVER-BURK TRANSFORMATION
Vmax 223.7
Km .... 3.73 m.M.
[K+] m.M.
SPECIFIC ACTIVITY (u.moles/mg.protein/hr.)
1/ACTIVITY
1/[K+] . . . m.M.-1
0 1.6 3.3 4.9 6.6 8.2 9.9 11.5 13.2 14.8 16.5
0 48.1 96.3
-0.05 0 0.31 0.60 0.89
0 0.16 0.32 0.47

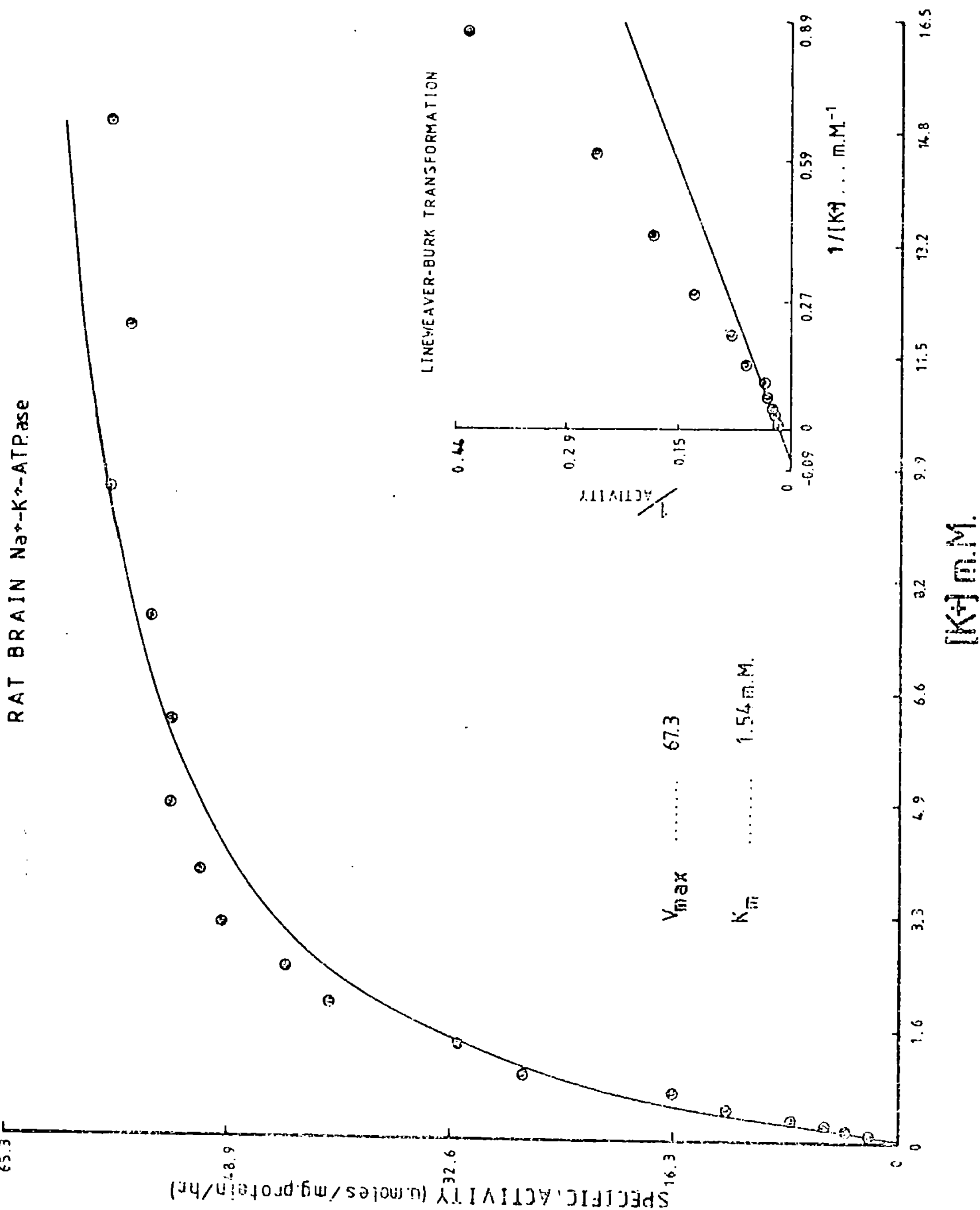


Fig. 5.2
RAT BRAIN Na+-K+-ATPase
LINEWEAVER-BURK TRANSFORMATION
Vmax 67.3
Km .... 1.54 m.M.
[K+] m.M.
1/[K+] . . . m.M.-1
1/ACTIVITY
SPECIFIC ACTIVITY (u.moles/mg.protein/hr)
0
16.3
32.6
48.9
65.3
1.6
3.3
4.9
6.6
8.2
9.9
11.5
13.2
14.8
16.5
-0.09
0.27
0.59
0.89
0.15
0.29
0.44

Figures 5.3 and 5.4 are typical sets of data to which sigmoid curves (Hill equation) have been fitted, using a computer assisted least squares minimisation procedure (see Appendix II).The values of the 'Hill parameters' defining the fitted sigmoid curves are listed in Table 5.1. The value returned for the $K_{0.5}$ for the rat brain preparation (1.35×10^{-3}M) is higher than that previously reported for this preparation (Robinson, 1967, 1970), but the value for the Hill exponent (1.4) compares favourably with the literature values for this preparation (Robinson, 1967, 1970). The disagreement between the $K_{0.5}$ obtained here and the available literature values can be accounted for by the higher sodium concentrations used in this study, since it has been reported that the potassium concentrations required for half maximal response of the sodium pump is sensitive to sodium ion concentration (Lindenmayer, Schwartz & Thompson, 1974; Fukushima & Tonomura, 1975). In spite of the good fit of the experimental data to the Hill equation (see inserts in figures 5.3 and 5.4), the fact that the equation is essentially empirical, means that any mechanistic interpretation of the results is precluded. At best, the Hill equation allows a numerical definition of the sigmoid curve, and as a consequence a numerical means of comparing different data sets. Thus the values quoted in Table 5.1 suggest that the trout brain preparation is less potassium sensitive than the rat brain preparation (trout $K_{0.5}$ > rat $K_{0.5}$), and that the degree of deviation from a rectangular hyperbola is smaller in the case of the trout (n for trout is closer to unity than n for the rat).

The observation that two moles of potassium ion are translocated across the erythrocyte membrane for each mole of ATP hydrolysed by the sodium pump (Garrahan & Glynn, 1967; Sen & Post, 1964) suggested a description of the Na^{+}-K^{+}-ATPase defining two potassium

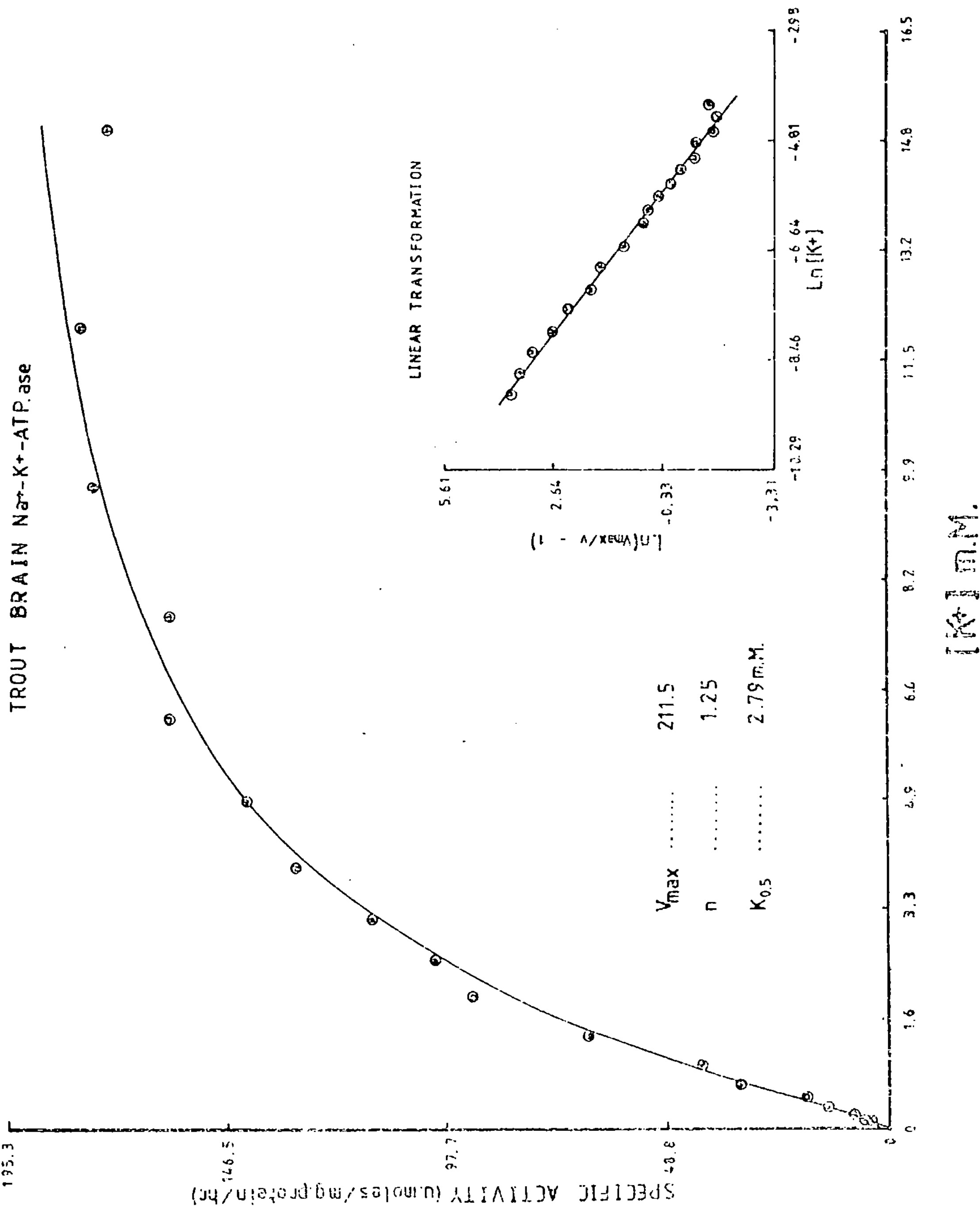


Fig.5.3
TROUT BRAIN Na+-K+-ATPase
Vmax 211.5
n 1.25
K0.5 .... 2.79 m.M.
LINEAR TRANSFORMATION
Ln(Vmax/v - 1)
Ln[K+]
SPECIFIC ACTIVITY (umoles/mg.protein/hr)
[K+] m.M.

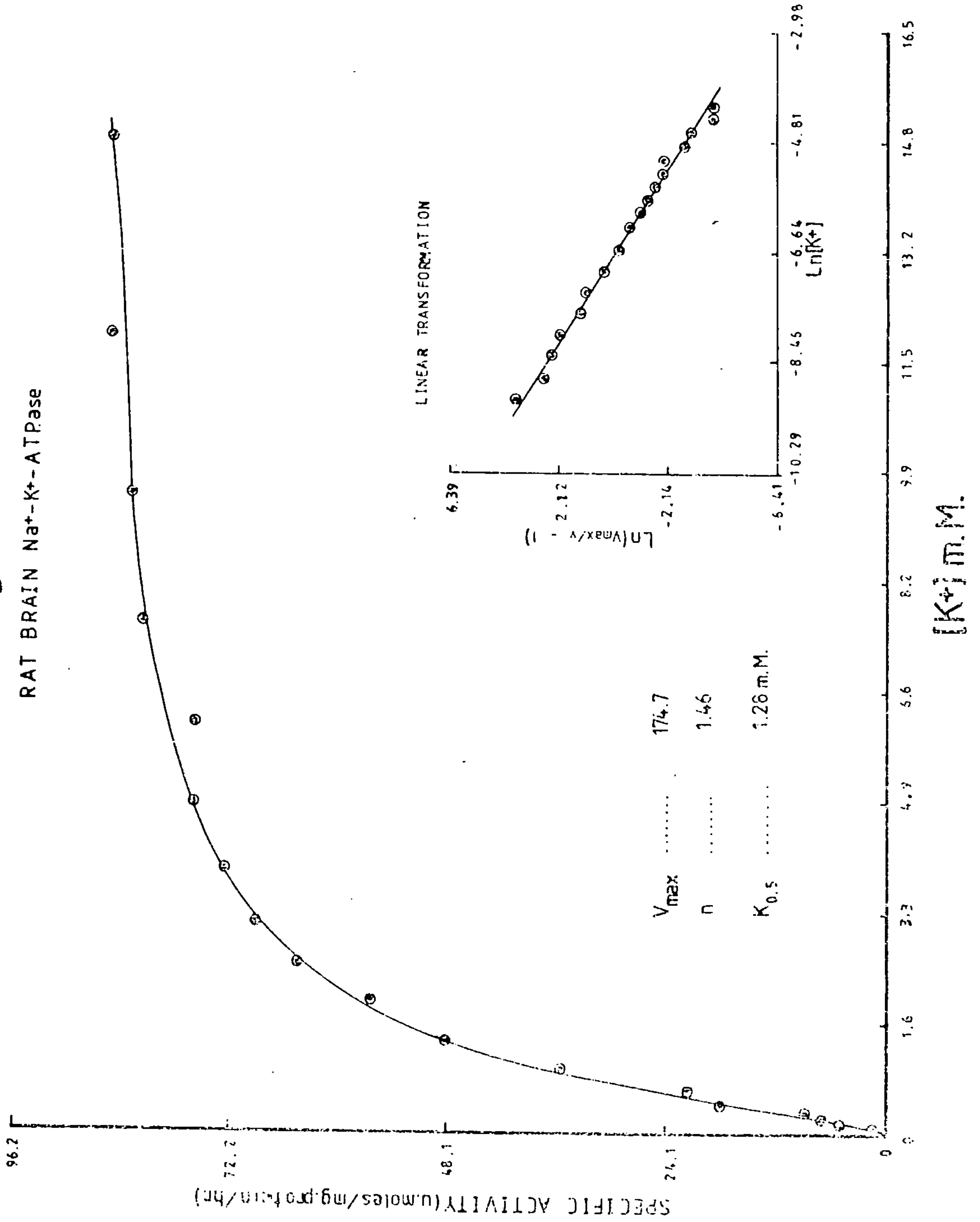


Fig. 5.4
RAT BRAIN Na+-K+-ATPase
LINEAR TRANSFORMATION
Ln(Vmax/v - 1)
Ln[K+]
6.39
2.12
-2.14
-6.41
-10.29
-8.45
-6.64
-4.81
-2.98
Vmax 174.7
n 1.46
K0.5 .... 1.26 m.M.
96.2
72.2
48.1
24.1
0
1.6
3.3
5.6
8.2
9.9
11.5
13.2
14.8
16.5
[K+] m.M.

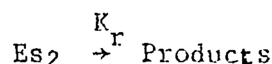
TABLE 5.1

Hill parameters defining the Sigmoid curves fitted to the K^+ activation of the Na^+-K^+-ATPase

Tissue source	n	$K_{0.5}$	Hill exponential
Rat Brain	4	$1.35\times10^{-3}\pm4.61\times10^{-5}$	1.41 ± 0.055
Trout brain	4	$2.95\times10^{-3}\pm1.55\times10^{-4}$	1.23 ± 0.02

Value means of n preparations $\pm$ 1 standard deviation

sites. This suggestion has been supported by recent kinetic measurements (Lindenmayer, Schwartz & Thompson, 1974; Robinson, 1975; Fukishima & Tonomura, 1975). Within this description, each catalytic unit of the functional enzyme is envisaged to contain two potassium sites, both of which must be occupied for the completion of the enzyme cycle. Thus assuming saturating concentrations of all other essential ligands, the potassium activation of the enzyme could be described by the following equations.



K_r .. Catalytic rate constant

S .. Potassium ion concentration

E .. 'Free' potassium sensitive species

Es_1.. Species with one potassium site occupied

Es_2.. rate limiting species with both potassium sites occupied.

Using the above equations, the conservation equation ($E_o = E + Es_1 + Es_2$ .. E_o = Sum of all the species present), and the saturation approximation (i.e. maximal activity = $V_{max} + K_r E_o$) the observed activity (V) can be shown to be defined by the equation

$$V = \frac{V_{max}}{1 + (K_b/S) + (K_a K_b/S^2)} \qquad (5a)$$

The type of curve described by equation (5a) is sigmoidal. At the higher potassium concentrations, the equation predicts that the curve should approach the form of a Michaelis-Menten rectangular hyperbola with an apparent K_m given by K_b (for $S >> K_a$ the term $\frac{K_a K_b}{S^2} \simeq 0$). Thus in this respect, the predicted behaviour agrees with the observations shown in figures 5(i) and 5(ii). Furthermore, the sigmoidal potassium activation kinetics arise naturally out of the description and offer a mechanistic interpretation of the data, since by definition the constants K_a and K_b represent the potassium concentrations needed to saturate the respective sites on the enzyme.

Figures 5.5 and 5.6 are typical sets of data to which sigmoid curves (as defined by equation (5a)) have been fitted using a computer assisted least squares minimisation procedure (see Appendix II) The defining parameters are listed in Table 5.2. In both cases (trout and rat) the model interprets the data as follows:

(a) There are two potassium sites.

(b) The first site (that defined by K_a) has a greater potassium sensitivity than the second site (that defined by K_b). This is because higher potassium concentrations are required to half saturate the second potassium site.

The differences between the Na^+-K^+-ATPase preparations from the rat and trout can be interpreted by the high affinity site of the trout preparation having a greater potassium sensitivity than that of the rat preparation (trout K_a < rat K_a); and the low affinity site of the rat preparation having a greater potassium sensitivity than that of the trout preparation (rat K_b < trout K_b). Since the value for the trout K_a is smaller than

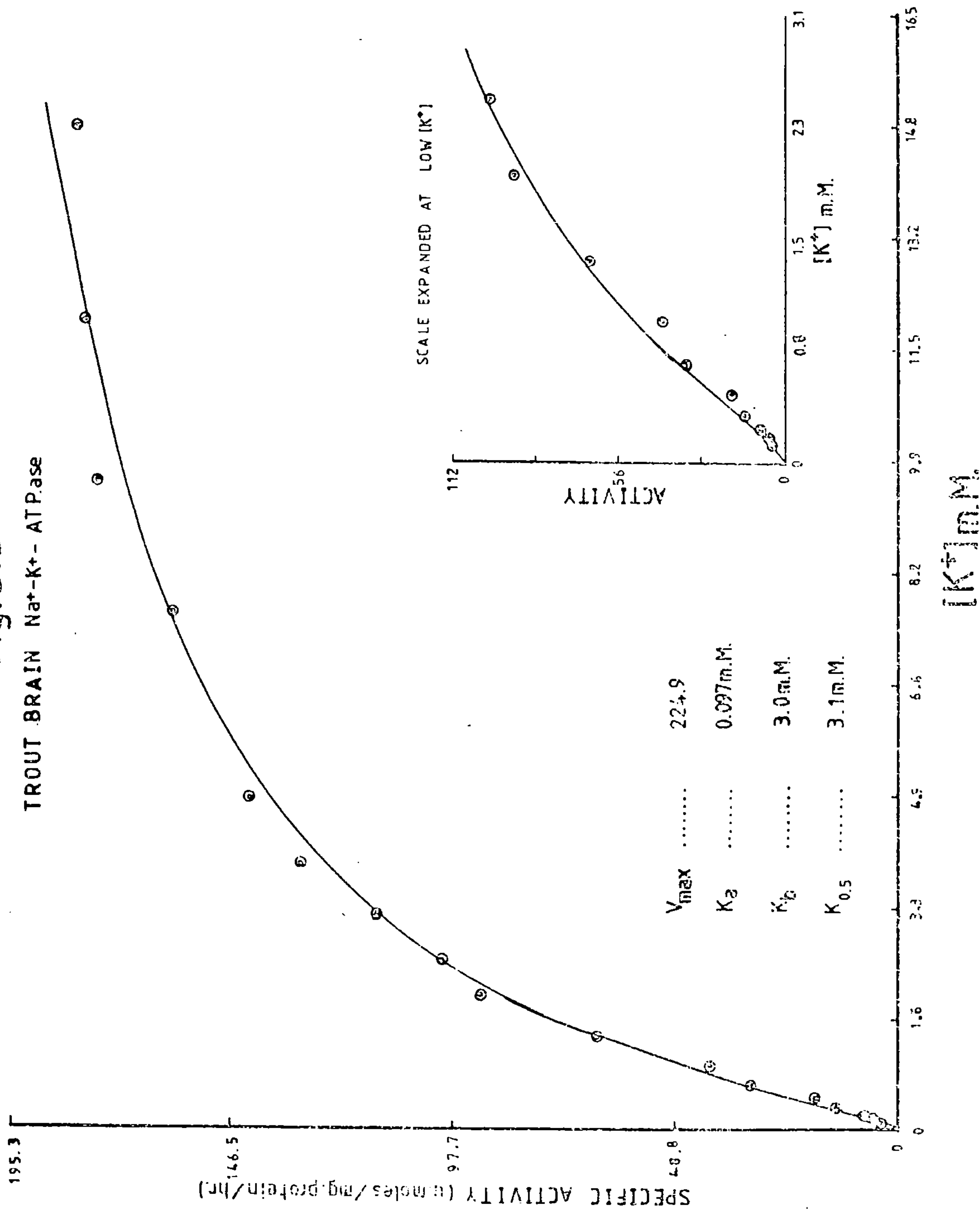


Fig. 5.5
TROUT BRAIN Na+-K+- ATPase
SPECIFIC ACTIVITY (u moles/mg.protein/hr)
195.3
146.5
97.7
48.8
0
1.6
3.3
4.9
6.6
8.2
9.9
11.5
13.2
14.8
16.5
[K+] m.M.
Vmax 224.9
Ka .... 0.097 m.M.
Kb .... 3.0 m.M.
K0.5 .... 3.1 m.M.
SCALE EXPANDED AT LOW [K+]
ACTIVITY
112
56
0.8
1.5
2.3
3.1
[K+] m.M.

Fig.5.6

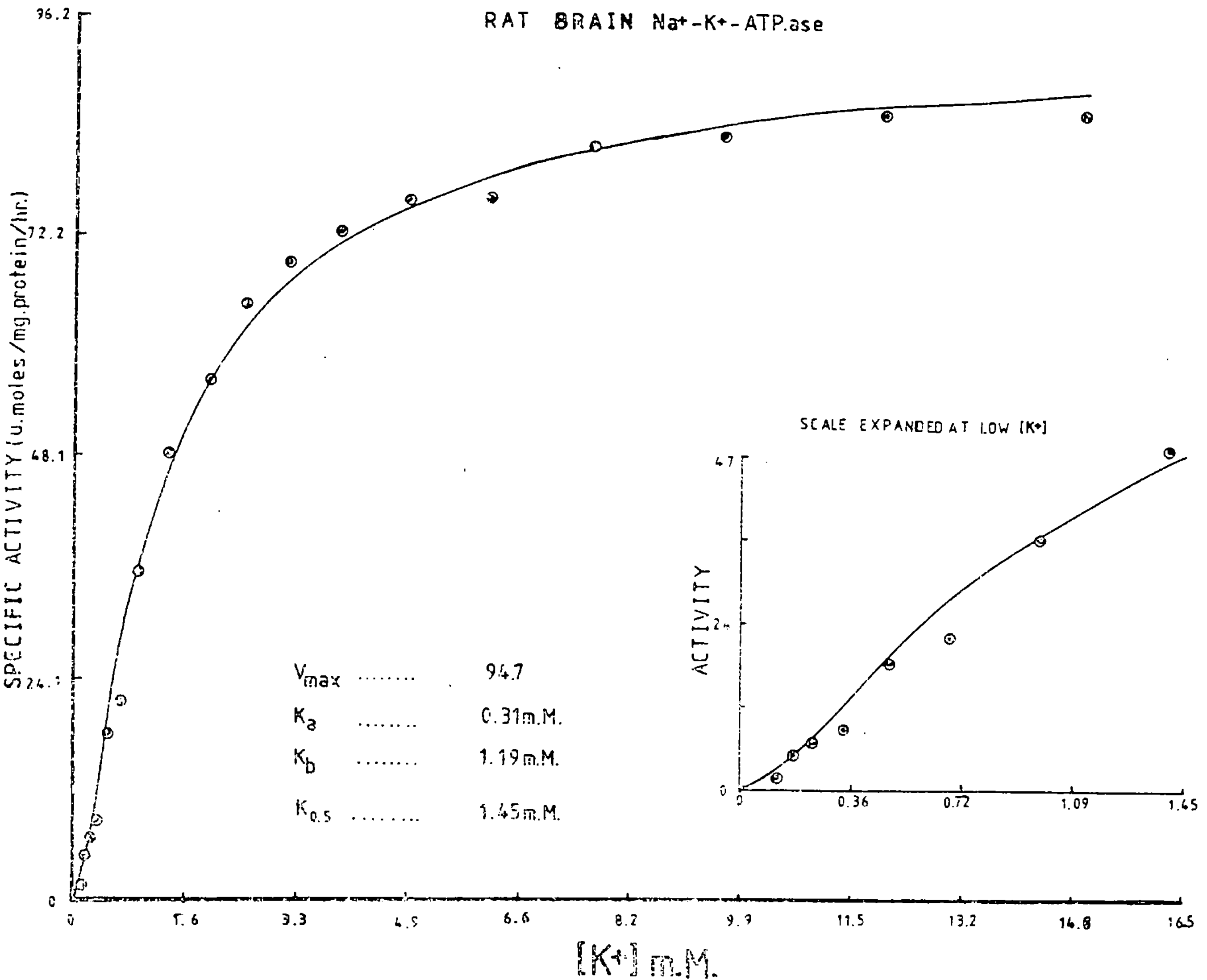


TABLE 5.2

Parameters defining the sigmoid curve drawn according to the stoichiometric model

Tissue source	n	K_a	K_b
Rat brain	4	$2.25x10^{-4}M \pm 6.67x10^{-5}M$	$1.26x10^{-3}M \pm 4.97x10^{-5}M$
Trout brain	4	$8.25x10^{-5}M \pm 1.45x10^{-5}M$	$3.10x10^{-3}M \pm 1x10^{-4}M$

Values - mean of n preparations $\pm$ 1 standard deviation

that of the rat, the results suggest that if the potassium activation kinetics of these two preparations are compared over any given potassium concentration range, the data described by the trout preparation would show a smaller deviation from a Michaelis-Menten rectangular hyperbola, since the degree of saturation of the high affinity potassium site would be greater for the trout preparation at any finite potassium concentration. Thus this description suggests a possible explanation for the smaller deviation from the rectangular hyperbola observed for the trout preparation (the Hill exponent for the trout was nearer to unity than that of the rat). Table 5.3 shows the values returned by both methods (Hill equation and stoichiometric model) for the potassium concentrations giving half maximal response, and the maximal activities (all maximal activities have been normalised against 100 for the Hill V_{max}). This shows that the values calculated are in broad agreement with each other though those returned by the stoichiometric description were consistently greater than those calculated by the Hill equation (≈ 5-8%).

CONCLUSIONS

The experimental observations suggest that the activation of the Na^{+}-K^{+}-ATPase by potassium ion is best described by sigmoid kinetics, and the accurate fits of the data to the two sigmoid curve types tested support this suggestion. Although the comparable parameters calculated by both methods (Hill equation and stoichiometric model), are in general agreement with each other, the stoichiometric model was preferred since, unlike the Hill equation, it is not empirical and the sigmoidal response to increasing potassium concentration arises naturally

TABLE 5.3

Comparison of appropriate parameters of the Hill and stoichiometric sigmoid curves.

Model	Rat brain preparation		Trout brain preparation	
	Δ V_{max}	▲ $K_{0.5}$	Δ V_{max}	▲ $K_{0.5}$
Hill	100	$1.35 \times 10^{-3}M \pm 4.61 \times 10^{-5}M$	100	$2.95 \times 10^{-3}M \pm 1.55 \times 10^{-4}M$
Stoichio-metric	106.8 ± 0.99	$1.45 \times 10^{-3}M \pm 1.13 \times 10^{-5}M$	104.8 ± 1.8	$3.15 \times 10^{-3}M \pm 5 \times 10^{-5}M$

Δ The V_{max} values are normalised against 100 for the Hill equation V_{max}

▲ $K_{0.5}$ - Potassium concentration that produces half maximal activity.

All values mean of 4 preparations $\pm$ 1 standard deviation.

out of the description. As previously mentioned, this stoichiometric description is compatible with the stoichiometry of the sodium pump and recent kinetic evidence. However, in its present form, the description is probably too simple to account for the complex activation of the Na^{+}-K^{+}-ATPase by monovalent cations, and consequently, the interpretation of the behaviour according to this model can only be taken as approximate. In spite of these limitations, the description allows a 'quasi-mechanistic' interpretation of the data, and as a result it was used as the framework for the interpretation of the potassium activation of the Na^{+}-K^{+}-ATPase.

CHAPTER 6

The effect of some partial purification procedures on the kinetic properties of the Na^+-K^+-ATPase

INTRODUCTION

The biological activity of the Na^+-K^+-ATPase appears to be heavily dependent on a firm association between the catalytic species (or assembly of species) and the lipid constituent of the biomembrane containing the enzyme. As a result, all attempts to prepare pure, biologically active samples of this enzyme, free of membrane lipid, have so far been unsuccessful. Thus the best that has been achieved is the preparation of membrane samples that are enriched with the enzyme by the removal of other protein species. The procedures for so doing usually exploit the firm association of this enzyme with the membrane, by the controlled use of surface-active agents and high ionic strength under conditions such that the protein species that are less firmly membrane bound than the Na^+-K^+-ATPase, are selectively removed.

A variety of procedures have been used in the enrichment of membrane fragments with respect to the Na^+-K^+-ATPase. These are based on the use of surface active agents and/or high ionic strength. In the latter procedures, high concentrations (>1.6M) of sodium iodide (Nakao, Nakao, Mizuno, Komatsu & Fujiata, 1973), on ammonium sulphate (Uesugi, Dulak, Dixon, Hexum, Dahl, Perdue & Hokin, 1971) are used to differentially solubilise protein species that are less firmly membrane bound than the Na^+-K^+-ATPase. These procedures usually form part of a more elaborate extraction process

in which a surface active agent is used. As a result the effects of these agents on the enzyme is difficult to evaluate.

A variety of surface-active agents have been used in the enrichment of membrane fractions with respect to the Na^+-K^+-ATPase. These include non-ionic detergents like lubrol (Uesegi, Durlak, Dixon, Hexum, Dahl, Perdue & Hokin, 1971; Nakao, Nakao, Hara, Nagai, Yagasaki, Koi, Nakagawa & Kawai, 1974) and Triton X-100 (Banerjee, Dowosh, Khanna & Sen, 1970), weakly anionic detergents like deoxycholate (Kyte, 1971b), and strongly anionic detergents like dodecyl-sulphate (Jorgensen, 1974b). The effectiveness of these materials in the enrichment of biomembranes with Na^+-K^+-ATPase appears to be the product of their interaction with membrane lipid rather than membrane protein, since they appear to exert optimal effects at concentrations near their critical micellar concentrations (Jorgensen & Skou, 1971). Thus in all cases where detergents have been used, the response of the enzyme was characterised by an apparent activation at low detergent concentrations, followed by a sharp loss of activity at the higher detergent concentrations. The initial apparent activation of the enzyme has been attributed to the exposure of occluded enzyme sites by the detergent since there was no significant change in the 'molecular activity' of the enzyme (as determined by ^{3}H-ouabain binding) on treatment with detergents (Jorgensen, 1974c). The loss of Na^+-K^+-ATPase activity at the higher detergent concentrations is generally believed to be the result of protein denaturation and or membrane solubilisation.

Given that the biological activity of this enzyme appears to depend on the association of the catalytic species with membrane lipid, and since some perturbation of the membrane structure must inevitably occur when biomembranes are enriched in Na^+-K^+-ATPase by the use of

surfactants, the possibility of the kinetic properties of the Na^{+}-K^{+}-ATPase being affected by the detergent induced perturbations of the membrane structure during such enrichment procedures had to considered. Thus the effects of the enrichment procedures on some of the kinetic properties of the Na^{+}-K^{+}-ATPase were investigated with the aim of determining whether the kinetic properties of the enzyme were significantly affected by the enrichment procedures.

MATERIALS AND METHODS

(1) Preparation of membranes containing Na^{+}-K^{+}-ATPase activity.

(a) Synaptic membranes from the brains of mammals.

These were prepared as described for the rat brain in Chapter 2. The brains of rats, mice and hamsters were used as sources of synaptic membranes.

(b) Microsomal membranes from rat and trout brain.

All operations were carried out at 0-4°C. The animals were killed and their brains excised into ice cold extraction medium (320mM Sucrose, 30mM Imidazole, 2mM EDTA, pH 7.2 at room temperature) as described in Chapter 2. The cerebral hemispheres were then dissected free of blood vessels and underlying tissue and then homogenised (≃ 10% tissue concentration) in the extraction medium with a teflon lined glass homogeniser. The homogenate was centrifuged at 900g (MSE 2L refrigerated centrifuge) for 10 minutes and the pellet was discarded. The supernatant was then centrifuged at 20,000g (MSE High speed 18 refrigerated centrifuge) for 30 minutes. The pellet was either discarded or used for the preparation of synaptic membranes (see Chapter 2), and the supernatant was recentrifuged at

20,000g for 30 minutes. The supernatant from this spin was then carefully removed from the pellet and centrifuged at 100,000g (MSE Prepspin 50 ultracentrifuge) for one hour. The supernatant was discarded and the surface of the pellet was washed free of loosely packed fatty material by a gentle swirling of low ionic strength buffer (10mM Imidazole, 1mM EDTA, pH 7.2 at room temperature)▼. The final pellet was then resuspended by homogenising in low ionic strength buffer at a protein concentration of 1-3 mg. cm^{-3} and stored on ice until required.

(c) Microsomal membranes from rat submaxillary gland.

All operations were carried out at 0 - 4°C. The animals were stunned by a blow to the head and killed by cervical dislocation. The submaxillary glands were then quickly excised into an ice-cold extraction medium (250mM Sucrose, 30mM Imidazole, 2mM EDTA, pH 7.2 at room temperature). The submaxillary glands were then dissected free of associated connective tissue and blood vessels, and then minced thoroughly with a pair of scissors. The tissue was then homogenised in the extraction medium (≃ 5% tissue concentration) with a teflon lined glass homogeniser, and the homogenate centrifuged at 900g (MSE 2L refrigerated centrifuge) for 10 minutes. The pellet

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▼ Note: The washing of the surface of the pellet (from 100,000g spin) was important especially in the preparation of trout brain microsomes where the supernatant (from 100,000g spin) was found to be a suspension of low density fatty material containing little or no Na^{+}-K^{+}-ATPase activity.

was discarded and the supernatant centrifuged at 20,000g (MSE High speed 18 refrigerated centrifuge) for 30 minutes. The supernatant from this spin was then carefully removed from the pellet, and the microsomal membranes sedimented from the supernatant by centrifugation at 100,000g for one hour (MSE Prepspin 50 ultracentrifuge). The supernatant was discarded and the pellet washed by resuspension in a low ionic strength buffer (10mM Imidazole, 1mM EDTA, pH 7.2 at room temperature) and resedimented by centrifugation at 100,000g for one hour. The final pellet was resuspended in the low ionic strength buffer at a protein concentration of 0.8 - 1.2 $mg.cm^{-3}$ and stored on ice until required.

(2) Extraction of membrane preparations with detergents.

(a) Determination of the optimal detergent concentration for the extraction of membranes with Sodium deoxycholate. The procedure adopted was adapted from that described by Jorgensen (1974b), for rabbit kidney microsomes.

Prepared solutions

(i) Buffered Medium . . . . pH 7.0 at 20°C (HCl)

2 x 2mM E.D.T.A.

2 x 88mM Sucrose

1.5 x 50mM Imidazole

(ii) Buffered deoxycholate media . . . pH 7.0 at 20°C (HCl)

8 solutions were prepared containing 50mM Imidazole and sodium deoxycholate at concentrations 4 x required concentration. The deoxycholate solutions were prepared freshly, so as to span a final concentration range of 0-800 $\mu g.cm^{-3}$ with an even spacing of concentrations.

(iii) Membrane preparation.

The stock membrane preparation was diluted (if necessary) to a membrane protein concentration of 4 x 200-300 $\mu g.cm^{-3}$, with the low ionic strength buffer.

Two volumes of the buffered medium were mixed with one volume of the membrane preparation and the mixture thermo-equilibriated at 20^oC for 5-10 minutes. One volume of a pre-warmed buffered deoxycholate medium was slowly added to the above mixture (with stirring) and the final mixture incubated at 20^oC for 20 minutes. The mixture was then diluted with twice its volume of ice cold low ionic strength buffer. An aliquot was stored on ice for enzyme assay and the remainder subjected to centrifugation at 100,000g (temperature $\approx$ 0-4^oC) for 45 minutes to sediment the microsomes. The pellet was then washed by two cycles of resuspension in ice cold low ionic strength buffer and centrifugation at 100,000g. The final pellet was resuspended in the low ionic strength buffer to a protein concentration of 0.8-1.2 $mg.cm^{-3}$ and stored on ice until required.

(b) Determination of the optimal detergent concentration for the extraction of membranes with sodium dodecyl sulphate. The procedure adopted was adapted from that described by Jorgensen (1974b) for rabbit kidney microsomes.

Prepared solutions

(i) Buffered medium . . . . . pH 7.5 at 20^oC (HCl)

4 x 2mM E.D.T.A.

3 x 50mM Imidazole

(ii) Adenosine Triphosphate . . pH 7.5 at 20^oC (Tris)

4 x 3mM A.T.P. (Sigma Grade I - Cat. No. A2383)

(iii) Buffered dodecyl sulphate media . . pH 7.5 at 20^oC (HCl)

8 solutions were prepared containing 50mM Imidazole and

sodium dodecyl sulphate at concentrations 4 x required concentration. The dodecyl sulphate solutions were prepared freshly, so as to span a final concentration range of 0 - 175 $\mu g.cm^{-3}$ with an even spacing of concentrations.

(iv) Membrane preparation.

This was prepared as described for the deoxycholate extraction above.

One volume of the buffered medium was mixed with one volume of ATP and one volume of the membrane preparation. The mixture was thermoequilibriated at $20^{o}C$ for 5-10 minutes. One volume of pre-warmed buffered dodecyl sulphate medium was slowly added to the above mixture and the final mixture incubated at $20^{o}C$ for 45 minutes. The mixture was then diluted with twice its volume of an ice cold washing buffer (50mM Imidazole, 2mM EDTA, 3mM ATP, pH 7.5 at $20^{o}C$). A sample was retained on ice for enzyme assays and the rest of the membranes sedimented by centrifugation at 100,000g (temperature ≈ $0-4^{o}C$) for 45 minutes. The pellet was then washed three times in the washing buffer and once in the low ionic strength buffer. The final pellet was resuspended in the low ionic strength buffer to a protein concentration of 0.8 - 1.2 $mg.cm^{-3}$ and stored on ice until required.

(c) Extraction of membranes with Cirrasol ALN-WF (Lubrol).

The procedure adopted was adapted from that described by Lewis (1974), for pig brain synaptic membranes. All operations were carried out at $0-4^{o}C$.

Prepared solutions

Iodide extraction medium . . . . pH 8.1 at $20^{o}C$ (HCl)

3.2M Sodium iodide

100mM tris

1mM E.D.T.A.

Adenosine Triphosphate . . . pH 7.5 at 20°C (Tris)

4 x 3mM A.T.P. (Sigma Grade I - Cat. no. A2383).

Membrane preparation.

The stock membrane preparation was diluted with low ionic strength buffer (if necessary) to a membrane protein concentration of 5.5 - 6.5 mg.cm^{-3}.

Cirrasol ALN-WF (Lubrol)

A solution of the detergent was made up in distilled water at a detergent concentration of 4mg.cm^{-3}.
One volume of the membrane preparation was mixed with one volume of ATP. The mixture so formed was gently stirred while two volumes of the iodide extraction medium were slowly added. The mixture was then stood on ice for 30 minutes, and then distilled water was added to dilute the iodide concentration to 0.6M. The membranes were then sedimented from the mixture by centrifugation at 100,000g for one hour. The pellet was then washed once in the low ionic strength buffer and once in distilled water, and was resuspended in distilled water to a protein concentration of 4mg.cm^{-3}. The suspension so obtained was then treated with an equal volume of lubrol solution (with stirring) and the mixture gently homogenised in a teflon lined glass homogeniser. It was then stood on ice for 15 minutes, and centrifuged at 100,000g for one hour. The supernatant was then carefully removed from the pellet and centrifuged for 24 hours at 100,000g. The final gelatinous pellet was resuspended in distilled water to a protein concentration of 1-1.5 mg.cm^{-3}.

(3) S.D.S. polyacrylamide gel electrophoresis.

The procedure adopted was adapted from that described by Weber & Osborn (1969).

Prepared solutions.

Running buffer . . . pH 7.0 at 20°C

100mM phosphate buffer (NaH_2PO_4, Na_2HPO_4) containing 0.1% Sodium dodecyl sulphate.

Protein solubilisation medium . . . pH 7.0 at 20°C

10mM phosphate buffer (NaH_2PO_4, Na_2HPO_4) containing 0.1% Sodium dodecyl sulphate and 1% (v/v)2-mercaptoethanol.

Preparation of polyacrylamide gels.

6.82g of acrylamide and 182mg of methylene bis acrylamide were dissolved in 95 cm^3 of the running buffer and the mixture was degassed by a water driven suction pump. 5 cm^3 of 5% (w/v) Ammonium persulphate were added to the degassed mixture. The gels were polymerised in 15 cm long glass tubes (10mm I.D.). The tubes were secured in the vertical position in a jacket thermostated at 20°C with their lower ends covered with sealing film. 0.05 cm^3 of were then added to 100 cm^3 of the acrylamide mixture which was quickly mixed and introduced into the glass tubes, to a level of 12 cm. Distilled water was then carefully layered on top of the acrylamide mixture in the tubes so as to obtain a smooth horizontal interface between the acrylamide and the water. The system was then left undisturbed at 20°C until the polymerisation was complete.

Preparation and electrophoresing of protein samples.

Two volumes of a protein sample (1.5-2mg cm^{-3}) were mixed with one volume of the protein solubilisation medium and the mixture incubated at 37°C for two hours. The mixture was then mixed with one tenth its volume of glycerol and 0.05 cm^3 of 1% Bromophenol blue

were added to the sample which was thoroughly mixed. The gels were then set up with the running buffer in both electrode compartments. A volume of sample (0.1 cm^3) containing approximately 100 μg of protein was then layered on the surface of the gel. The electrophoresis was run at a current of 8mA per gel until the marker dye (Bromophenol blue) had travelled for 9 cm. The gels were then removed from the tubes and transected at the marker dye position. The gels were then stained for total protein in a staining solution (0.1% (w/v) Coomassic Brilliant Blue G250 in 50% Methanol (v/v), 7% acetic acid (v/v) for 12 hours. Excess stain was removed by a continuous washing in 10% Methanol (v/v)-7% Acetic acid (v/v). The gels were finally stored in 7% Acetic acid (v/v).

(4) Assay of Na^+-K^+-ATPase activity.

(a) Standard assay

The procedure has been described in Chapter 2.

(b) Assays at varying temperatures.

The procedure has been described in Chapter 4.

(c) Assays at non-saturating ouabain concentrations.

The procedure has been described in Chapter 3.

(d) Assays at non-saturating potassium concentrations.

The procedure has been described in Chapter 5.

(5) Determination of protein concentration.

The procedure has been described in Chapter 2.

RESULTS

(a) The enrichment of membranes in Na^+-K^+-ATPase by extraction with detergents.

The effects of sodium deoxycholate on the specific activity of the Na^+-K^+-ATPase from rat brain synaptic membranes are shown in figure 6.1. It is clear that, under the stated conditions, the incubation of the membranes with deoxycholate at concentrations up to 400 μg cm^{-3} results in an increase in total Na^+-K^+-ATPase activity which at the peak is about 60% greater than the crude preparation. At higher detergent concentrations (>400 μg cm^{-3}), there is a progressive decline in total Na^+-K^+-ATPase activity. The specific activity of the enzyme in membranes sedimented from the detergent incubation medium was greater than the apparent specific activity of the enzyme, whilst in the incubation medium, at all detergent concentrations investigated. However, the response curve described by the membranes sedimented from the incubation medium had a broader peak, showing virtually no change in specific activity for detergent concentrations in the range 400-600 μg cm^{-3}. At higher detergent concentrations (>600 μg cm^{-3}), there was a sharp decline in the specific activity of the membranes sedimented from the detergent incubation medium.

The response of the Na^+-K^+-ATPase from the rat brain synaptic membranes to incubation with sodium dodecyl sulphate is shown in figure 6.2. Like that observed for the deoxycholate, there was an increase in the total Na^+-K^+-ATPase activity at low detergent concentrations, followed by a progressive decline in total activity

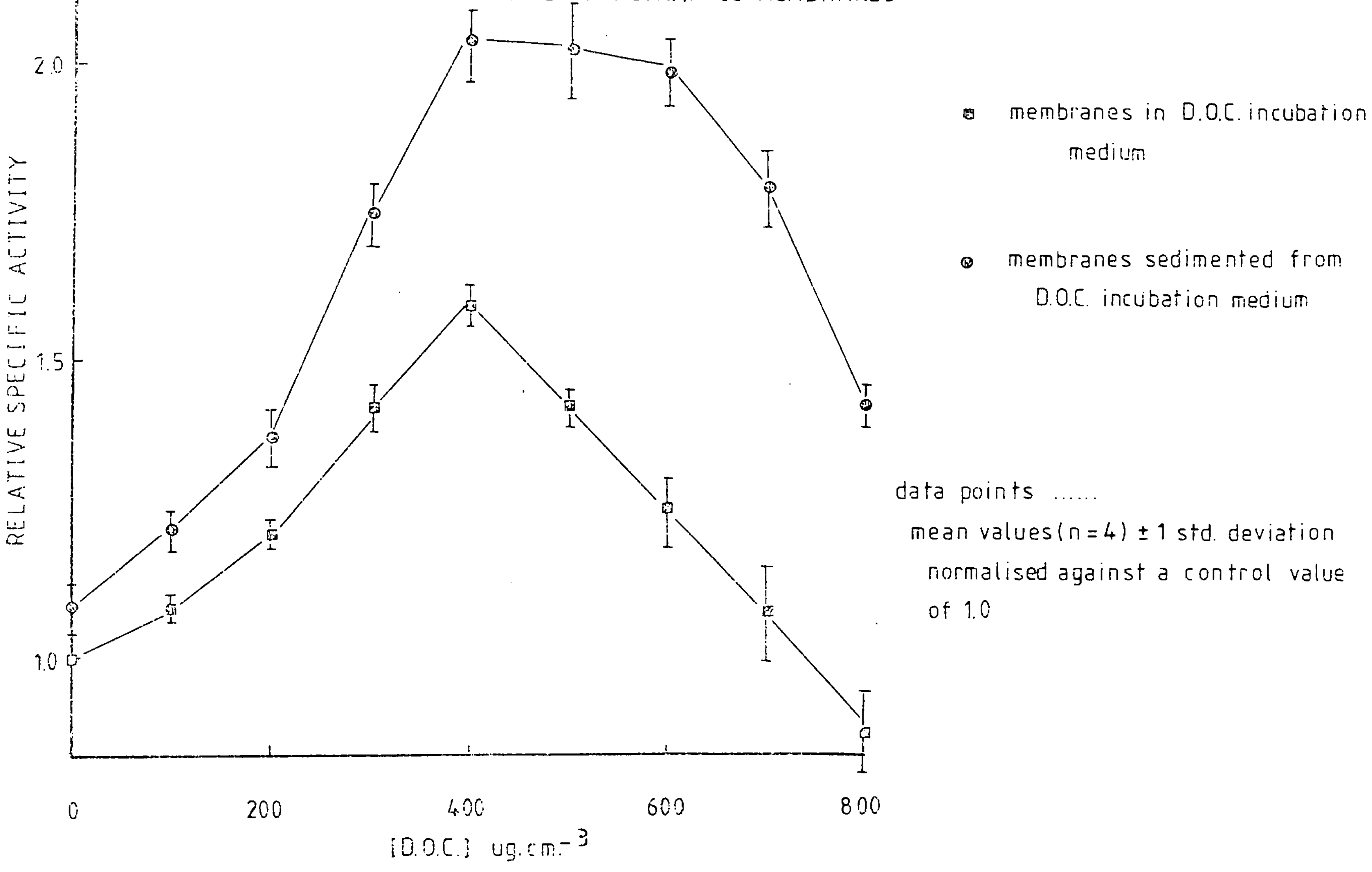


Fig. 6.1
DEOXYCHOLATE RESPONSE PROFILE OF THE
Na+-K+-ATPase FROM RAT BRAIN SYNAPTIC MEMBRANES
RELATIVE SPECIFIC ACTIVITY
2.0
1.5
1.0
0
200
400
600
800
[D.O.C.] ug.cm.-3
membranes in D.O.C. incubation medium
membranes sedimented from D.O.C. incubation medium
data points ......
mean values (n = 4) ± 1 std. deviation
normalised against a control value
of 1.0

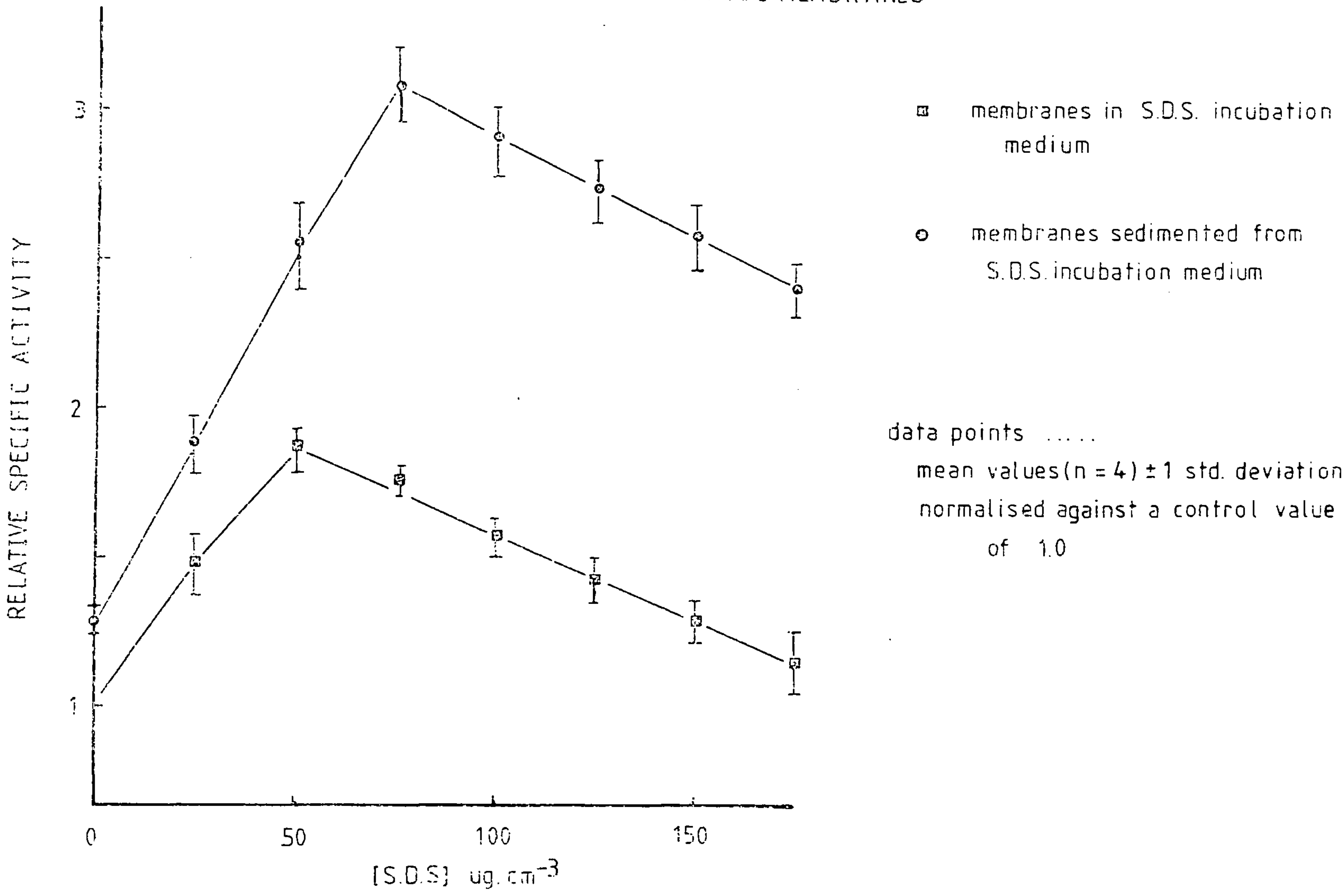


Fig.6.2
DODECYL SULPHATE RESPONSE PROFILE OF THE
Na+-K+-ATPase FROM RAT BRAIN SYNAPTIC MEMBRANES
RELATIVE SPECIFIC ACTIVITY
3
2
1
0
50
100
150
[S.D.S] ug.cm-3
membranes in S.D.S. incubation medium
membranes sedimented from S.D.S. incubation medium
data points .....
mean values(n = 4) ± 1 std. deviation
normalised against a control value
of 1.0

at the higher detergent concentrations. However the effects of the detergent occurred at lower detergent concentrations (mass and molar) in the case of the dodecylsulphate, and the peak increase in total activity (≈83%) was greater than that observed for deoxycholate. The specific activities of the enzyme in the membranes sedimented from the incubation media were also greater than their apparent specific activities whilst in the incubation media, and their absolute peak values obtained, were about 50% greater than those obtained from similar samples incubated in deoxycholate. Unlike the deoxycholate incubated samples, the response profile described by the samples sedimented from dodecyl sulphate had a narrow peak which occurred at a higher detergent concentration (75 $\mu g.cm^{-3}$) than that observed for the membranes in the incubation medium (50 $\mu g.cm^{-3}$).

The above results are broadly similar to those previously reported for rabbit kidney microsomes (Jorgensen & Skou, 1971). Since it has been shown that detergent treatment similar to that used here does not affect the 'molecular activity' of the Na^+-K^+-ATPase (Jorgensen, 1974a), the increase in total activity by incubation with low detergent concentrations was attributed to the exposure of occluded Na^+-K^+-ATPase sites by the interaction of the detergent with the membranes, while the increased specific activity of the membrane preparations sedimented from the detergent was attributed to the extraction of 'inactive' protein from the membrane preparations. Thus the above results suggested that deoxycholate concentrations of 400 $\mu g.cm^{-3}$ and dodecylsulphate concentrations of 75 $\mu g\ cm^{-3}$ were optimal for the preparation of membranes enriched in Na^+-K^+-ATPase under the given conditions. However, since it has been established that the

detergent concentration producing optimal effects is dependent on the protein concentration in the incubation medium, and other factors (see Jorgensen &Skou, 1971), the above detergent concentrations were not considered suitable for the routine reproducible preparations of membrane samples enriched by these detergents, on account of the practical problems of reproducing the exact incubation conditions. Thus the routine extraction of membranes was done at detergent concentrations of 500 $\mu g.cm^{-3}$ for deoxycholate and 100 $\mu g.cm^{-3}$ for dodecylsulphate.

The specific activities of crude and detergent extracted rat brain synaptic membrane preparations with respect to Na^{+}-K^{+}-ATPase activity are given in Table 6.1. This shows that the deoxycholate extraction procedure results in a two fold increase in specific Na^{+}-K^{+}-ATPase activity over that of the control, while a three fold increase in specific activity was observed on dodecylsulphate extraction. The preparations yielding the highest specific activity were the lubrol extracted preparations. However, unlike the crude, deoxycholate extracted, and dodecylsulphate extracted preparations, the lubrol extracted preparation was found to be labile (half life ≃ 12 hours at 0-4°C). The other preparations were found to be stable at 0-4°C for at least 25 days. The absolute specific activity values obtained after detergent extraction of the rat brain synaptic membranes were considerably lower than those of some of the highly active samples prepared in other laboratories (Uesegi _et al_, 1971; Nakao _et al_, 1974; Jorgensen, 1974b; Kyte, 1971b. These other preparations have been made from other tissue sources and by different methodology for detergent extraction of the membranes, and

TABLE 6.1

Absolute and relative specific activities of the Na^{+}-K^{+}-ATPase from rat brain synaptic membranes before and after mild extraction with detergents.

SAMPLE	$^{\Delta}$SPECIFIC ACTIVITY	*RELATIVE ACTIVITY
CRUDE	55 - 80	100
D.O.C. extract	110 - 130	200±12
S.D.S. extract	180 - 200	308±18
Lubrol extract	†150 - 550	370±123

Δ Activity units ..μMoles Pi mg.protein^{-1}. hr^{-1} at 37°C

* Relative activity.... values normalised against 100 for the crude preparation, mean of 4 preps ± 1 Standard deviation.

† Samples were labile... activities were measured soon after preparation of the sample.

Range of activity values established from a minimum of 4 observations.

it is generally found that the success of any given procedure in producing a sample of high specific Na^{+}-K^{+}-ATPase activity is difficult to reproduce in other laboratories (see Schwartz, Lindenmayer & Allen, 1975). Furthermore, it is also generally found that the success of any given procedure in 'purifying' the Na^{+}-K^{+}-ATPase is largely dependent on the initial tissue source (see Schwartz, Lindenmayer & Allen, 1975). Thus the effectiveness of the detergent extraction procedures used and the probable influence of tissue source were investigated.

The effect of extracting various membrane preparations with Sodium dodecylsulphate (100 $\mu g.cm^{-3}$) is shown in Table 6.2. It is clear that the increase in total activity and the specific activity of the final washed membrane preparation are dependent on the specific activity of the initial preparation, since higher specific activity samples are obtained if the initial preparations are themselves rich in Na^{+}-K^{+}-ATPase activity. Furthermore, the extent of enrichment of any given sample by the procedure adopted, varied from one sample to the next, as evidenced by the varying relative increases in the specific activity of the final preparations over that of the membranes in the detergent incubation medium (rat submaxillary gland microsomes $\simeq$ x 1.4 vs hamster brain synaptic membranes $\simeq$ x 2.5). The above results supported the view that the source of any given membrane sample is a big factor in determining how effective any 'purification' method can be as far as the Na^{+}-K^{+}-ATPase is concerned.

The effectiveness of the detergent extraction procedures used in 'purifying' the Na^{+}-K^{+}-ATPase from rat brain synaptic membranes was

TABLE 6.2

Comparison of the specific activities of the Na^{+}-K^{+}-ATPases from crude and S.D.S. extracts of membranes from different sources.

Membrane source	CRUDE SAMPLE		SAMPLE A		SAMPLE B	
	Range	n	Range	n	Range	n
Rat brain microsomes	18-25	8	60-80	4	160-180	4
Rat brain synaptic membranes	55-80	16	110-140	5	180-200	14
Mouse brain synaptic membranes	60-80	9	120-150	4	220-260	9
Hamster brain synaptic membranes	70-90	11	130-150	3	320-360	9
Trout brain synaptic membranes	160-210	7	300-400	3	600-800	7
Trout brain microsomes	60-80	6	180-210	3	300-350	3
Rat submaxillary gland microsomes	20-25	4	50-60	3	70-80	3

Range of specific activities established from n observations.

Specific activities were determined at 37°C under standard assay conditions.

1 unit of specific activity = 1μMole Pi. $mg.protein^{-1}$. hr^{-1}.

SAMPLE A - Membranes in S.D.S. incubation medium (see Text)

SAMPLE B - Washed membranes sedimented from the S.D.S. incubation medium (see Text)

further investigated by comparing the distribution of protein species in the final membrane samples by S.D.S. polyacrylamide gel electrophoresis (see Plate 6(I). The results showed that the extraction of the membranes with deoxycholate and dodecylsulphate did not appear to have had major effects on the distribution of polypeptide species in the membrane preparations, since most of the high and low molecular weight polypeptide species observed in the crude sample were also present in the detergent extracted preparations. However, there was evidence that the deoxycholate and dodecylsulphate extraction procedures resulted in the removal of a few low molecular weight polypeptide species and that deoxycholate was more effective than dodecylsulphate in this respect. Furthermore, there was also evidence of a significant alteration in the distribution of the polypeptide species in the lubrol extracted sample since several high and low molecular weight species were absent from the lubrol extracted preparation when compared with the crude membranes.

(b) Effects of detergent extraction on some of the kinetic properties of the Na^{+}-K^{+}-ATPase.

The kinetic measurements were done on the enzyme found in crude, deoxycholate extracted (@ 500 $\mu g.cm^{-3}$) and dodecylsulphate extracted (@ 100 $\mu g.cm^{-3}$) rat brain synaptic membranes. The Na^{+}-K^{+}-ATPase activity from lubrol extracts of these synaptic membranes were too labile for the taking of meaningful kinetic measurements. The dose response ouabain sensitivities of the three preparations are shown in figure 6.3. It is clear that the response of the preparations to increasing ouabain concentrations is biphasic, and that the curves

PLATE 6(1)

S.D.S. electrophoresis of rat brain synaptic membranes.

A Crude membranes

B Dodecylsulphate extracted membranes

C Deoxycholated extracted membranes

D Lubrol extracted membranes

Arrow shows the direction of migration of the polypeptides.

A

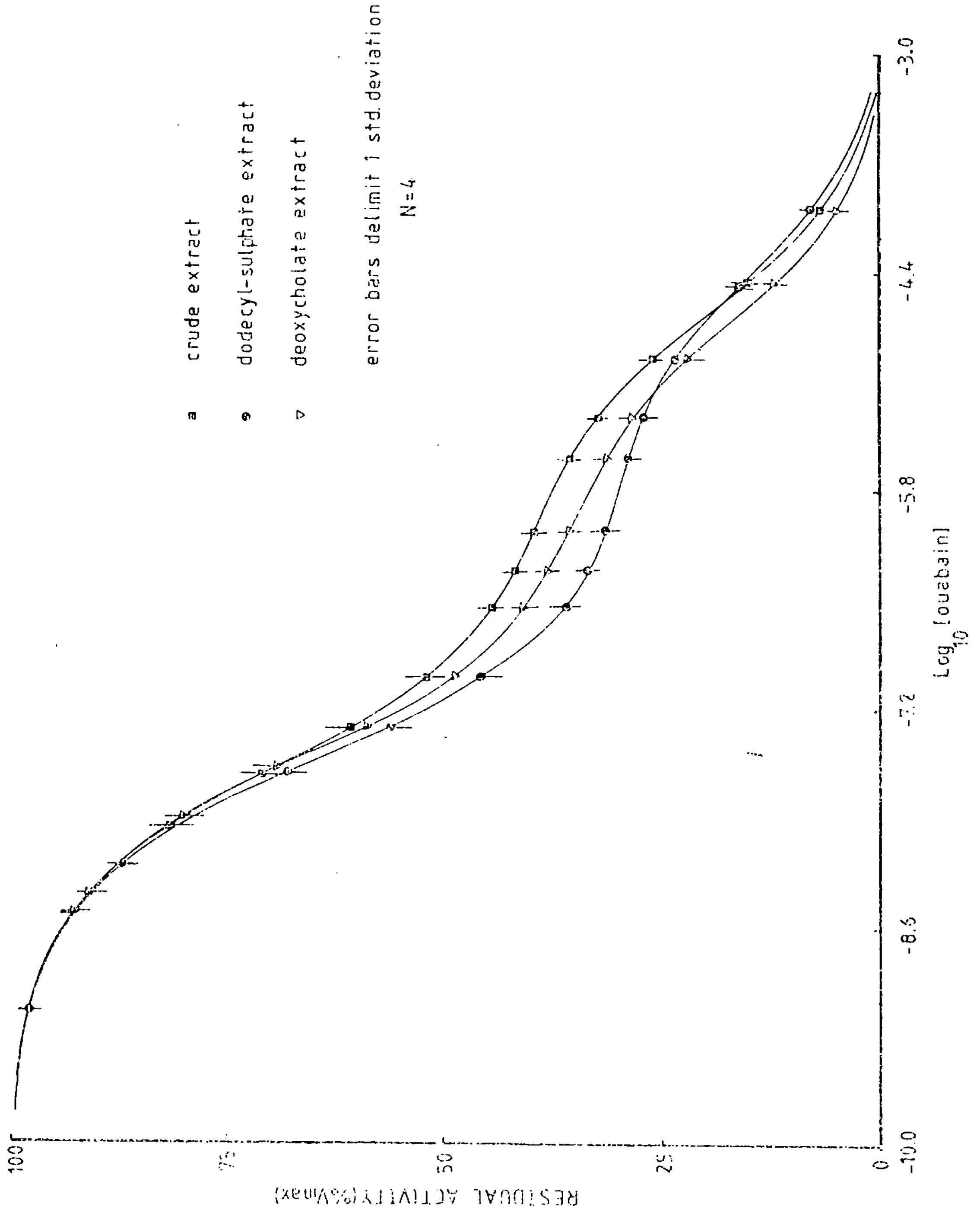
B

C

D

Fig.6.3

OUABAIN INHIBITION OF THE Na^{+}-K^{+}-ATPase FROM RAT BRAIN SYNAPTIC MEMBRANES



tend to converge at low ouabain concentrations ($\leq 10^{-8}$M) and at the high ($>10^{-4}$M). However the curves diverge significantly from each other at ouabain concentrations where the inflection in the curves is obvious (10^{-7} - 10^{-5}M). The parameters defining these curves (see Table 6.3a) show the detergent extraction has had very small effects on the K_i parameters ($p>0.05$), and that the significant effects of the detergent treatments appear to be on the co-operativity parameters ϕ and β (see Chapter 3). The deoxycholate preparation appeared to be less modified than the dodecylsulphate extract since smaller changes (relative to the crude preparation) were observed. The results also suggested that the apparent ouabain binding affinities at both ouabain binding sites were unaffected by the detergent extraction since all the K_i values and the products βK_i were not significantly different from each other ($p>0.05$). Thus the small but significant differences in the apparent ouabain sensitivities of the samples ($p>0.02$) can be solely attributed to the effects of the detergent extraction on the catalytic co-operativity parameters ϕ.

Figure 6.4 shows the potassium activation kinetics of the Na^+-K^+-ATPase from the crude and detergent extracted membranes. The pattern of activation described by these three samples was sigmoidal and the deoxycholate extract appeared slightly less potassium sensitive than the others. The parameter lits defining the fitted curves (see Table 6.3b) show that the detergent extraction had little or no effect on the K_a values (potassium concentrations for half-saturation of the high affinity sites), since those of the detergent extracted samples were not significantly different from that of the crude preparation ($p>0.05$). The K_b values (potassium concentrations for

TABLE 6.3

Effects of detergent treatment on rat brain Na^{+}-K^{+}-ATPase

A. OUABAIN INHIBITION

Parameter	Crude	D.O.C. extracted	S.D.S. extracted
* K_i (M)	$2.32\times10^{-8} \pm 3.5\times10^{-9}$	$2.72\times10^{-8} \pm 2.07\times10^{-9}$	$2.76\times10^{-8} \pm 1.5\times10^{-9}$
* β	866 ± 25	632 ± 78	1282 ± 43
* ϕ	0.79 ± 0.031	0.716 ± 0.028	0.601 ± 0.016
apparent pI_{50}	6.95 ± 0.003	7.03 ± 0.007	7.16 ± 0.008

* See Chapter 3

B. POTASSIUM ACTIVATION

Parameter	Crude	D.O.C. extracted	S.D.S. extracted
▲ K_a (M)	$2.25 \pm 10^{-4} \pm 6.67\times10^{-5}$	$2.19\times10^{-4} \pm 7.1\times10^{-5}$	$4.09\times10^{-4} \pm 1.34\times10^{-4}$
▲ K_b (M)	$1.26\times10^{-3} \pm 4.97\times10^{-5}$	$1.65\times10^{-3} \pm 1.35\times10^{-4}$	$1.16\times10^{-3} \pm 1.0\times10^{-4}$
$K_{0.5}$ (M)	$1.45\times10^{-3} \pm 1.13\times10^{-5}$	$1.85\times10^{-3} \pm 2.1\times10^{-4}$	$1.47\times10^{-3} \pm 5.65\times10^{-5}$

▲ See Chapter 5

C. TEMPERATURE DEPENDENCE

Parameter	Crude	D.O.C. extracted	S.D.S. extracted
† μ ($K.J.mol^{-1}$)	67.7 ± 4.4	65.6 ± 2.4	75.2 ± 5.8
† ΔH ($K.J.mol^{-1}$)	-170.7 ± 9.0	-142.2 ± 6.4	-176 ± 19
† ΔS ($J.K.^{-1}mol^{-1}$)	-593 ± 32	-491 ± 12	-611 ± 66

† See Chapter 4

All values mean of 4 preparations $\pm$ 1 standard deviation

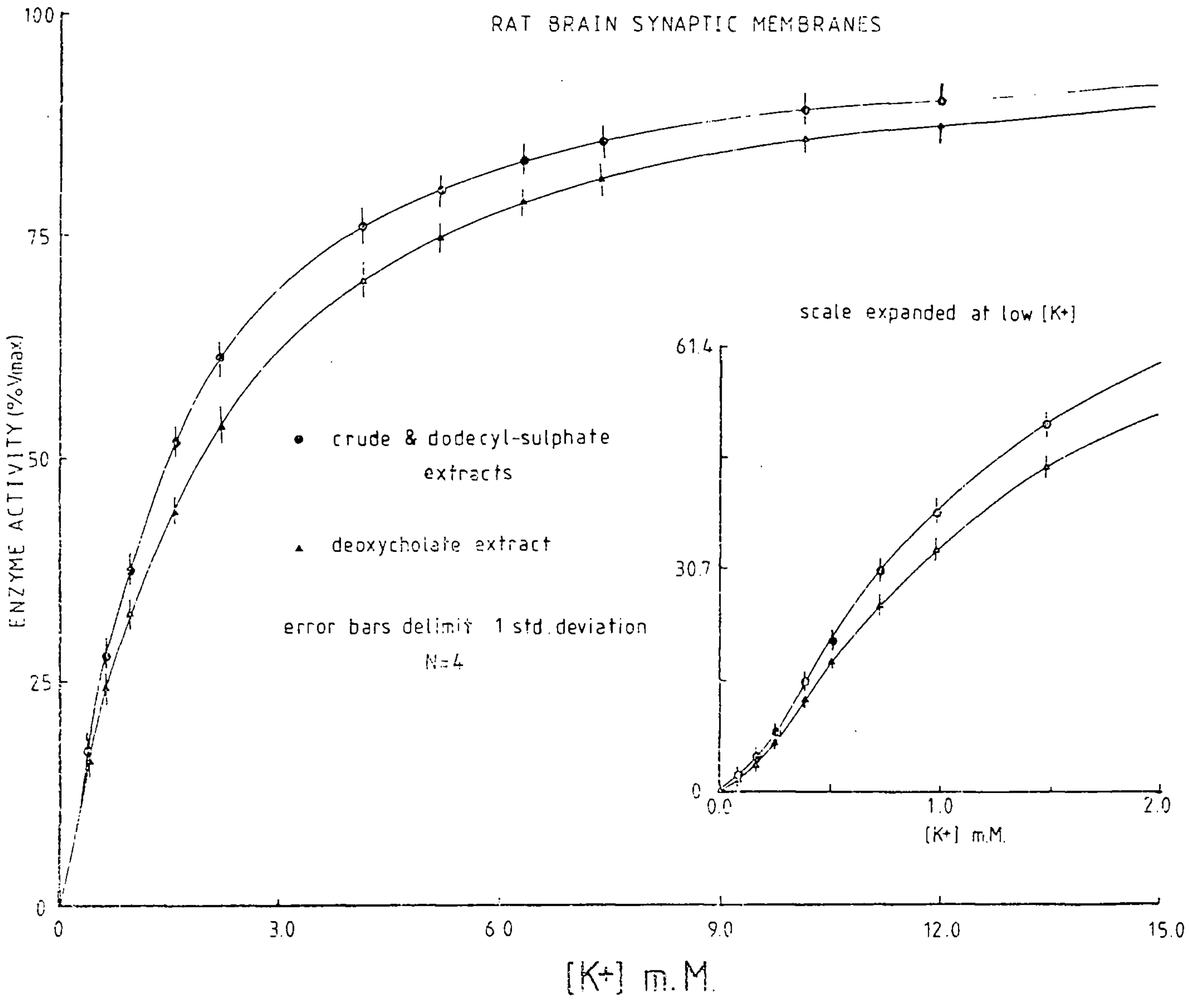


Fig. 6.4

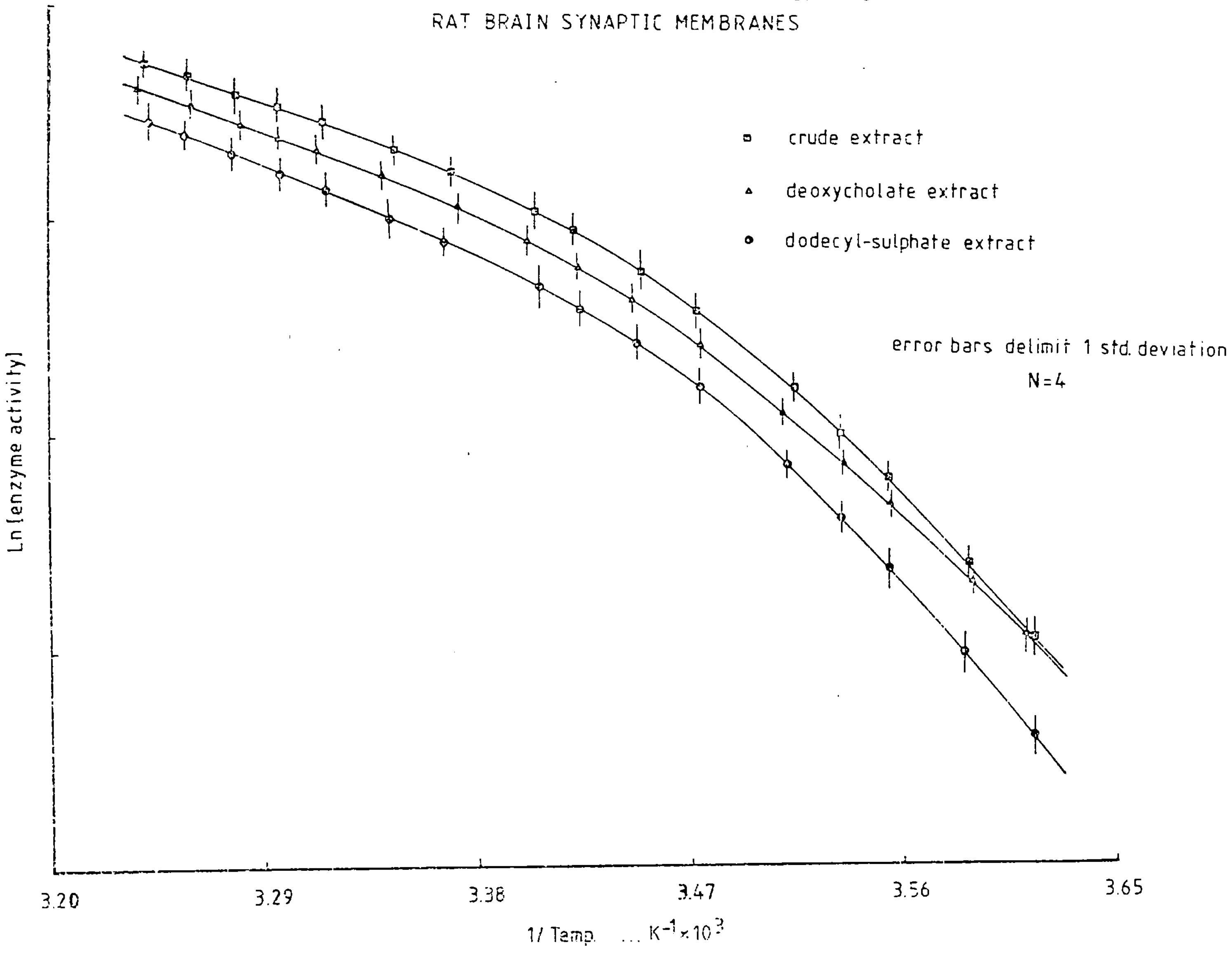
POTASSIUM ACTIVATION OF THE Na+-K+-ATPase FROM RAT BRAIN SYNAPTIC MEMBRANES

half saturation of the low affinity sites) showed that dodecylsulphate extraction had no significant effect on the potassium activation kinetics of the enzyme and a suggestion further underlined by the relevant $K_{0.5}$ values (potassium concentrations at half maximal activity) which are almost identical. In the case of the deoxycholate extracted sample, for which the K_b and $K_{0.5}$ values were slightly but significantly greater ($p<0.02$), the extraction with the detergent resulted in a decreased sensitivity to potassium ion.

The temperature kinetics of the enzyme from the three membrane samples are shown as Arrhenius plots in figure 6.5. It is clear that the crude and detergent extracted samples all describe non-ideal Arrhenius plots, and that the curvature of the plot described by the deoxycholate preparation is considerably less than that described by the crude and dodecylsulphate extracted preparations. The parameter lists defining the curves drawn (see Table 6.3c) show that dodecylsulphate treatment has not significantly affected the temperature kinetics of the enzyme since none of the defining parameters (μ, ΔH, ΔS) were found to be significantly altered (relative to the crude preparation) by extraction with this detergent. On comparing the defining parameters of the deoxycholate extracted sample with those of the crude sample, no significant difference was found between the apparent activation energies of their active states. However, the lower values returned for the ΔH and ΔS parameters of the deoxycholate extracted samples, suggested that, the transition to the inactive state was affected by extraction with this detergent, being broader in the case of the deoxycholate extracted sample.

Fig. 6.5

TEMPERATURE KINETICS OF THE Na+-K+-ATPase FROM RAT BRAIN SYNAPTIC MEMBRANES



DISCUSSION

The above results underlined some important points about the methodology of preparing membrane samples highly enriched in Na^{+}-K^{+}-ATPase activity. First, the effectiveness of any enrichment procedure is heavily dependent on the nature of the crude membrane source. This is evident from the large differences in specific Na^{+}-K^{+}-ATPase activity recorded here for sodium dodecylsulphate extracts of the various membrane sources. The absolute specific activity values measured here, for the S.D.S. extracts of mammalian brain are of the same order of magnitude as that reported for SDS extracts of canine brain (Sweadner, 1978). These values, however, are considerably smaller than that found when a similar procedure was applied to rabbit kidney (Jorgensen, 1974b). Furthermore, the application of similar extraction procedures to different mammalian kidney preparations produced samples of different specific Na^{+}-K^{+}-ATPase activities (Jorgensen, 1974a). This dependence of the specific activity of the final preparation on the source of the crude membrane sample is further emphasised by the observation that highly 'purified' samples have been successfully prepared only from a narrow functional range of tissue types (e.g. tissues specialised in electrical phenomena like brain (Nakao, *et al*, 1974) and ell electric organ (Dixon & Hokin, 1974) and tissues specialised in osmoregulatory function like mammalian kidney (Jorgensen 1974b) and elasmobranch rectal gland (Hokin, Dahl, Deupree, Dixon, Hackney & Perdue, 1973).

Secondly, the extent of 'purification' of the enzyme from a given membrane source varies considerably with the detergent extraction

procedure used. This is suggested by the large differences in absolute specific activities of the enzyme in the various detergent extracts of rat brain synaptic membranes. S.D.S. polyacrylamide gel electrophoresis also showed that the extent to which membrane bound protein was extracted from the membranes varied with the nature of the detergent used, since there were fewer polypeptide species detected in the lubrol extracted membranes. This probably accounted for the higher specific Na^+-K^+-ATPase activities recorded for the lubrol extract. The variations in the response of the rat brain synaptic membranes to the different detergents thus suggested that surface tension effects (see Jorgensen & Skou, 1971) was not the only factor operating, and that the different ionic properties of the surfactants used probably had a significant influence on the effectiveness of the given detergent in the 'purification' procedure. This would probably account for the different increases in peak total Na^+-K^+-ATPase activity observed when rat brain synaptic membrane samples were incubated with deoxycholate and dodecylsulphate. Given that such increases in total activity have been attributed to the exposure of occluded enzyme sites (Jorgensen, 1974c; Sweadner, 1978), the results apparently suggest that sodium dodecyl sulphate exposed the occluded Na^+-K^+-ATPase sites by a mechanism less deleterious to Na^+-K^+-ATPase activity than that of sodium deoxycholate. Such would be compatible with the observation that although the specific Na^+-K^+-ATPase activity of the dodecylsulphate extract was greater than that of the deoxycholate extract, fewer polypeptide species were detected by S.D.S. polyacrylamide gel electrophoresis of the latter extract when compared with the dodecylsulphate extract.

The results also suggested that some aspects of the properties of the Na^+-K^+-ATPase may be sensitive to the detergent extraction procedure used. This is especially so in the case of the kinetic stability of the enzyme to denaturation. In this case, lubrol extraction severely affected the ability of the enzyme to retain its biologically active conformational states. Since lubrol type detergents have been used to produce 'soluble' membrane extracts containing Na^+-K^+-ATPase activity (Lewis, 1974; Nakao _et al_, 1974; Uesegi _et al_, 1971) the target of lubrol action is generally believed to be the membrane lipids and thus the instability of the lubrol extracts reported here, and elsewhere (Nakao _et al_, 1974; Atkinson, Gatenby & Lowe, 1971) can be explained by the delipidation of the membranes by the detergent, to the extent that the retention of biological activity is endangered. The relative stability of the deoxycholate and dodecylsulphate extracts thus suggested that the conditions under which these two detergents were used, did not result in a delipidation of the membrane sufficient to affect the stability of the enzyme.

The kinetic measurements suggested that the deoxycholate and dodecylsulphate extraction procedures used had very small effects on the observed kinetic properties of the enzyme. Such effects were apparently smaller in the case of the dodecylsulphate extract where significant effects were only found in the ouabain inhibition co-operativity parameters. In the case of the deoxycholate extract, all the kinetic properties measured were affected. This supported the suggestion for a different mechanism of action of the deoxycholate.

Given that there is evidence that sodium dodecyl sulphate is primarily a protein targeted surfactant (Chan, 1967), deoxycholate can be assumed to be membrane lipid targeted like lubrol, but milder than the latter under the conditions used. If such is the case, then the data can possibly be interpreted as indicating a role for the membrane in the control of kinetic properties. The above considerations however, are subject to some ambiguity. Given that the specific activity of the deoxycholate extracted sample is lower than that of the dodecylsulphate extracted sample, in spite of the latter containing more polypeptide species than the deoxycholate extract, it seems probable that deoxycholate extraction resulted in a partial denaturation of the functional Na^+-K^+-ATPase. Since as shown in Chapter 9, a partial denaturation of the Na^+-K^+-ATPase has significant effects on the kinetic properties measured, the observed effects of deoxycholate extraction could be the product of such partial denaturation and not be indicative of a role for the membrane in the control of kinetic properties.

Finally, the stability of the dodecylsulphate extract and the absence of major effects on its kinetic properties as a result of detergent extraction, suggested that the use of such preparations in further preparations should not be subject to any major ambiguities. The properties of this preparation are thus in agreement with the reported failure to detect significant changes in the kinetic properties of Na^+-K^+-ATPase after 'purification' by extraction with detergents in cases where stable detergent extracts are obtained (Kline, Hexum, Dahl & Hokin, 1971; Ratanabanangkoon, Dixon & Hokin, 1973) and as a result this procedure was adopted for the preparation of membrane samples for further studies.

CHAPTER 7

A study of the thermal inactivation of the Na^{+}-K^{+}-ATPase

INTRODUCTION

A role for membrane lipid in the maintenance of the biological activity of the Na^{+}-K^{+}-ATPase was first indicated by the reported loss of activity from erythrocyte ghosts after enzymic delipidation of those membranes (Schatzmann, 1962). This was later supported by reports that Na^{+}-K^{+}-ATPase activity could be partially restored to delipidated biomembranes by incubation with phospholipids (Tanaka & Strickland, 1965; Fenster & Copenhaver, 1967; Taniguchi & Tonomura, 1971). The implicit suggestion of a special role for phospholipids in the functioning of this enzyme, has been confirmed by a large number of workers using a variety of tissue sources and experimental techniques with the result that the requirement for phospholipids in the sustenance of the kinetic stability of the Na^{+}-K^{+}-ATPase is generally accepted.

The experimental evidence supporting the role of phospholipids in maintaining the biological activity of the Na^{+}-K^{+}-ATPase is almost completely dependent on the use of potent membrane disrupting reagents or procedures. These procedures include:

(a) The delipidation of membranes by the use of phospholipases (Schatzmann, 1962; Taniguchi & Tonomura, 1971; Sun, Sun & Samorajski, 1971), and organic solvents (Jarnefelt, 1972; Noguchi & Freed, 1971).

(b) The 'fluidising' of membranes by the use of aliphatic alcohols (Sun & Samorajski, 1970; Grisham & Barnett, 1972, 1973).

(c) The 'solubilisation' of membranes with high concentrations of lipid targeted detergents (Tanaka & Strickland, 1965; Fenster & Copenhaver, 1967; Atkinson, Gatenby & Lowe, 1971).

Whenever such procedures were applied, the membrane phospholipid dependence of the Na^{+}-K^{+}-ATPase, was made obvious by a loss of catalytic activity or by the relative thermo-lability of the resultant preparation, and by the partial restoration or stabilisation of Na^{+}-K^{+}-ATPase activity by post-incubation of the treated samples with phospholipids. Thus, although the disruption of the membrane by the above procedures is too severe for a direct extrapolation to the in-vivo situation, these reports clearly link the kinetic stability of the Na^{+}-K^{+}-ATPase to the lipid composition and physical properties of the membrane.

It is generally recognised that the lipid composition of tissues of hibernators and ectothermic animals is responsive to the environmental temperatures experienced (see Hazel & Prosser, 1974). The composition of membrane lipids, and the physical properties of the membranes have been shown to be dependent on the thermal history of the individual (Johnson & Roots, 1964; Robb, Hammond & Bieber, 1972; Nozawa, Iida, Fukushima, Ohki & Ohnishi, 1974). In more recent work, significant variations in the membrane lipid compositions of ectothermic animals living in divers thermal habitats were observed. The membrane lipids isolated from the animals living in low temperature habitats were found to contain a higher proportion of unsaturated fatty acid residues than those isolated from animals that live at higher temperatures (Cossins, 1977; Cossins & Prosser, 1978; Cossins, Christiansen & Prosser, 1978). These variations in membrane lipids are considered to be adaptive allowing the maintenance of a functional

membrane fluidity at the given environmental temperature.
Given that there is evidence that the species variations in the protein component of the Na^+-K^+-ATPase enzyme system are very small (Hokin, 1974; Hopkins, Wagner & Smith, 1976), it seemed likely that a study of the kinetic stability of the Na^+-K^+-ATPase in membranes from species of markedly different thermal histories, might be a useful method of evaluating the sensitivity of the enzyme to variations in the lipid composition and physical properties of the membrane. This was approached by a comparative study of the kinetic stability of the Na^+-K^+-ATPase from various sources, along with a study of the changes in the kinetic stability of the enzyme as a result of physiological compensation to low temperatures by hibernators and ectothermic animals. The sources were chosen so as to exploit the properties of membranes naturally adapted for functioning at 'high' temperatures (e.g. birds ≃ 41°C, non-hibernating mammals ≃ 37°C), the 'low' temperatures (e.g. *Notothenia* - an antarctic fish ≃ 1°C), as well as the changes in the properties of membranes that can be induced by changes in the environmental temperature (e.g. trout acclimated at 5°C and 20°C; hibernating hamsters (≃4°C) vs non-hibernating hamsters (≃37°C)). These membrane sources were thus expected to hold the enzyme environments of different lipid composition with respect to the distribution of lipid classes and the degree of acyl chain unsaturation, and consequently different micro-viscosities. Thus, if membrane lipids are responsible for defining the potential energy well within which the Na^+-K^+-ATPase retains biological activity, this approach would inevitably probe the relationship between the enzyme and its lipid environment. The kinetic stability of the Na^+-K^+-ATPase was studied in such membranes and in membranes subjected to mild lipid perturbations.

MATERIALS AND METHODS

(i) Preparation of brain synaptic membranes.

(a) Brain synaptic membranes from mammals:

These were prepared from the brains of rats, mice, hamsters, guinea-pigs and rabbits by the procedure described for rat brain in Chapter 2.

(b) Brain synaptic membranes from non mammalian vertebrates:

These were prepared from the brains of starlings, pigeons, frogs, _Xenopus_, perch, _Notothenia_ and rainbow trout by a procedure similar to that described for rainbow trout in Chapter 3.

(ii) Sodium dodecylsulphate extraction of brain synaptic membranes.

The procedure adopted was that described in Chapter 6.

(iii) Thermal inactivation of the Na^+-K^+-ATPase in brain synaptic membranes.

Unless otherwise stated, the thermal inactivation was studied in a low ionic strength buffer (10mM Imidazole, 1mM E.D.T.A. pH 7.2 @ 20°C), and at a membrane protein concentration of between 50-100μg.cm^{-3}.

(a) Thermal inactivation at varying temperatures.

These studies were conducted at a fixed incubation period of 15 minutes, and in a temperature range such that the decay of Na^+-K^+-ATPase activity should span the range 5%-95% completion for that given incubation period. The temperature range thus varied with the source of synaptic membranes. The temperature ranges chosen were:

Membrane source	Temperature range
Mammals and birds	35°C - 50°C
Non-mammalian vertebrates (except Notothenia)	30°C - 48°C
Notothenia neglecta	18°C - 45°C

The thermal inactivation was carried out in clean thin-walled glass tubes which were thermo-equilibrat ed at the study temperature(s) ($\pm$0.1^{o}C).0.5 cm^{3} of a suitably diluted, cold enzyme preparation was quickly added to the test tube and shaken. The inactivation was quenched at the end of the incubation period by quickly cooling the tubes to 0°C by immersing in an ice bath. The enzyme samples were then stored on ice at 0-4°C until assayed.

(b) Isothermal inactivation studies

These studies were carried out at a temperature at which the apparent half life of the Na^{+}-K^{+}-ATPase activity was between 10-20 minutes. The temperature ranges used were 46-47°C for rat brain and 41-42°C for rainbow trout brain membranes. These studies were done at incubation times varying in the range 1 min - 240 min, and the inactivation procedure was the same as that described in (a) above.

(c) Thermal inactivation in the presence of 1mM n-octanol

A stock enzyme preparation was diluted to a membrane protein concentration of between 100-200μg cm^{-3}, with ice cold low ionic strength buffer. An equal volume of the low ionic strength buffer containing 2mM octanol was then slowly added to the enzyme preparation, and the final mixture stored on ice for 30 minutes before use. These studies were carried out over the appropriate temperature range as given in (a) above.

(d) Assay of Na^{+}-K^{+}-ATPase activity

The assay procedure adopted was the standard Na^{+}-K^{+}-ATPase described in Chapter 2. Unless otherwise stated, all assays were done at 37°C.

(e) Acclimation of rainbow trout at $5^{o}C$ and $20^{o}C$.

The procedure adopted was the same as that described in Chapter 8.

(f) Induction of hibernation in hamsters

The procedure adopted was the same as that described in Chapter 8.

RESULTS

The initial measurements were aimed at the defining of the temperature ranges appropriate for taking measurements on the enzyme from the various membrane sources studied. Inactivation was allowed to proceed for a fixed period of 15 minutes, at various temperatures, and the residual Na^{+}-K^{+}-ATPase activities were assayed at $37^{o}C$ in all cases except the antarctic species (<u>Notothenia</u>) which was assayed at $25^{o}C$. A summary of the results is shown in figure 7.1, from which it was clear that the Na^{+}-K^{+}-ATPase from the endothermic species studied (birds and mammals) were of greater thermal stability than those samples prepared from the ectothermic species. The most thermo-stable preparations were obtained from the two birds studied, for which the decay from 90% V_{max}-10% V_{max} occurred in the range $44^{o}C$ - $50^{o}C$. The samples obtained from themammalian sources studied were all slightly more thermo-labile than those of the birds, and were generally similar to each other, decaying from 90% V_{max}-10 V_{max} at temperatures between $42^{o}C$ - $49^{o}C$. The most thermo-labile preparation was obtained from the antarctic species <u>Notothenia</u>, forwhich the decay from 90% V_{max}-10% V_{max} occurred in the range $30^{o}C$ - $44^{o}C$. The samples obtained from the other ectothermic species were of greater thermal stability than the antarctic

species, but more thermo-labile than the endothermic species studied. These samples were generally similar as regards their apparent thermal stability since they all decayed from 90% V_{max} - 10% V_{max} in the temperature range between 38°C - 48°C. It is also apparent from the data summarised in figure 7.1 that the inactivation processes all occurred over a narrow range of temperature, and that the 'temperature width' of the various inactivations decreased as the thermal stability of the samples increased. Thus the broadest temperature range covering the decay from 90% V_{max} - 10% V_{max} was observed in the case of the antarctic fish (≈ 14°C) and the narrowest such range was observed in the case of the birds (≈ 6°C).

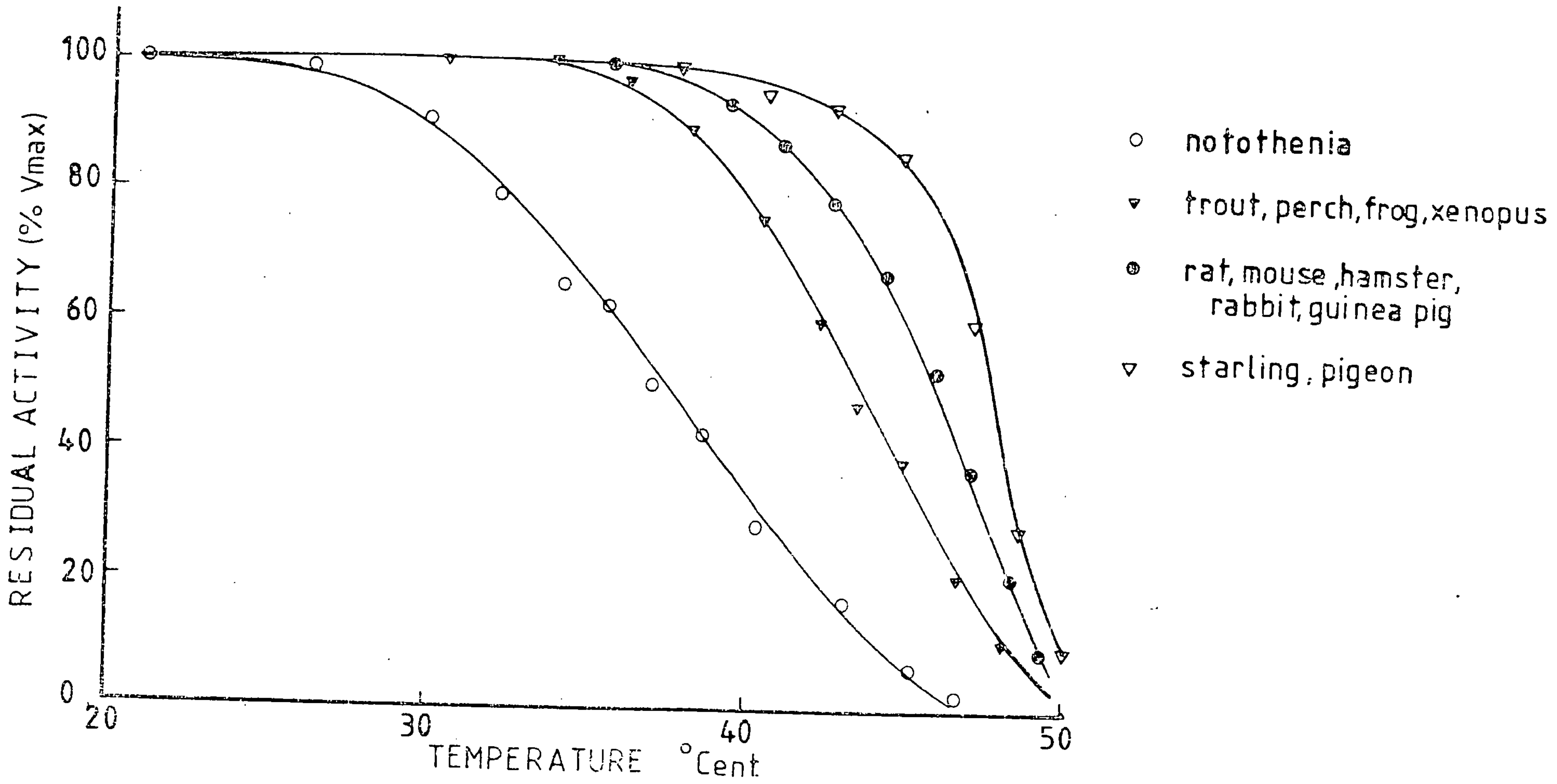
The narrow temperature range spanned by the inactivation processes, precluded the experimental approach of a precise measurement of the effect of temperature on the kinetics of thermal inactivation of the enzyme, since there would be too few temperature points for making reliable measurements. However, such information was potentially extractable from the above data, if the pattern of the decay kinetics of this enzyme were known. Thus the next set of measurements were aimed at the defining of the kinetics of inactivation of the enzyme at constant temperature.

The kinetics of isothermal inactivation of the Na^+-K^+-ATPase

The inactivation of the samples from rat brain and trout brain was studied at 46.7°C and 41.6°C respectively. At these temperatures, the apparent half-lives of the respective samples was expected to be near 15 minutes (see figure 7.1). The results of these experiments (see figures 7.2 and 7.3, each typical of three preparations) showed that the isothermal decay of Na^+-K^+-ATPase activity was not definable

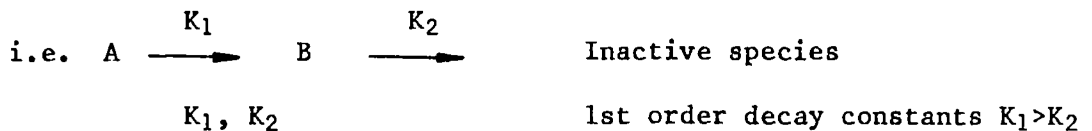
Fig.7.1

COMPARATIVE THERMAL INACTIVATION PROFILES OF THE Na^+-K^+-ATP.ase



in terms of the pseudo-first order decay kinetics normally expected of such processes (see Joly,1965). This was evident since a plot of the logarithm of residual activity (v) against time (t) was definitely non-linear. The plot was curvi-linear, describing a biphasic pattern with an initial rapid phase followed by a slower phase. The latter phase in the decay process, however, approached the form expected of first order decay kinetics (i.e. the graph of $\log_e(v)$ vs time was linear at large t). The biphasic nature of the decay process and the approach to first order decay kinetics at 'large' t suggested that there were two processes taking place and as such the kinetics of the decay process might be describable by one of the following decay schemes:

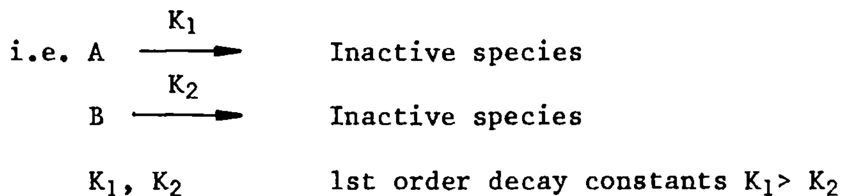
(a) A sequential decay scheme in which a relatively thermo-labile active species (A), rapidly decays to a relatively thermo-stable active species (B) which is then more slowly inactivated.



$$\text{i.e. } A \xrightarrow{K_1} B \xrightarrow{K_2} \text{Inactive species}$$

K_1, K_2 — 1st order decay constants $K_1 > K_2$

(b) A parallel decay scheme in which the initial sample is a mixture of a relatively thermo-labile active species (A), and a relatively thermo-stable active species (B), with the components of the mixture being inactivated at different rates.



$$\text{i.e. } A \xrightarrow{K_1} \text{Inactive species}$$

$$B \xrightarrow{K_2} \text{Inactive species}$$

K_1, K_2 — 1st order decay constants $K_1 > K_2$

If it is assumed that enzymic activities were measured at saturating concentrations of all essential ligands, then in both decay schemes, the measured activity (v) can be given by the following equation:

$$v = \alpha A + \beta B \tag{7a}$$

α . . . Catalytic rate constant of species A

β . . . Catalytic rate constant of species B

A, B . . . Concentrations of the respective species

Thus if A_o and B_o represent the initial concentrations of the two species, then the initial activity (V_{max}) will be given by the following equation:

$$V_{max} = \alpha A_o + \beta B_o \tag{7b}$$

Since the absolute values of the concentrations of the defined species, and their catalytic rate constants are not generally accessible, the relative parameters Θ and ϕ are defined thus:

$$\Theta = \frac{B_o}{A_o}$$

$$\phi = \frac{\beta}{\alpha}$$

Decay scheme (a)

In this scheme the concentration of the species A at any time t is obtainable from the integral of the following equation

$$\frac{d}{dt}(A) = -K_1 A$$

$$\text{i.e.} \quad A = A_o e^{-K_1 t} \tag{7c}$$

The concentration of the species B at any time t is obtainable from the following:

$$\frac{d}{dt}(B) = K_1A - K2B$$

i.e.

$$B = A_o \frac{K_1}{(K_1 - K_2)} (e^{-K_1t} - e^{-K_2t}) + B_o e^{-K_2t} \qquad (7d)$$

The expression describing the observed activity (v) can thus be obtained by substituting the above values for A and B (see equation 7c and 7d) into equation (7a).

$$\text{i.e. } v = \alpha A_o \left\{ (1 - \frac{\beta}{\alpha} \frac{K_1}{K_1 - K_2}) e^{-K_1t} + \frac{\beta}{\alpha} (\frac{K_1}{(K_1 - K_2)} + \frac{B_o}{A_o}) e^{-K_2t} \right\} \qquad (7e)$$

Equation (7e) can then be simplified by expressing it in terms of the initial activity (V_{max}) and the previously defined parameters ϕ and Θ .

$$\text{i.e. } V = \frac{V_{max}}{(1+\phi\Theta)} \left\{ (1 - \phi \frac{K_1}{(K_1 - K_2)}) e^{-K_1t} + \phi (\frac{K_1}{(K_1 - K_2)} + \Theta) e^{-K_2t} \right\} (7f)$$

Decay scheme (b)

In this scheme the concentrations of the species A and B at any time t is obtainable from the integral of their respective decay equations

$$\frac{d}{dt}(A) = - K_1A$$

$$\frac{d}{dt}(B) = - K_2B$$

i.e.

$$A = A_o e^{-K_1t}$$

$$B = B_o e^{-K_2t}$$

Thus the observed activity (v) can be given by the equation:

$$V = \alpha A_o e^{-K_1 t} + \beta B_o e^{-K_2 t} \qquad (7g)$$

Equation (7g) can then be expressed in terms of the initial activity (V_{max}) and the previously defined parameters ϕ and Θ.

$$\text{i.e. } V = \frac{V_{max}}{1 + \phi\Theta} \left(e^{-K_1 t} + \phi\Theta e^{-K_2 t} \right) \qquad (7h)$$

The behaviour predicted by both decay schemes (see equations (7f) and (7h) is similar in that a graph of $\log_e(v)$ vs time is expected to be curvi-linear approaching the form of first order (or pseudo first order) decay kinetics at large t, when the observed decay constant would approach that of the thermo-stable species (B). Since in both decay schemes, the system is mathematically described by the sum of two exponential terms, they are indistinguishable by the decay kinetics experiments described above. However, the results of the combined thermal inactivation/ouabain inhibition studies (see Chapter 3) argued against a description of the system as a mixture of two independent populations of Na^+-K^+-ATPase enzymes. Consequently, the parallel decay scheme was considered unlikely and the sequential decay scheme was adopted for describing the kinetics of isothermal inactivation of the enzyme.

The typical data sets shown in figures 7.2 and 7.3 have been fitted to the sequential rate equation (7f) by a computer assisted non-linear least squares minimisation procedure. These show a good agreement between the experimental and fitted points. The defining parameters of the fitted curves are listed in Table 7.1. It is clear that the values calculated for the ϕ and Θ parameters were reproducable while those calculated for the first order rate constants were subject to significant variation. The variation in the values of the rate constants was attributed to the practical problems of reproducing the

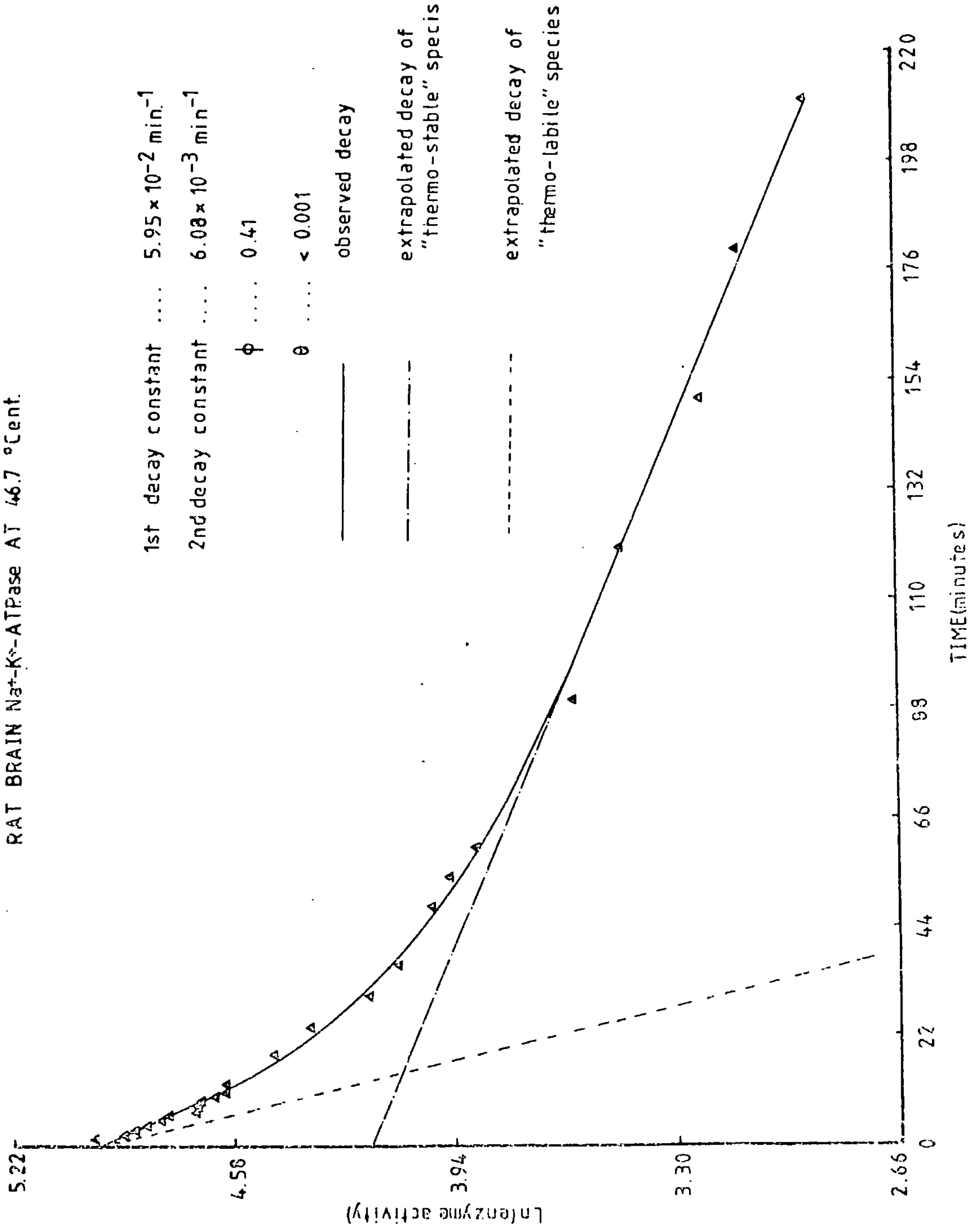


Fig. 7.2
KINETICS OF INACTIVATION OF RAT BRAIN Na^{+}-K^{+}-ATPase AT 46.7 °Cent.

Fig. 7.3

KINETICS OF INACTIVATION OF
TROUT BRAIN Na^+-K^+-ATPase AT 41.6 °Cent.

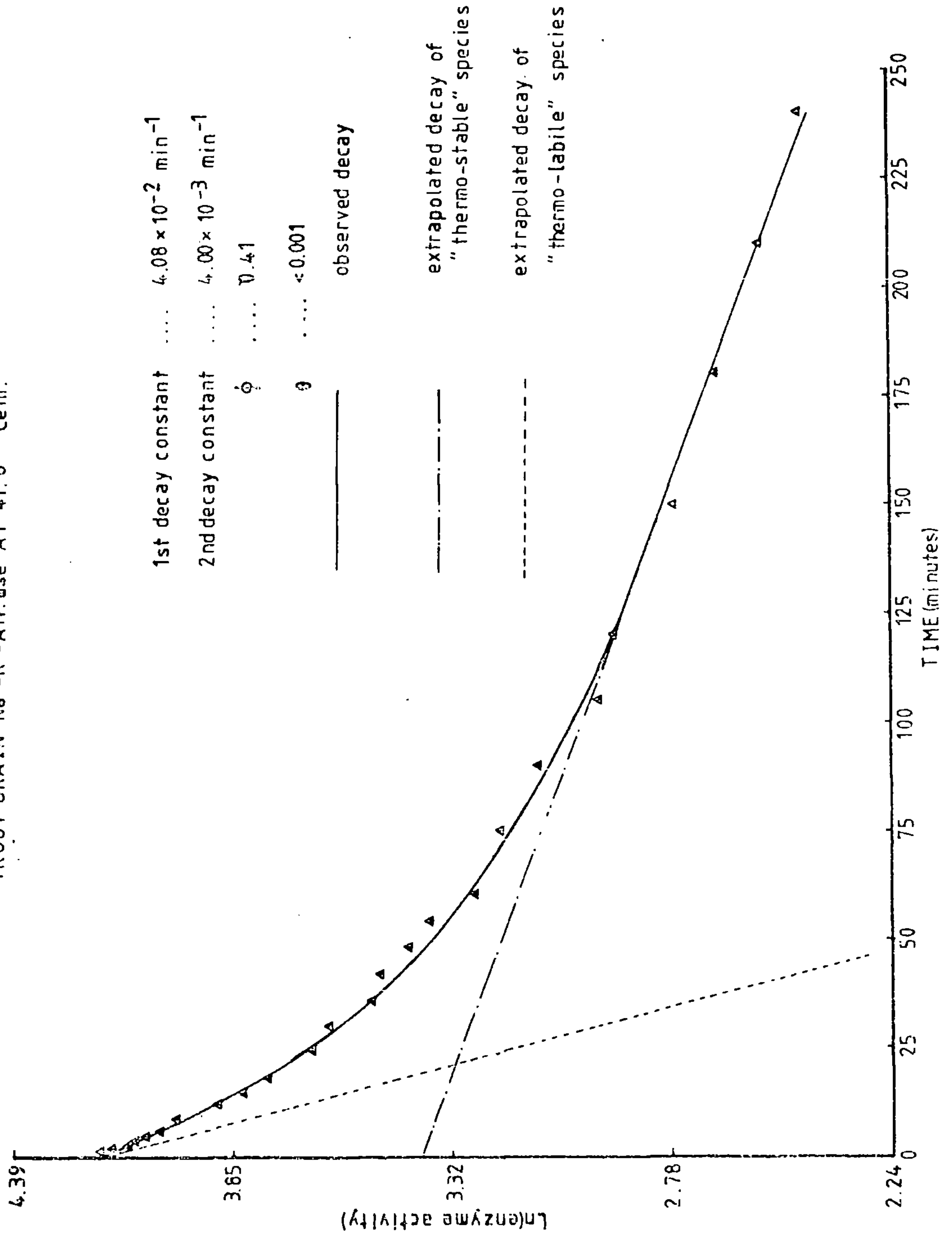


TABLE 7.1

Parameters describing the decay of Na^{+}-K^{+}-ATPase activity in crude brain synaptic membrane preparations from rat and trout.

*Parameters	Rat at 46.7°C	Trout at 41.6°C
1st decay constant	$4x10^{-2}$-$7x10^{-2}Min^{-1}$	$3x10^{-2}$-$6x10^{-2}Min^{-1}$
1st half life	10 - 17 Min	12 - 23 Min
2nd decay constant	$5x10^{-3}$-$7x10^{-3}Min^{-1}$	$3.5x10^{-3}$-$4.5x10^{-3}Min^{-1}$
2nd half life	100 - 135 Min	154 - 198 Min
ϕ	0.4 - 0.45	0.38 - 0.42
θ	≈0	≈0

Range above established from observations on 3 preparations

* See Text

conditions under which the inactivation was studied. In spite of this, it is clear that the pattern of inactivation was reproducible and that the respective 'thermo-stable' species was approximately ten times as stable as the 'thermo-labile' species at the temperatures studied (i.e. $\frac{\text{half life of B}}{\text{half life of A}} \simeq 10$). The results also suggested that the catalytic efficiency of the thermo-stable species was less than that of the thermo-labile species ($\phi \simeq 0.4$) and that the conditions under which the enzyme was stored resulted in little inactivation of the enzyme ($\Theta \simeq 0$).

The complexity of the kinetics of thermal inactivation of the Na^+-K^+-ATPase, precluded a reliable extraction of data pertinent to the temperature coefficients of the inactivation process(es), from the type of data summarised in figure 7.1. Although such information was theoretically accessible, it required the solution of a numerical problem of seven adjustable parameters, and given the aforementioned problems of reproducing experimental conditions, and the narrow temperature range accessible for kinetic measurements, such an approach was not considered likely to return reliable information. An alternative approach will be discussed later.

The effects of changes in lipid composition and physical properties of the membrane:

One approach in these studies was to determine the effect of the temperature induced membrane lipid changes expected of ectothermic animals and hibernating mammals. Thus the stability of the Na^+-K^+-ATPase in membranes prepared from rainbow trout acclimated at 5°C and 20°C were compared, along with the enzyme in membranes prepared from active and hibernating hamsters. The data shown in figure 7.4 is a summary

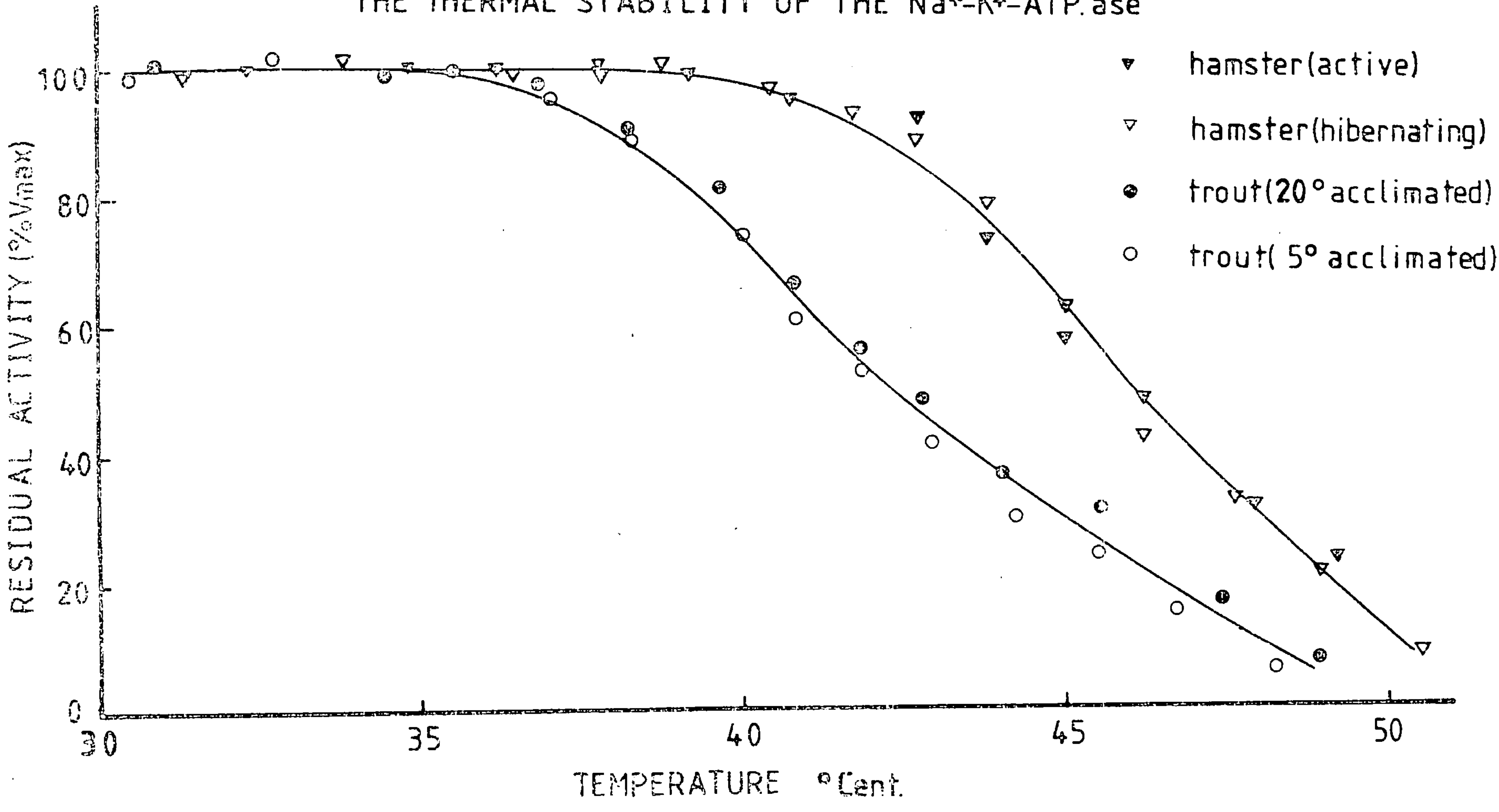


Fig. 7.4

EFFECT OF TEMPERATURE ACCLIMATION ON THE THERMAL STABILITY OF THE Na^{+}-K^{+}-ATP.ase

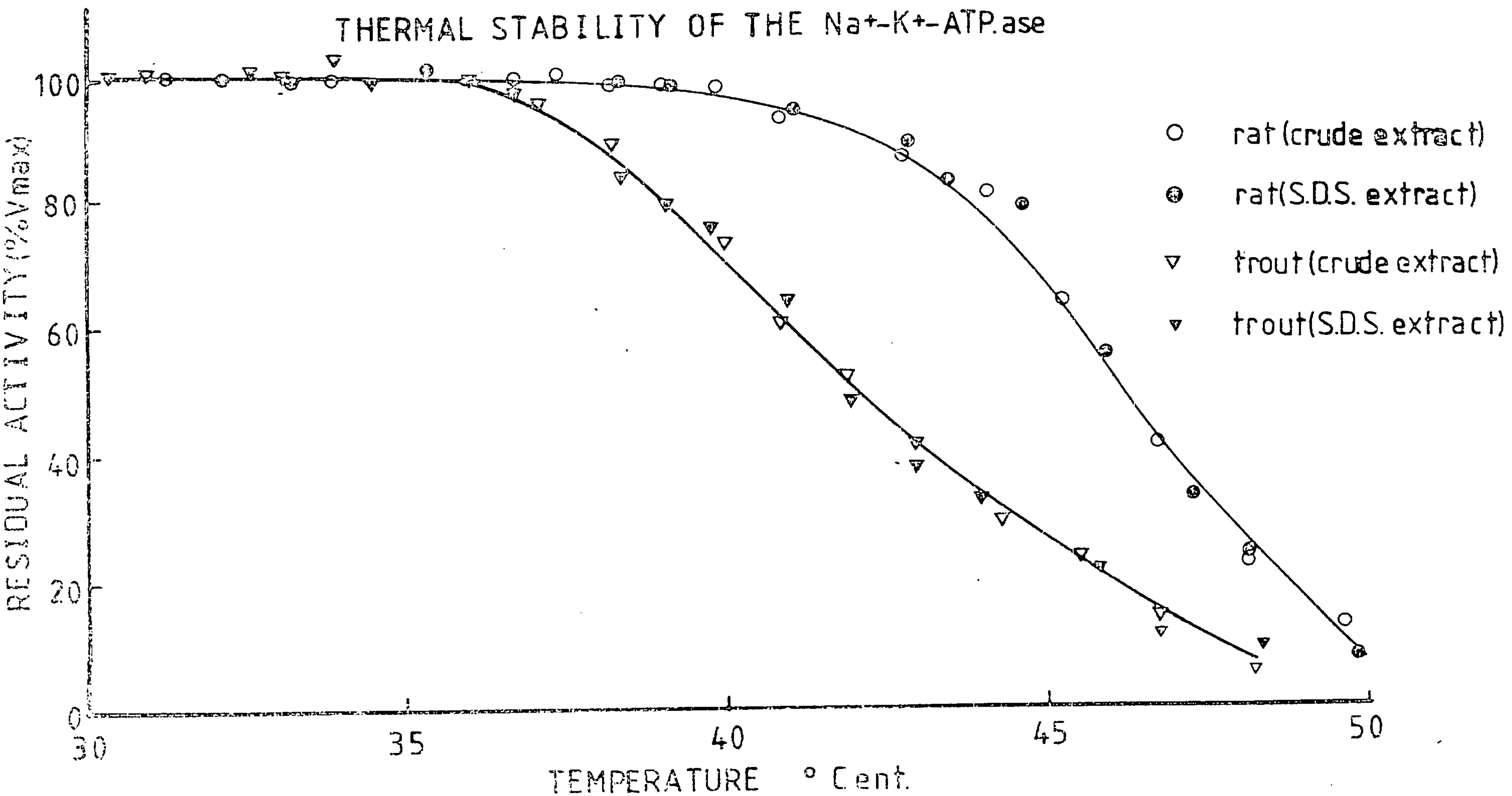
of observations on 3-4 preparations (see legend to fig. 7.4). It is clear from these observations that no significant difference was detected between the kinetic stabilities of the Na^+-K^+-ATPases in membranes prepared from active and hibernating hamsters. In both cases, the decay of the Na^+-K^+-ATPase activity with increasing temperature (Incubation period = 15 minutes) occurred over the same temperature range (42^oC - 50^oC), and the same temperature width ($\approx 8^oC$) described the decay from 90% V_{max} - 10% V_{max}.

The pattern describing the thermal inactivation of the Na^+-K^+-ATPases in the membranes from 5^oC and 20^oC acclimated trout was generally similar to that described for the hamsters above. The same temperature range (38^oC - 48^oC) described the decay of enzymic activity, and no significant difference could be claimed for the width of the decay from 90% V_{max} - 10% V_{max} (10^oC). However, as is obvious from figure 7.4, the data points obtained from the animals acclimated at 20^oC, showed a slight bias towards the higher temperatures, but given the previously mentioned uncertainties as regards the reproducibility of experimental conditions, no significance was attributed to this observed bias.

The other approach to these investigations involved a study of the effect of mild membrane perturbations on the kinetic stability of the enzyme. Hence, the effects of sodium dodecylsulphate extraction of the membranes and the effect of lmM octanol on the stability of the Na^+-K^+-ATPases in membranes prepared from rat and trout brain were investigated. The data shown in figure 7.5 is a summary (4 preparations) of the effects of dodecylsulphate extraction on the 'thermal inactivation profiles' of the Na^+-K^+-ATPases from the two preparations. It is clear that no significant effect on either preparation was

Fig. 7.5

EFFECT OF S.D.S EXTRACTION ON THE THERMAL STABILITY OF THE Na+-K+-ATP.ase



detected, since neither the temperature range nor the 'temperature width' of the thermal inactivation of the dodecyl-sulphate extracted samples were significantly different from their respective control preparations.

The data shown in figure 7.6 summarises the thermal inactivation profiles of rat and trout brain preparations (4 preparations each) in the presence and absence of 1mM octanol. The assays of residual Na^{+}-K^{+}-ATPase activity were done at 35°C, and the control assays were done in the presence of 100μM octanol. These results showed that the incubation of the samples in the presence of 1mM octanol, resulted in a decreased thermal stability of both preparations, since the effective temperature ranges describing the thermal inactivations were at lower temperatures in the presence of the alcohol. In the case of the rat samples, the lowered temperature range (42-49°C control vs 37-46°C octanol) was accompanied by a slightly broader temperature width (7°C control vs 9°C octanol), while the lowering of the temperature range for the inactivation of the trout brain samples (38°C - 48°C control vs 35 - 43°C octanol), was accompanied by a narrowed temperature width (10°C control vs 8°C octanol).

DISCUSSION

The results presented above, show that the kinetics of isothermal denaturation of the Na^{+}-K^{+}-ATPase cannot be adequately described by simple first order (or pseudo first order) decay kinetics. In this respect, the results compare favourably with previously reported observations (Atkinson, Gatenby & Lowe, 1971; Gladwell, 1975). However, the interpretation of the kinetics proposed here, differs in some respects from

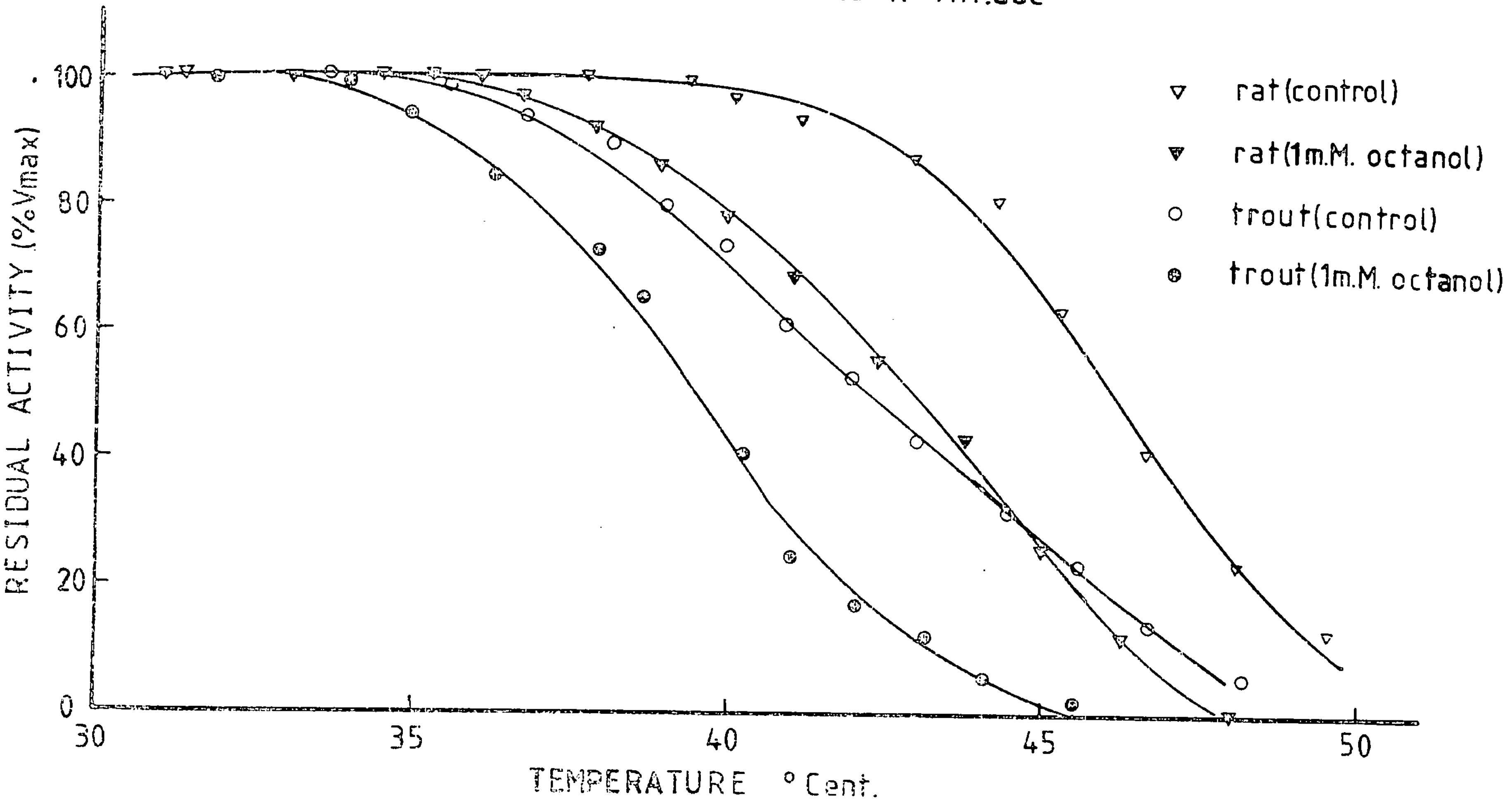
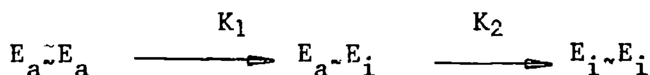


Fig. 7.6
EFFECT OF OCTANOL ON THE
THERMAL STABILITY OF THE Na+-K+-ATPase
rat(control)
rat(1mM. octanol)
trout(control)
trout(1mM. octanol)
RESIDUAL ACTIVITY (%Vmax)
100
80
60
40
20
0
30
35
40
45
50
TEMPERATURE °Cent.

that proposed earlier (Atkinson et al. 1971). These earlier proposals described the kinetics of inactivation of the enzyme as a first order decay to an equilibrium between active and inactive forms of the enzyme. Since those measurements were taken at a lower temperature (37°C) than those described here (41°C), it is possible that Atkinson et al. (1971) were only observing the initial phase of the decay process, and that at the lower temperature used, the final phase of the decay process was proceeding too slowly to be detected. Thus, despite the difference between the interpretations proposed, it seems unlikely that there is any fundamental difference between the observations reported by those workers and those presented here.

As mentioned earlier, the framework adopted for the description of the kinetics of isothermal inactivation of the enzyme is not supported by the data to the exclusion of all other schemes. However, this sequential decay scheme is consistent with the description of the functional Na^+-K^+-ATPase as a co-operative kinetic dimer (see Chapter 3). If this is assumed, then the decay scheme can be described by the following system:



$$E_a{\sim}E_a \xrightarrow{K_1} E_a{\sim}E_i \xrightarrow{K_2} E_i{\sim}E_i$$

$E_a{\sim}E_a$ - dimeric species with both subunits active

$E_a{\sim}E_i$ - dimeric species with one inactive subunit

$E_i{\sim}E_i$ - dimeric species with both subunits inactive

K_1 K_2 - first order decay constants $K_1 > K_2$

As described, the thermal energy imported to the above system would be distributed between both subunits. The distribution of the energy between the two subunits is expected to be symmetrical in the case of the native species (E_a-E_a), but in the case of the species E_a-E_i the distribution of the thermal energy imported is expected to be assymetric with more of it distributed to the lower energy inactive subunit. Thus the probability of the active subunit of the species E_a-E_i being inactivated should be smaller than that of either of the subunits of the species E_a-E_a, with the result that the latter species should be less thermo-stable than the former.

It was also apparent from the results, that given the mild conditions used here (low ionic strength, near neutral pH), the enzyme was inactivated at relatively low temperatures (>50°C). It is generally observed that under comparable conditions, higher temperatures (>60°C) are usually required to inactivate soluble proteins at rates similar to those observed here (see Joly, 1965; Bull & Breese, 1973a, 1973b). This suggests that differen forces are probably operating in the maintenance of the biologically active conformational states of the Na^{+}-K^{+}-ATPase when compared with the soluble proteins. Given the above mentioned phospholipid requirement for Na^{+}-K^{+}-ATPase activity, the low incidence of disulphide bridges, and the high incidence of hydrophobic amino acid residues in the protein component of this enzyme (see Hopkins, Wagner & Smith, 1976), it seems probable that the forces that maintain the biologically active conformational states of this enzyme may largely be the Van der Waal's forces between the hydrophobic amino acid side chains and membrane lipids. If such is the case, then the stability of this enzyme ought to be heavily dependent on the properties of the membrane.

The comparative studies presented here, show a general correlation between the stability of the enzyme and the temperature to which the source species have been naturally adopted. The enzyme from sources naturally adapted to function at 'high' temperatures (e.g. birds and non-hibernating mammals) was found to be more thermo-stable than those of the ectothermic species which generally function at much lower temperatures. However, this was only apparent when the natural thermal environments of the source animals were markedly different. Thus, while significant differences were found between the systems naturally adapted to function at $1^{o}C$ (Notothenia), the ectothermic species normally adapted to the temperature range $5^{o}C$ - $25^{o}C$, and the 'high' temperature endothermic species ($T_b 37^{o}C$), significant differences were not detected within any of the four groups illustrated in figure 7.1. The data also suggested that the stability of the enzyme was not sensitive to the changes co-incident with physiological compensation by a species to environmental temperature changes. Thus despite the changes expected as a result of hibernation in hamsters, and acclimation of trout to different temperatures ($5^{o}C$ & $20^{o}C$), the kinetic stabilities of the respective Na^{+}-K^{+}-ATPases were apparently unaffected.

As mentioned previously, there is evidence that the protein component of the Na^{+}-K^{+}-ATPase enzyme system has undergone little change during the evolution of the various groups (see Hokin, 1974; Hopkins, Wagner & Smith, 1976). If such is assumed, then the differences in the kinetic stabilities of the enzyme preparations illustrated in figure 7.1, can be interpreted in terms of the dif ferent lipid composition and physical properties of the respective

membranes. Given that membrane lipids isolated from animals living in low temperature habitats have been shown to contain a higher proportion of unsaturated fatty acid residues, and to be less viscous than those isolated from animals that live at higher temperatures (Cossins, 1977; Cossins & Prosser, 1978; Cossins, Christiansen & Prosser, 1978), the differences in the kinetic stability of the Na^{+}-K^{+}-ATPase samples illustrated in figure 7.1 can be correlated with the differences in the lipid composition and fluidity expected of the various membrane samples. The results would also suggest that the stability of the enzyme is insensitive to the changes in membrane lipid composition and fluidity of the magnitude coincident with physiological compensation by a species to environmental temperature changes.

The relative insensitivity of the stability of the Na^{+}-K^{+}-ATPase to the small membrane perturbations expected of the sodium dodecyl-sulphate extraction procedure adopted, supported the observations described above in suggesting the stability of the enzyme was sensitive only to large changes in the properties of the membrane. That such large changes, do affect the stability of the enzyme was evident from the results of the octanol experiments, in which the aliphatic alcohol was found to reduce the stability of the enzyme at alcohol concentrations below those previously reported to inhibit the enzyme (Grisham & Barnett, 1973b). Since octanol has been shown to increase the 'fluidity' of the membrane (Grisham & Barnett, 1973b)it seems likely that this increased membrane fluidity was responsible for the greater thermo-lability of the enzyme in the presence of the alcohol, especially since the aliphatic alcohols like octanol have been shown

to be lipid targeted (Grisham & Barnett, 1972). This would correlate with the general observations of the comparative studies described above.

As discussed previously, the complex kinetics of inactivation of the enzyme precluded a rigorous and reliable treatment of the data. Given the practical problems of reproducing the experimental conditions, the best that was practical was to obtain rough estimates of the values estimated for the enthalpy (ΔH) describing the rate of inactivation. This was done by estimating the temperatures for 90% V_{max} and 10% V_{max} and calculating values for the enthalpy by the integrated form of the Van't Hoff isotherm.

$$\text{i.e. } \log_e 90 - \log_e 10 = -\frac{\Delta H}{R} \left(\frac{1}{T_{90\%}} - \frac{1}{T_{10\%}} \right)$$

R - gas constant; T - absolute temperature

The values calculated (Table 7.2) were used as an estimate of the enthalpy term. The results of these calculations underlined the general observations of the comparative study in that the more thermo-stable samples were associated with the larger ΔH values, (e.g. birds ≈ 270-320 $K.J.Mol^{-1}$ vs Notothenia ≈ 120-160 $K.J.Mol^{-1}$). Since the ΔH is an estimate of the temperature coefficient of the rate of inactivation it follows that as a general trend the more thermo-stable preparations are likely to be characterised by sharper thermal inactivation profiles. Such is therefore consistent with the suggestion that membrane fluidity is a significant determinant of the stability of the enzyme, since it is generally observed that the more ordered lipid membranes are characterised by sharper thermal transitions (see Trauble, 1971).

TABLE 7.2

Apparent 15 minute half life temperatures of the Na^+-K^+-ATPase from the brains of different species.

Tissue source	n	*T(°C)	ΔT½ (°C)	θU_{app} (K.J.Mol^{-1})
Starling	3	41	47.5-48.5	270-320
Pigeon	2	41	47.5-48.5	270-320
Rat	5	37	45.5-46.5	230-280
Mouse	3	37	46 - 47	230-280
Guinea pig	2	37	46 - 47	230-280
Rabbit	2	37	45.5-46.5	230-280
Hamster (non-hibernating)	4	37	47 - 48	230-280
Hamster (hibernating)	4	4	47 - 48	230-280
Perch	2	15	42 - 43	180-220
Trout (20° Acclimated)	3	20	41.5-42.5	180-220
Trout (5° Acclimated)	4	5	41 - 42	180-220
†<u>Notothenia</u>	1	1	36 - 38	120-160
<u>Xenopus</u>	3	23	42 - 43	180-220
Frog	2	4	41 - 42	180-220

* Mean body temperature

Δ Temperature at which half life of Na^+-K^+-ATPase is ≃ 15 minutes. T½ range established from n observations.

† Range established from 3 repeats on one preparation.

θ Apparent activation energy of the thermal inactivation process. Values give the range expected (see Text).

The interpretation of the data presented here, is in general support of the view that membrane lipids play an important part in maintaining the biologically active state(s) of the Na^+-K^+-ATPase. However, this is based on the assumption that the differences in the Na^+-K^+-ATPase proteins are too small to account for the observations. Since the currently available experimental evidence does not give unambiguous support to such an assumption, a precise interpretation of the above data must await comparative studies on the structure of this enzyme. However, within the context of the above assumption, it seems likely that the membrane lipids are responsible for defining the potential energy well within which the biological activity of the Na^+-K^+-ATPase is maintained.

CHAPTER 8

The effect of lipid targeted modulators on the kinetic properties of the Na^{+}-K^{+}-ATPase

INTRODUCTION

A role for membrane lipid in the maintenance of the biological activity of the Na^{+}-K^{+}-ATPase is generally acknowledged and has been previously discussed in Chapter 7. However, there is some uncertainty as to whether these lipids are involved in the regulation and fine control of this enzyme. That they might play a role was implied from reports that the enzyme had a specific requirement for phosphatidyl serine (Fenster & Copenhaver, 1967; Wheeler & Whittam, 1970), though such a requirement has been questioned (DePont, Van Prooijen-Van Eeeden & Bonting, 1973). Other workers have presented evidence supporting a special role for negatively charged phospholipids (Palatint, Dabbeni-Sala, Pitotti, Bruni & Mandersloot, 1977), but such has also been questioned (DePont, Van Prooijen-Van Eeden & Bonting, 1978). A recent report, however, has presented evidence which suggests that phosphatidyl inisitol may be an endogenous activator of the Na^{+}-K^{+}-ATPase (Mandersloot, Roelofsen & DeGier, 1978).

The bulk of the evidence supporting a regulatory role for membrane lipid in this enzyme system, has been obtained from kinetic studies on the temperature dependence of its activity. The effect of temperature on Na^{+}-K^{+}-ATPase activity is generally described by a non-linear Arrhenius graph (see Chapter 4) and this has been interpreted within the context of temperature dependence lipid phase transitions (see Barnett & Parlazzotto, 1974). Other workers have shown that the non-linear Arrhenius graph, characteristic of the temperature

kinetics of this enzyme, can be made linear by the disruption of the biomembranes with phospholipases (Taniguchi & Iida, 1972b; Charnock, Cook, Almeida & To, 1973), and that the effect can be reversed by post incubation of the phospholipase treated membranes with phospholipids. Such observations were supported by a report that the shape of the Arrhenius graph of the Na^{+}-K^{+}-ATPase from Ehrlich Ascites tumour cells can be altered by changes in the lipid composition of those cell membranes (Solomonson, Liepkalns & Spector, 1976), and an earlier observation that enzymic peroxidation of membrane lipids resulted in an alteration of the sensitivity of the Na^{+}-K^{+}-ATPase to both ATP and potassium ion (Sun, 1971). Against such evidence are reports that the non linear Arrhenius graph of the Na^{+}-K^{+}-ATPase can be 'straightened' by alterations in magnesium ion concentration (Kimelberg, 1975; Boldyrev, Ruuge, Smirnova & Tabak, 1977) and that the loss in Na^{+}-K^{+}-ATPase activity coincident with the treatment of brain synaptic membranes with phospholipase C was not accompanied by any significant change in the apparent Km of the enzyme with respect to ATP (Sun, Sun & Samorajski, 1971). These reports, and the observation that the ADP binding affinity of catalytically inactive delipidated Na^{+}-K^{+}-ATPase preparations was unchanged after reactivation by phospholipids (Jensen & Ottolenghi, 1976), have argued against a regulatory role for lipids with respect to the Na^{+}-K^{+}-ATPase.

The experiments designed to investigate a probable regulation of the Na^{+}-K^{+}-ATPase by membrane lipids, usually employ methods which involve a severe disruption of the membrane and an almost complete loss of Na^{+}-K^{+}-ATPase activity. Consequently, it is difficult to interpret such data within the context of an in-*vivo* regulation of the Na^{+}-K^{+}-ATPase by membrane lipid. In this study, an attempt was made to investigate the effect of small lipid perturbations on the kinetic properties of the enzyme. The lipid modulation procedures employed, primarily exploited the changes in membrane lipid composition

coincident with temperature acclimation by fish (Johnson & Roots, 1964; Roots, 1968; Kemp & Smith, 1970), and the onset of hibernation in hamsters (Goldman, 1975). In addition the effect of a more severe membrane lipid perturbant (i.e. lipid peroxidation) was investigated.

MATERIALS AND METHODS

(1) Preparation of brain synaptic membranes containing Na^+-K^+-ATPase activity.

These were prepared by the methods previously described in Chapter 2. The brains of rats, hamsters and rainbow trout were used as sources of synaptic membranes.

(2) S.D.S. extraction of synaptic membranes

The procedure adopted was the same as that described in Chapter 6.

(3) Lipid peroxidation of crude rat brain synaptic membranes

Unless otherwise stated, all operations were carried out at 0-4°C. The procedure adopted was adapted from that described by Dobretsov, Borchevskaya, Petrov & Vladimirov (1977).

The crude membranes prepared as described above (1), were sedimented from the low ionic strength buffer (10mM Imidazole,1mM E.D.T.A. pH 7.2 at room temperature) by centrifugation at 100,000g (M.S.E. prepspin 50 ultracentrifuge) for 30 minutes. The pelleted membranes were then washed free of E.D.T.A. by three cycles of homogenisation in an E.D.T.A. free low ionic strength buffer (10mM Imidazole pH 7.2 at room temperature), and centrifugation at 100,000g. The washed membranes were resuspended in the E.D.T.A. free low ionic strength buffer to a protein concentration of 2-3 mg.cm^{-3}, and used immediately for lipid peroxidation.

The washed membranes were added to a solution containing ascorbic acid and ferrous sulphate (thermoequilibriated at 25°C) so as to obtain final concentrations of 10^{-4}M ascorbate, 5 x 10^{-6}M ferrous sulphate and a membrane phospholipid concentration of between 175–225μg.cm^{-3}. The mixture was stirred by a magnetic stirrer at 25°C for 2 hours, and the peroxidation reaction terminated by the addition of disodium E.D.T.A. to a final concentration of about 10^{-3}M. The peroxidised membranes were then sedimented by centrifugation at 100,000g, and washed by two cycles of homogenisation in low ionic strength buffer (10mM Imidazole, 1mM E.D.T.A. pH 7.2 at room temperature), and centrifugation at 100,000g. The membranes were finally resuspended in the low ionic strength buffer (containing E.D.T.A.) and stored on ice at 0-4°C until required.

(4) Assay of Na^{+}-K^{+}-ATPase activity

(i) Standard assay procedure:

This was the same as that described in Chapter 2.

(ii) Assays at non-saturating ouabain concentrations:

The procedure adopted is described in Chapter 3.

(iii) Assays at non-saturating potassium concentrations:

The procedure adopted is described in Chapter 5.

(iv) Assays at varying temperatures:

The procedure adopted is described in Chapter 4.

(5) Assay of proteins

The procedure adopted is described in Chapter 2.

(6) Assay of cholesterol

The procedure adopted was adapted from that described by Watson (1960).

Preparation of chromagenic solution

The chromagenic solution was prepared by carefully mixing 3 volumes of Acetic anhydride, with one volume of glacial acetic acid and one volume of 0.25M 2,5,dimethyl-benzene sulphonic acid in glacial acetic acid. The mixture obtained was stored in a dark bottle at 0-4°C and had an indefinite shelf-life under those conditions.

All operations were carried out at room temperature. An aliquot of a membrane sample containing 0.1-1 μ.mole of cholesterol was evaporated to dryness in an oven at 110-120°C. The sample was then cooled and then treated with 0.2 cm^3 of distilled water and 0.2 cm^3 of glacial acetic acid. 5 cm^3 of the chromagenic solution were then added to the above mixture and thoroughly mixed. An exothermic reaction resulted and the tubes were allowed to cool for 20 minutes at room temperature. 0.6 cm^3 of concentrated sulphuric acid were added to the cooled mixture, and the final mixture was quickly and thoroughly mixed so as to ensure the solubilisation of any precipitated protein. The mixture was then stored at room temperature for 20 minutes to allow complete colour development and its extinction determined at 620 n.m.

The mixture was found to maintain its colour intensity for at least 10 minutes after the completion of colour development. The extinction was interpreted in terms of cholesterol content by calibrating the same with cholesterol. The absorbance was found to be linear with cholesterol content in the range 0.1 - 1μ.Mole of cholesterol (see Fig. 8.1).

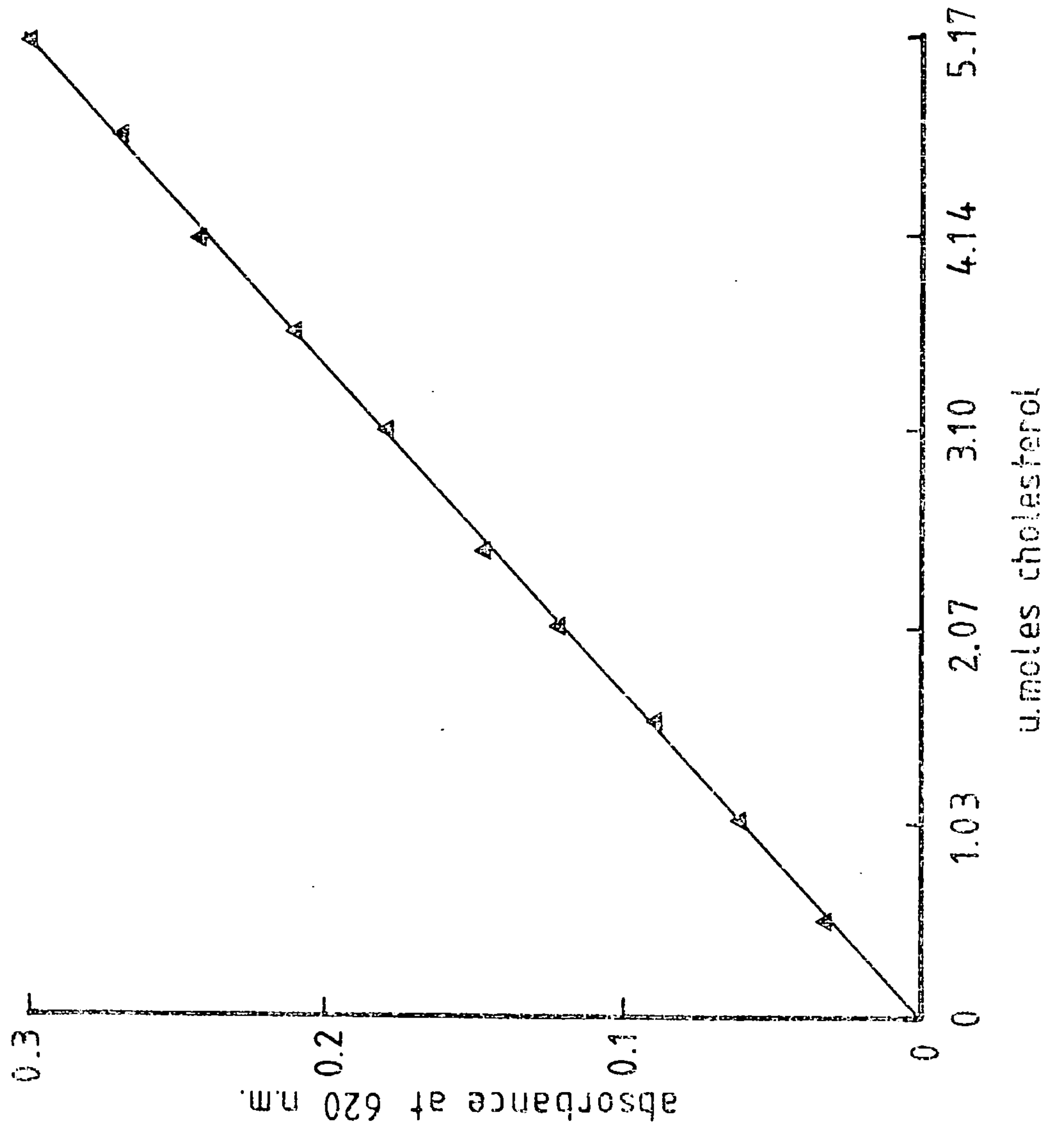


Fig.8.1
CHOLESTEROL CALIBRATION GRAPH

(7) Assay of Total Phospholipid

The procedure adopted was adapted from that described by Raheja, Kaur, Singh & Bhatia (1973).

Preparation of chromagenic solution

Prepared Solutions:

(i) Ammonium Molybdate

16g of ammonium molybdate were dissolved in 120 cm^3 of distilled water. A few drops of concentrated hydrochloric acid were added to the mixture to ensure the complete dissolution of the ammonium molybdate.

(ii) Solution I

10 cm^3 of redistilled mercury were added to a mixture of 40 cm^3 of concentrated hydrochloric acid and 80 cm^3 of the ammonium molybdate solution above (i). The mixture was stirred on a magnetic stirrer for 45 minutes and filtered. A red-brown filtrate was formed.

(iii) Solution II (Acid Molybdate)

This was prepared by carefully adding 200 cm^3 of concentrated sulphuric acid to 40 cm^3 of the ammonium molybdate above (i).

The chromagenic solution was prepared by carefully adding Solution II to the reddish-brown Solution I with stirring. 25 volumes of the dark green solution formed were then mixed with 45 volumes of methanol, 5 volumes of chloroform and 20 volumes of distilled water. The dark green mixture so formed was stored at 0-4°C under which conditions it had a shelf-life in excess of 3 months.

An aliquot of a sample containing 0.03-0.3 μ.moles of lipid phosphorous was introduced into a clean test-tube and the solvent/ dispersant evaporated in an oven at 100-110°C. 0.5 cm^3 of chloroform

were then added to the sample to disperse the lipids and the chloroform was then removed by evaporation at 100°C. After the tubes had cooled, 0.4 cm^3 of chloroform and 0.2 cm^3 of the chromagenic solution were added to the sample, which was then heated in a boiling water bath for 3 minutes. After the tubes had cooled, 3 cm^3 of chloroform were added to each of the samples, which were shaken and then centrifuged (≃500g) to separate the two phases. The extinction of the organic layer was then determined at 716 n.m.

The final colour, which was stable for at least 3 hours, was interpreted in terms of phospholipid content, by calibrating the same with phosphatidyl choline. The calibration graph (see Fig. 8.2) was found to be linear with phospholipid content in the range 0.03 - 0.3 μ.moles of lipid phosphorous.

(8) Assay of Sphingolipids

Sphingolipids were determined by the estimation of sphingosine after methanolysis by a procedure adopted from that described by Lauter & Trams (1962).

Prepared Solutions

(i) Ethyl acetate saturated acetate buffer:

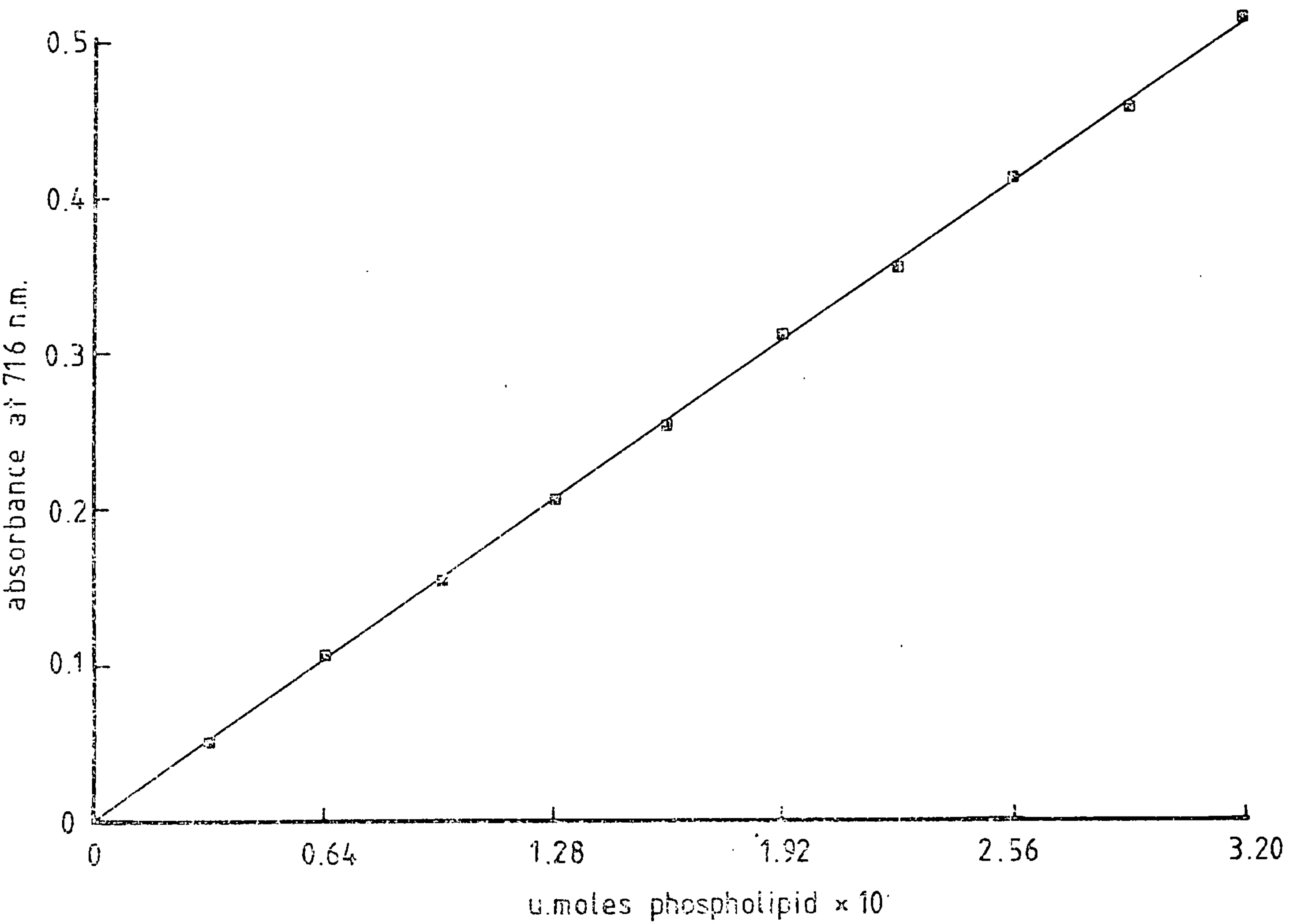
185 volumes of 10mM acetic acid were mixed with 15 volumes of 10mM sodium acetate and 50 cm^3 of ethyl acetate. The final mixture was thoroughly mixed and had a pH of 3.6 - 3.8.

(ii) Chloroform washed methyl orange:

500mg of methyl orange were warmed with 100 cm^3 of distilled water until the solid dissolved. The warm aqueous solution was then extracted three times with 20 cm^3 of chloroform. The final aqueous phase was stored at room temperature and had an indefinite shelf-life.

Fig. 8.2

PHOSPHOLIPID CALIBRATION GRAPH



An aliquot of a sample containing 0.01-0.1 μ.moles of sphingolipid was introduced into a clean test-tube and the solvent/dispersant evaporated at 100-110°C. After the samples were dry, 0.2 cm^3 of Boron Trifluoride/methanol were added, and the samples heated to dryness at 100-110°C to ensure completion of the methanolysis. After the samples had cooled, 5 cm^3 of ethyl acetate were added so as to dissolve the lipids, and the solution obtained was washed with 2 cm^3 of 0.5M sodium hydroxide. The organic phase was separated from the aqueous phase by centrifugation (≈500g). The aqueous phase was discarded and the organic phase washed twice with distilled water. After the removal of the final aqueous phase, 2 cm^3 of ethyl acetate saturated acetate buffer were added to the organic phase, followed by 0.1 cm^3 of methyl orange reagent. The mixture was thoroughly mixed, centrifuged so as to separate the two phases, and the extinction of the organic phase determined at 416 n.m.

The extinction readings were interpreted in terms of sphingosine content by calibrating the same with sphingosine. The calibration graph (see Fig. 8.3) was found to be linear for sphingosine contents in the range 0.01-0.1 μ.moles.

(9) Assay of Plasmalogens

Plasmalogens were determined by the estimation of long chain aldehyde after acid hydrolysis by a procedure adapted from that described by Wittenberg, Korey & Swenson (1956).

Chromagenic solution

20mM p-nitrophenyl hydrazine in 95% ethanol - prepared freshly.

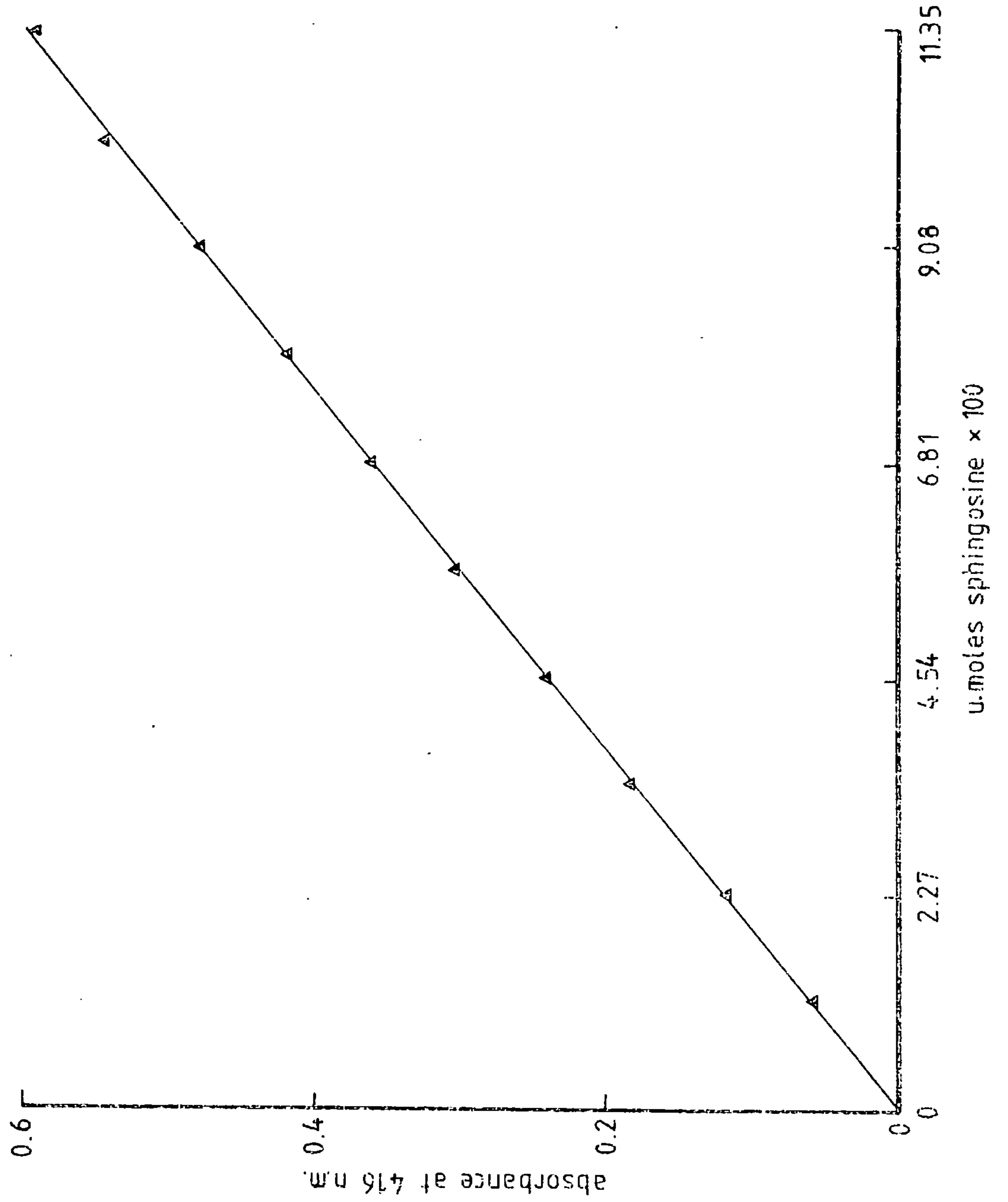


Fig.8.3

SPHINGOSINE CALIBRATION GRAPH

An aliquot of a sample containing .01-.13μ.moles of plasmalogen was introduced into a 25 cm^3 tube and the solvent/dispersant evaporated by heating at 100-110^oC. 0.2 cm^3 of concentrated hydrochloric acid were added to the sample to release the aldehydes, and the mixture was then heated to dryness at 100-110^oC to ensure completion of the hydrolysis. After the tubes had cooled, 0.7 cm^3 of chloroform, 0.2 cm^3 of 0.5M sulphuric acid and 0.2 cm^3 of para-nitrophenyl hydrazine reagent were added to the sample. The tubes were then stoppered and heated in a boiling water bath (with shaking), for 20 minutes. After the tubes had cooled, 5 cm^3 of petroleum ether (boiling point ≈70^oC) were added and thoroughly mixed to dissolve the lipid contents. The mixture was then treated with 5 cm^3 of 95% ethanol and 3 cm^3 of distilled water. The mixture was thoroughly mixed and stood at room temperature so as to allow the organic and aqueous phases to separate. An aliquot of the organic phase (0.75 - 2 cm^3) was then 'dried down' in a boiling water bath and the residue dissolved in 3 cm^3 of 95% ethanol. The extinction of the solution obtained was determined at 390 n.m.

The absorbance readings were interpreted in terms of fatty aldehyde content by calibrating the same with hexadecanal. The final colour was found to be stable for at least 3 hours at room temperature and it was linear with aldehyde content in the range 0.01-0.13μ.moles, (see Fig. 8.4).

(10) Extraction of lipids from synaptic membrane fractions

The procedure adopted was that described by Folch, Lees & Sloane-Stanley (1957) and later modified by Rouser, Kritchevsky & Yamamoto (1967). 2-6-ditertiary butyl-para cresol (B.H.T) was used as an antioxidant (see Wren & Szczepanowska, 1963).

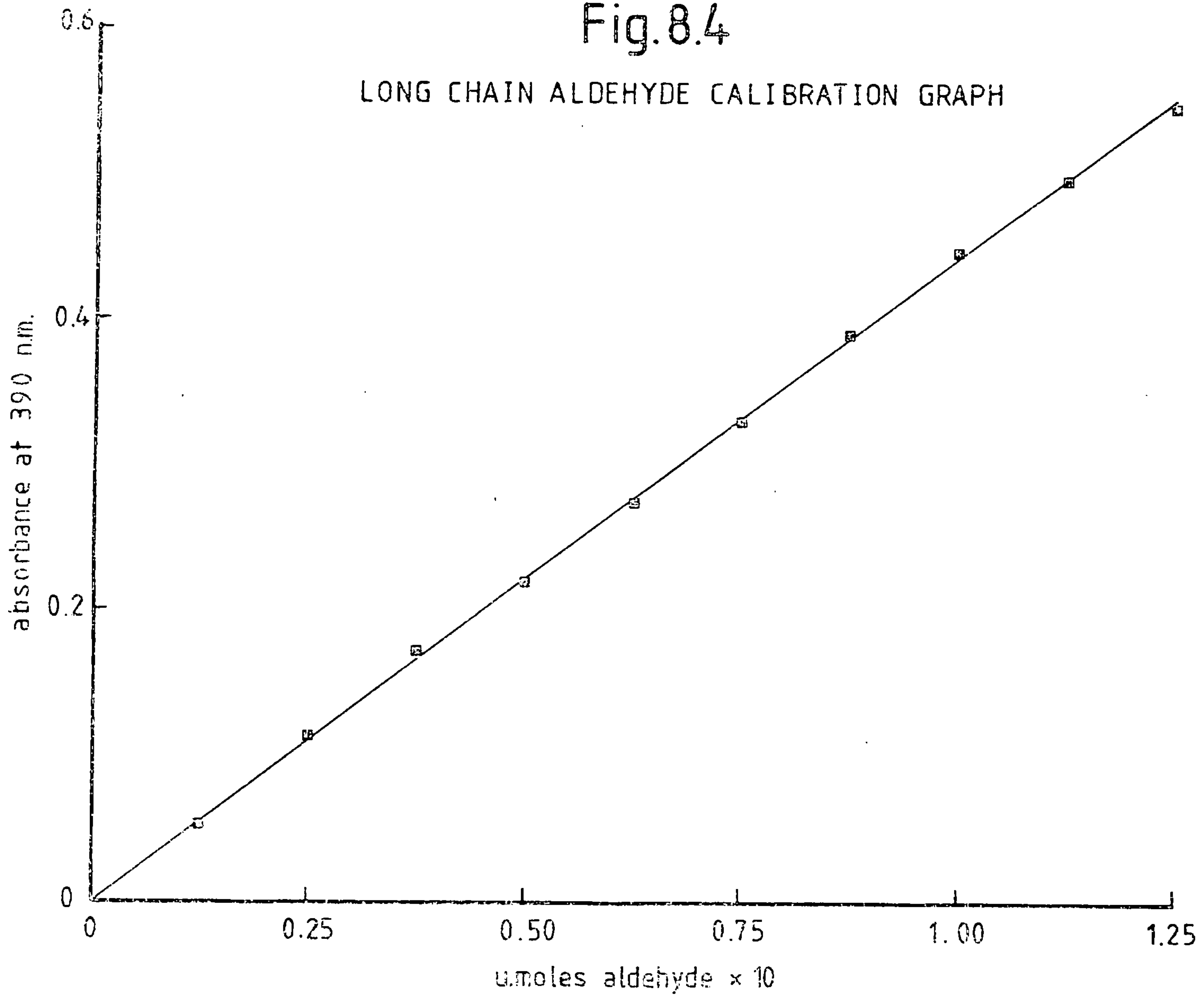


Fig.8.4

LONG CHAIN ALDEHYDE CALIBRATION GRAPH

Prepared solutions: (All solutions contained 0.1% w.v B.H.T)

(i) Chloroform/Methanol.

This was made by mixing two volumes of chloroform with one volume of methanol.

(ii) Chloroform/Methanol/Hydrochloric acid

This was made by preparing a 1% (w;v) solution of hydrochloric acid in a mixture of equal volumes of chloroform and methanol.

(iii) Chloroform/Methanol/Ammonia.

This was made by preparing a 0.5% (w:v) solution of ammonia in a mixture of equal volumes of chloroform and methanol.

The membranes were sedimented by centrifugation at 100,000g, and then homogenised in chloroform/methanol. The solid was sedimented by centrifugation (≃3000g) and the supernatant retained. The pellet was then re-extracted once in chloroform/methanol, twice in chloroform/methanol/hydrochloric acid and twice in chloroform/methanol/ammonia by repeated cycles of homogenisation and centrifugation. The supernatants from each step were pooled and then extracted with a 0.79% potassium chloride. The organic and aqueous phases were separated by centrifugation and the organic phase was re-extracted with 0.79% potassium chloride. The organic phase was again separated from the aqueous phase by centrifugation. Water was removed from the aqueous phase by standing the latter in anhydrous sulphate overnight. The dried lipid solution so formed was filtered free of solid sodium sulphate through a scintered glass funnel and the solid eluted with chloroform. The filtrate and washings were concentrated in a rotary evaporator and stored in small aliquots (1-1.5 cm^3) in sealed glass ampoules at -25^oC.

(11) Thin layer chromatographic separation of phospholipid fractions

The procedure adopted was adapted from that described for mammalian phospholipids by Veerkamp & Broekhuyse (1976).

(i) Preparation of thin layer plates.

A slurry of silica gel H (Kieselguhr H - Trp 60) was made in 0.1% aqueous solution of sodium acetate (45gm silica gel per 180 cm^3 solution). The slurry was coated on 20 cm square glass plates to a thickness of 0.5m.m. The plates were air dried, and then activated by oven drying at 110-120^oC for at least two hours before use.

An aliquot of the lipid sample containing up to 1 mg of total lipid was applied to the plate as aspot and the plate was dried at 110-120^oC for 5-10 minutes. The plate was first developed in a solvent containing chloroform (65 volumes), methanol (30 volumes), and 28% ammonia (4 volumes). The plate was then dried at 110-120^oC for 5-10 minutes, rotated through 90° and developed in a solvent containing chloroform (135 volumes), methanol (65 volumes), glacial acetic acid (18 volumes) and distilled water (3 volumes). The plate was then dried at 110-120^oC for 5-10 minutes before being treated with the locating agent.

(ii) Location and identification of phospholipid fractions after thin layer chromatography.

The procedure used for location of the phospholipid fractions was varied according to the experimental procedures intended after the locations of the fractions.

(a) Location of fractions intended for gas chromatographic analysis of fatty acid methyl esters.

This was done according to the procedure described by Gitler (1972). The plates were lightly sprayed with 0.1% 8-anilino-1-napthalene sulphonate (ANS) and irradiated with a U.V. lamp using

a 350 n.m. filter. The phospholipid fractions appeared as bright fluorescent spots against a dark background.

(b) Location of fractions intended for lipid phosphorous determinations.

It was found that the A.N.S. locating agent above (a) severely interfered with the phospholipid assay procedure of Raheja _et al_ (1973). Consequently, the fractions intended for further lipid phosphorous determinations were located by immersing the dry plate in a chromatography tank containing iodine vapour. The fractions appeared as yellow spots against a white background. After successfully locating the target fraction(s), the iodine was removed by warming the plate at 110-120°C for 5-10 minutes.

NOTE: The ANS locating procedure was prefereed to iodine for the location of the fractions intended for gas chromatographic analysis of fatty acid methyl esters, because the oxidising power of iodine was considered likely to interfere with the carbon-carbon double bonds in the unsaturated fatty acids.

The fractions located were identified by removing authentic phospholipid standards under comparable conditions.

(12) <u>Gas liquid chromatography of fatty acid methyl esters</u>

The samples used for these analyses were usually those phospholipid fractions separated by thin layer chromatography as described above. The samples were methanolysed and separated on a polyethylene adipase stationary phase.

(a) Preparation of fatty acid methyl esters.

The phospholipid fractions were methanolysed in a bottle sealed with a screw cap lined with a tuf-bond seal.

The silica gel containing the target phospholipid fraction was scraped off the plate into a bijou bottle, and then a quantity of Boron Trifluoride/methanol was added so as to 'wet' the entire sample. The bottle was then sealed and the sample heated at 100°C for 15 minutes so as to complete the methanolysis. After the sample had cooled, a stream of dry nitrogen was blown over the sample so as to remove the bulk of the low boiling point contaminants and to volatalise any residual Boron trifluoride/methanol. The fatty acid methyl esters were then eluted from the residue with n-hexane. The solution obtained was then concentrated to a very small volume in a rotary evaporator, and used immediately for gas liquid chromatographic analysis.

(b) Preparation of the chromatographic column

20 grams of Gaschrom Q (100-120 mesh) were added to 100 cm^{3} of a 3% solution of polyethylene glycol adipase in chloroform (w/v). The mixture was then shaken and filtered through a scintered glass funnel. The residue was first air dried and then heated at 100°C for 1 hour to ensure complete volatilisation of any residual chloroform. The solid so formed was poured in a meter long glass column and packed tightly with the aid of a vacuum pump. The column so formed was then conditioned by heating in a stream of dry nitrogen (flow rate 20-25 cm^{3} min^{-1}) at 250°C for 48 hours before use.

(c) Gas liquid chromatography of phospholipid methanolysates, and the identification and quantification of the resolved methyl esters.

The gas liquid chromatographic separation of the samples was done on the above column (b) in a Pye 104 series gas chromatograph at 180°C. Dry white spot nitrogen was used as the carrier gas at a

flow rate of 45 cm^3 min^{-1}. The resolved samples were detected by flame ionisation (combustion system Hydrogen/Air) and the signal from the detector was used to trace a 'chromatograph' on a flat bed chart recorder. In cases where fatty acid methyl ester stands were available, the peaks were tentatively identified by comparing their retention times with those of the standards under the same conditions. However, where such standards were unavailable, a tentative identification was done by comparing the observed retention times with those calculated according to Ackman (1963a, 1963b). The quantification procedure used (Carroll, 1961), enabled an estimation of the relative proportions of the resolved materials. Since this procedure assumed a complete separation of the resolved samples, it was necessary to correct the observed peak heights for interference from neighbouring peak(s), in cases where resolution was incomplete. This correction was calculated according to the principles described by Bartlett & Smith (1960). The product of the peak height (or corrected peak height) and the retention time gave a value proportional to the area of the peak and consequently the mass of the sample forming the peak. On account of the variations in the day to day operating conditions, the mass estimate obtained was normalised by expressing the same as a percent of the total sample applied (i.e. the sum of all the peak areas).

(d) 'Short hand' notation used to identify fatty acids.

The descriptive notation used to refer to fatty acids is the same as that described by Farquhar, Insull, Rosen, Stoffel & Ahrens (1959). This describes the fatty acid in terms of the length

of the carbon chain, the number of double bonds, and the length of the 'end carbon chain' (i.e. the length of the carbon chain starting at the methyl end of the fatty acid and ending at the middle of the first double bond). The system is adequate for the description of fatty acids normally found in animal tissues. It assumes that the fatty acids are unbranched and, in the case of polyunsaturated fatty acids, it also assumes that the olefinic bonds are separated by methylene groups (i.e. methylene interrupted polyunsaturated fatty acids). Thus the notation 22:6:w3 represents a fatty acid of carbon length, 22, which contains six methylene interrupted carbon: carbon double bonds, and with an end carbon chain three carbon atoms long.

(13) Radio-isotope dilution assay of the phospholipid fractions.

(a) Preparation of ^{14}C labelled phospholipids.

^{14}C was incorporated into the brain phospholipids of young rats by 5 daily injections of 10uC of 2 ^{14}C sodium acetate. The injections were done during the period of most rapid post-natal brain growth (5-10 days after birth) so as to maximise the incorporation of the isotope into the brain lipids. The animals were killed and lipids extracted from the brain as described above (10). The phospholipid fractions were then separated as described above (11). The procedure was found to yield approximately 10uC of ^{14}C phospholipid per mC of ^{14}C acetate used, and the specific activity of the prepared ^{14}C phospholipid was between 50-100 nano Curies per micro mole of phospholipid.

Samples of the labelled phospholipid (of known specific activity) containing between 500-1000 disintegration per minute were added to the membrane lipid extract containing a known amount of lipid phosphorous.

The components of the mixture were then separated by thin layer chromatography as described above (11). The specific activities of the separated fractions was then determined, and the amount of each fraction present in the original membrane was calculated from the dilution of the label.

(14) Induction of hibernation in Hamsters

Twelve week old hamsters were placed singly in cages with a generous supply of nesting material (shredded paper), food and water. The animals were then conditioned in the dark at 5°C with the minimum of disturbance for 6 weeks. After the conditioning period, they were transferred to a dark room of ambient temperature 1°C, and the animals usually went into their hibernation cycles within 6 weeks of their being transferred to the lower temperature. The animals had a hibernation cycle of 3-4 days and they were sacrificed for experiments only during their periods of 'hibernating sleep'.

(15) Acclimation of Rainbow Trout to different temperatures

The animals (5-7" long) were purchased from a commercial fish farm and acclimated to the laboratory conditions for 4 weeks. Groups of 40-50 animals were then transferred to tanks of ambient temperatures 20°C and 5°C, and were kept under these conditions for at least 8 weeks before being sacrificed for experiments. During their period of acclimation, the animals were fed once every three days on trout pellets, and they were maintained in well aeriated running water. The 20°C acclimated animals were maintained at 16 hr light - 8 hr dark photoperiod while those at 5° C were kept in a 8 hr light - 16 hr dark photoperiod.

RESULTS

(a) A comparison of the kinetic properties of the Na^+-K^+-ATPases in the brain synaptic membranes of 5°C and 20°C acclimated rainbow trout.

The membrane preparations used for these studies were the sodium dodecyl sulphate extracts of the brain synaptic membranes of the experimental animals. The specific activities of the Na^+-K^+-ATPases from the membranes prepared from both the 5°C and 20°C acclimated animals were found to be between 650 and 745 units when measured at 37°C (see Table 8.1). Thus no significant differences were found between the specific activities of the two samples.

TABLE 8.1

Specific Activities of the Na^+-K^+-ATPases in SDS extracts of brain synaptic membranes from 5°C and 20°C acclimated trout

SAMPLE	Specific Activity (μmoles Pi mg.protein^{-1}. hr^{-1} at 37°C	
5°C Acclimated	683 ± 31	(n=4)
20°C Acclimated	695 ± 44	(n=3)

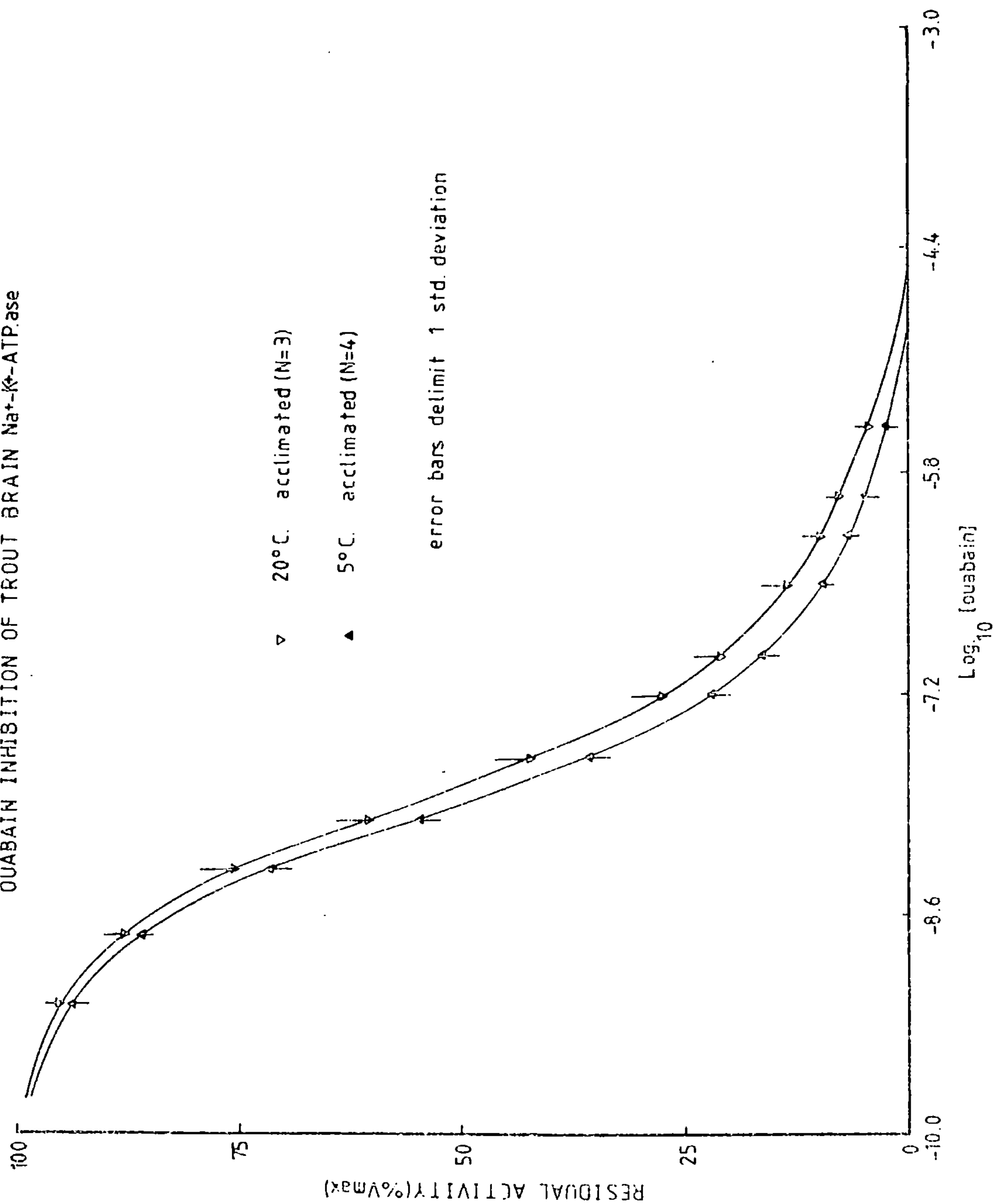
Values - mean of n preparations ± 1 standard deviation.

Each preparation was extracted from the brains of 7-10 animals

The effect of the temperature acclimation of the ouabain inhibition of the enzyme is shown in Fig. 8.5. In both cases, the pattern of the decay of activity described a decay curve which, while appearing to be monophasic, showed significant deviation from simple uncompetitive inhibition kinetics at ouabain concentrations greater than 10^{-7}M.

Fig.8.5

EFFECT OF TEMPERATURE ACCLIMATION ON THE OUABAIN INHIBITION OF TROUT BRAIN Na^+-K^+-ATPase



From the decay curves, it is apparent that the preparation extracted from the 20°C acclimated animals showed some bias towards a lower ouabain sensitivity. The numerical description of the decay patterns was done according to the model adopted in Chapter 3, and from the list of the defining parameters (see Table 8.2A), the 'lower ouabain sensitivity bias' observed for the preparations from the 20°C acclimated animals, was defined in terms of slightly higher values for the ouabain binding parameter (5° $K_i = 9.74 \times 10^{-9}$ vs 20° $Ki = 1.28 \times 10^{-8}$), the catalytic co-operativity parameter (5° $\phi = 0.187$ vs 20° : $\phi = 0.239$) and the inhibitor co-operativity parameter (5°:$\beta = 119$ vs 20°: $\beta = 129$). However, despite this bias, the differences between the respective values of these primary parameters were not found to be significantly different ($p>0.05$). The apparent lower ouabain sensitivity of the preparations from the 20°C acclimated animals was also reflected in the difference between the respective apparent pI_{50} values (5°: $pI_{50} = 7.93$ vs 20°: $pI_{50} = 7.78$), differences which were statistically significant ($p<0.02$). However, since the apparent pI_{50} is a parameter derived from the other three by calculation, it seemed likely that the observed difference only represented the cumulative effect of the observed, but insignificant, differences between the other parameters, and no significance was attached to this observation. Thus it was concluded that the changes coincident with the acclimation to the different temperatures did not affect the ouabain sensitivity of the Na^{+}-K^{+}-ATPase.

Figure 8.6 shows the effect of increasing potassium ion concentration on the activity of the Na^{+}-K^{+}-ATPases extracted from the differently acclimated animals. It is clear that the activities of

TABLE 8.2

Effect of temperature acclimation on the kinetic properties of the Na^+-K^+-ATPase from rainbow trout brain synaptic membranes.

A. OUABAIN INHIBITION

*Parameters	5°C Acclimated (n=4)	20°C Acclimated (n=3)
Ki(M)	$9.737 \times 10^{-9} \pm 8.25 \times 10^{-10}$	$1.275 \times 10^{-8} \pm 2.01 \times 10^{-9}$
ϕ	0.187 ± 0.031	0.239 ± 0.069
β	119 ± 32	129 ± 48
apparent pI_{50}	7.93 ± 0.007	7.78 ± 0.02

*See Chapter 3

B. POTASSIUM ACTIVATION

†Parameters	5°C Acclimated (n=4)	20°C Acclimated (n=3)
Ka (M)	$1.36 \times 10^{-4} \pm 3.63 \times 10^{-5}$	$1.94 \times 10^{-4} \pm 5.1 \times 10^{-5}$
Kb (M)	$4.06 \times 10^{-3} \pm 5.23 \times 10^{-4}$	$4.11 \times 10^{-3} \pm 6.01 \times 10^{-4}$
$K_{0.5}$ (M)	$4.18 \times 10^{-3} \pm 5.25 \times 10^{-4}$	$4.31 \times 10^{-3} \pm 6.05 \times 10^{-4}$

†See Chapter 5

C. TEMPERATURE KINETICS

▲Parameters	5°C Acclimated (n=4)	20°C Acclimated (n=3)
U ($K.J.mol^{-1}$)	38.6 ± 5.3	38.4 ± 5.1
ΔH ($K.J.Mol^{-1}$)	-70.9 ± 7.3	-65.8 ± 6.2
ΔS ($J.K.^{-1}Mol^{-1}$)	-248 ± 28	-229 ± 31

▲See Chapter 4

All values mean of n preparations $\pm$ 1 standard deviation

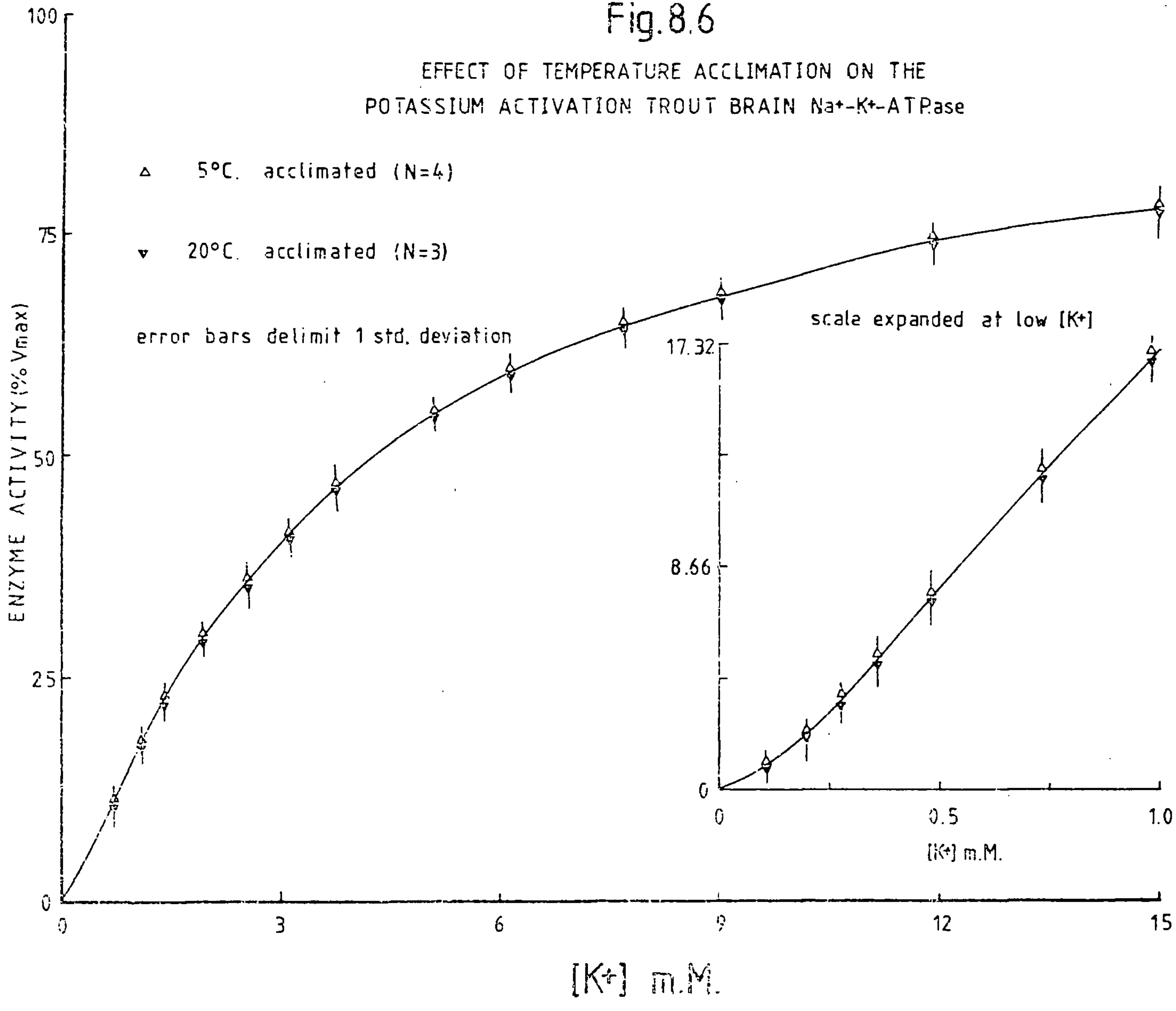


Fig.8.6
EFFECT OF TEMPERATURE ACCLIMATION ON THE
POTASSIUM ACTIVATION TROUT BRAIN Na+-K+-ATPase
5°C. acclimated (N=4)
20°C. acclimated (N=3)
error bars delimit 1 std. deviation
scale expanded at low [K+]
ENZYME ACTIVITY (% Vmax)
100
75
50
25
0
0
3
6
9
12
15
[K+] m.M.
17.32
8.66
0
0
0.5
1.0
[K+] m.M.

both preparations show a sigmoidal concentration dependence, and that there is little or no difference between this aspect of both preparations. This is further emphasised by the list of parameters (see Table 8.2B) defining the stoichiometric sigmoid curves fitted to the points (see Chapter 5). The parameters calculated for the extracts from the 20°C acclimated animals showed a slight bias towards a lower potassium sensitivity in that larger values were returned for its Ka parameter (5°: Ka = 1.36×10^{-4} vs 20°: Ka = 1.94×10^{-4}), the Kb parameter (5°:Kb = 4.06×10^{-3} vs 20°: Kb = 4.11×10^{-3}) and the derived $K_{0.5}$ value (5°: $K_{0.5}$ = 4.18×10^{-3} vs 20°: $K_{0.5}$ = 4.31×10^{-3}). However, since the differences between the respective potassium activation parameters were not found to be significant ($p>0.1$), this aspect of the Na^{+}-K^{+}-ATPase was not considered responsive to the regime of temperature acclimation used.

The temperature kinetics of the Na^{+}-K^{+}-ATPases prepared from the two groups of fish are shown in figure 8.7. In both cases, the temperature dependence of the catalytic activity of the preparations described non-linear Arrhenius plots which, when interpreted within the framework adopted in Chapter 4, returned similar values for the defining parameters (see Table 8.2C). The values returned for the apparent activation energy parameter (5°: U = 38.6 vs 20°: U = 38.4), the enthalpy of transition (5°: ΔH = 70.9 vs 20°: ΔH = 65.8) and the entropy transition (5°: ΔS = 248 vs 20°: ΔS=229), were not significantly different ($p>0.05$) and as a result, it was concluded that the temperature kinetics of the Na^{+}-K^{+}-ATPase was not responsive to the changes coincident with the temperature acclimation by these animals.

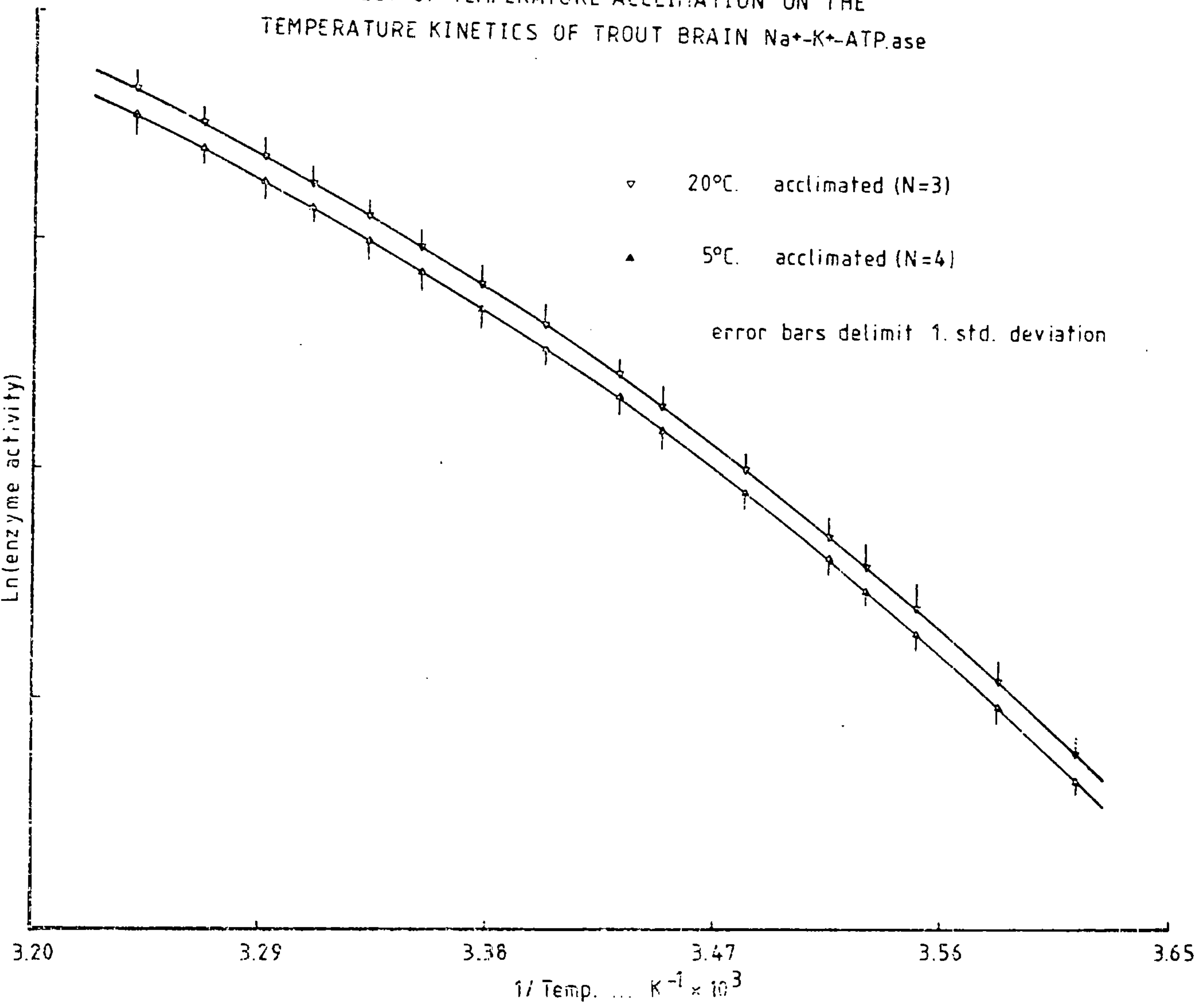


Fig. 8.7
EFFECT OF TEMPERATURE ACCLIMATION ON THE
TEMPERATURE KINETICS OF TROUT BRAIN Na+-K+-ATP.ase
20°C. acclimated (N=3)
5°C. acclimated (N=4)
error bars delimit 1. std. deviation
Ln(enzyme activity)
3.20
3.29
3.38
3.47
3.56
3.65
1/ Temp. ... K-1 x 10 3

(b) Effect of hibernation on the Na^+-K^+-ATPase and the lipids present in the synaptic membranes prepared from hamster brain.

The experimental measurements were done on the sodium dodecyl sulphate extracts of the synaptic membranes prepared from the brains of the respective animals. The specific activity of the Na^+-K^+-ATPases contained in those membranes are shown in Table 8.3 below. In both cases the values were in the range expected for the enzyme when prepared from this source (see Table 6.2).

TABLE 8.3

Specific activities of the Na^+-K^+-ATPases in SDS extracts of brain synaptic membranes from active and hibernating hamsters

SAMPLE	Specific activity	(µmoles Pi.mgprotein^{-1}.hr^{-1} at 37°C)
Active animals	338$\pm$15	(n=4)
Hibernating animals	349$\pm$11	(n=4)

Values mean of n preparations $\pm$ 1 Standard deviation

Figure 8.8 shows the ouabain dose response curves of the enzyme extracted from the active and hibernating animals. The dose response curves described by both preparations were biphasic showing a pronounced inflection at ouabain concentrations of between 10^{-7}-10^{-5}M. It is also clear that little or no difference was observed between the ouabain sensitivities of both preparations. Thus, after fitting the data to the model described in Chapter 3, no significant differences were found between the values of the ouabain binding parameters as well

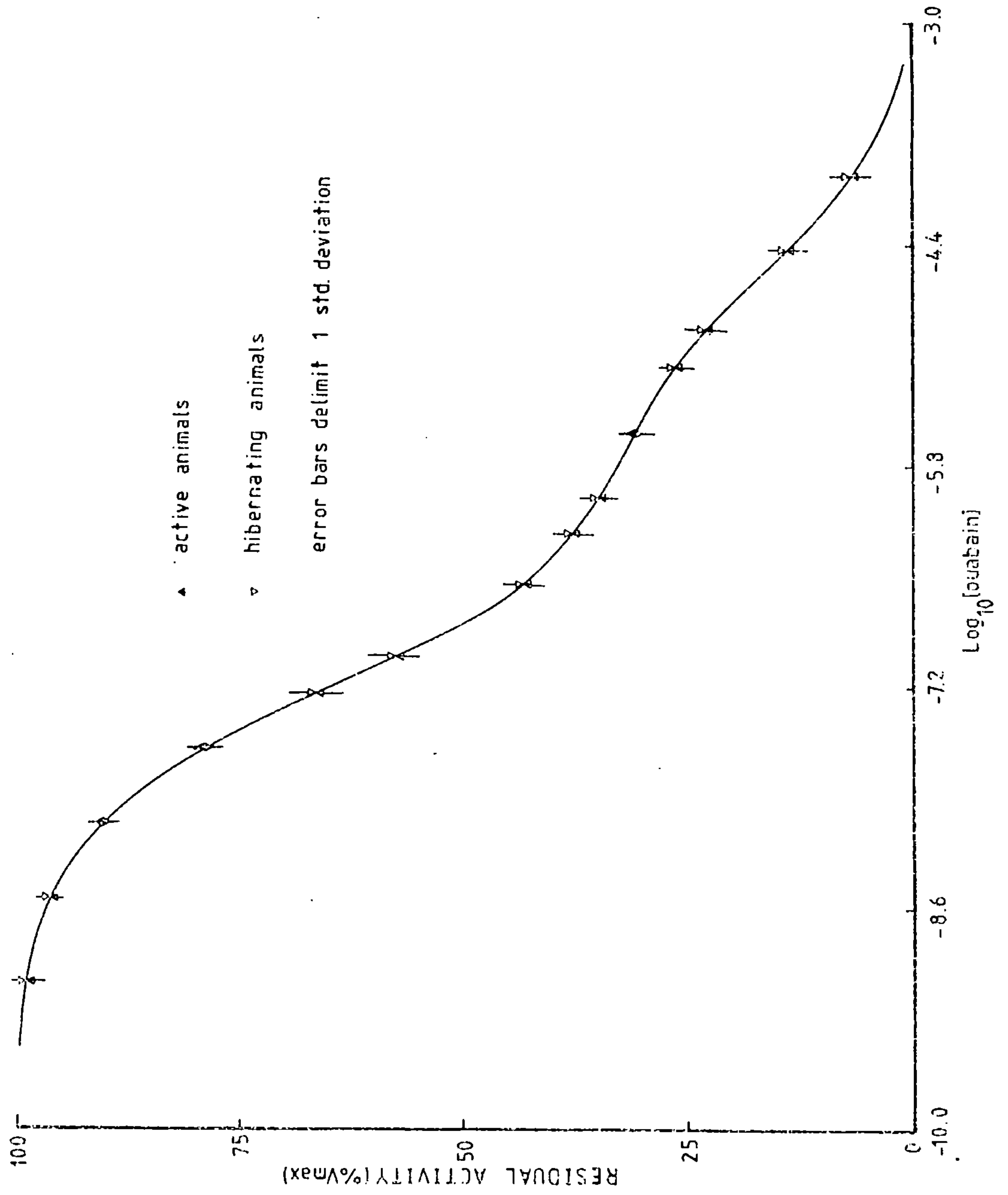


Fig. 8.8

EFFECT OF HIBERNATION ON THE OUABAIN INHIBITION OF HAMSTER BRAIN Na^{+}-K^{+}-ATPase

as the co-operativity parameters (see Table 8.4A). Although the values for the Ki and β parameters of the preparation from the hibernating animals were somewhat higher than those obtained from the active animals, the larger errors in the estimation precluded the attachment of any statistical significance to these results ($p>0.1$). Thus it seemed unlikely that this aspect of the hamster brain Na^+-K^+-ATPase was affected by any changes coincident with hibernation.

The response of the two enzyme preparations to increasing concentrations of potassium ion is shown in Figure 8.9. This shows that both preparations exhibit a sigmoidal response to increasing potassium concentrations and that the preparation obtained from the active animals was slightly more potassium sensitive than that of the hibernating animals since the latter showed slightly lower levels of activity (relative to V_{max}) than that of the active animals at all non-saturating potassium concentrations. This observation, when quantified by the stoichiometric sigmoid curves described in Chapter 5, was found to be entirely attributable to the differences in the apparent high affinity potassium binding constants (i.e. Ka - see Table 8.4B). The results showed that there was no significant difference between the values of low affinity potassium binding constants (Kb), but in the case of the extracts from the active animals, the Ka value was slightly lower than that of the hibernator and that the difference was statistically significant ($p\leq0.5$). This difference was obviously the reason for the difference between the $K_{0.5}$ values, since there was no significant difference between the Kb values and the $K_{0.5}$ is derived from both these primary parameters (Ka and Kb) by calculation.

TABLE 8.4

Effect of hibernation on the kinetic properties of the Na^+-K^+-ATPase from Hamster brain synaptic membranes.

A. OUABAIN INHIBITION

*Parameters	Active animals	Hibernating animals
Ki (M)	$4.97 \times 10^{-8} \pm 4.89 \times 10^{-9}$	$5.70 \times 10^{-8} \pm 1.28 \times 10^{-8}$
ϕ	0.65 ± 0.027	0.617 ± 0.16
β	399 ± 12	585 ± 214
apparent pI_{50}	6.83 ± 0.003	$6.83 \pm .008$

* See Chapter 3

B. POTASSIUM ACTIVATION

†Parameters	Active animals	Hibernating animals
Ka (M)	$8.68 \times 10^{-5} \pm 9.1 \times 10^{-6}$	$3.11 \times 10^{-4} \pm 1.33 \times 10^{-4}$
Kb (M)	$1.85 \times 10^{-3} \pm 7.46 \times 10^{-5}$	$1.90 \times 10^{-3} \pm 2.62 \times 10^{-4}$
$K_{0.5}$ (M)	$1.93 \times 10^{-3} \pm 8.17 \times 10^{-5}$	$2.16 \times 10^{-3} \pm 2.44 \times 10^{-4}$

† See Chapter 5

C. TEMPERATURE KINETICS

▲Parameters	Active animals	Hibernating animals
U. $(K.J.mol^{-1})$	69.9 ± 4.4	66.1 ± 6.2
ΔH $(K.J.mol^{-1})$	-150 ± 10	-161 ± 17
ΔS $(J.K.^{-1}mol^{-1})$	-519 ± 36	-558 ± 52

▲See Chapter 4.

All values mean of 4 preparations $\pm$ 1 Standard deviation

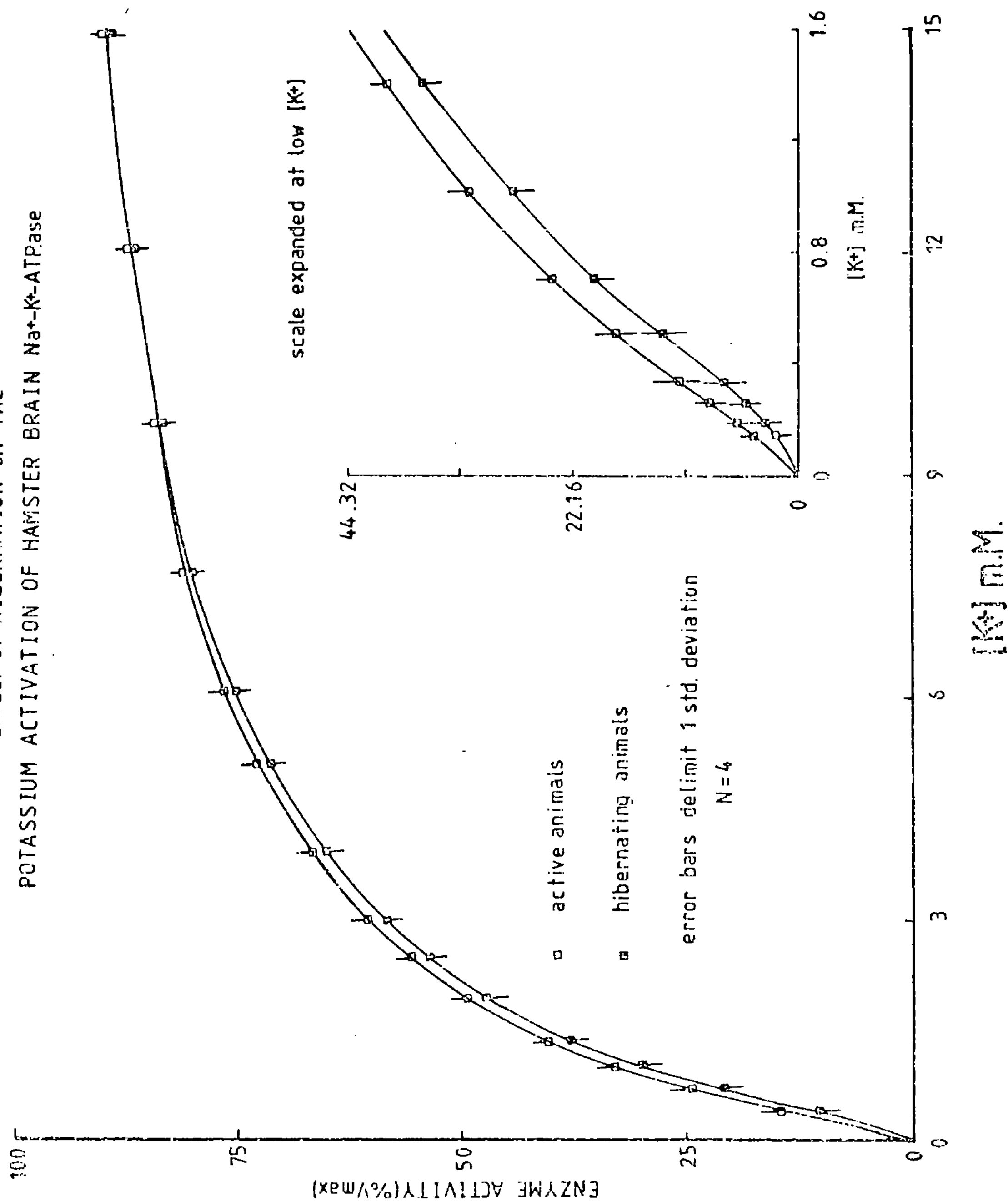


Fig.8.9

EFFECT OF HIBERNATION ON THE POTASSIUM ACTIVATION OF HAMSTER BRAIN Na+-K+-ATPase

Figure 8.10 compares the temperature dependence of the activity of the Na^{+}-K^{+}-ATPases extracted from both active and hibernating animals. It is clear that both preparations describe non-linear Arrhenius plots, and from the figure (8.10) very little difference can be detected in the curves fitted to the data (see Chapter 4). This is supported by the list of the parameters defining the fitted curves (see Table 8.4C), since the differences between the apparent activation energy values (active ≈69.6 vs hibernating ≈66.1), the enthalpy parameter (ΔH active ≈150 vs hibernating ≈161), and the entropy values (∇S active ≈519 vs hibernating ≈558), were all found not to be statistically significant ($p>0.05$) - see Table 8.4C.

The results of the above kinetic measurements showed that, with the probable exception of one of the potassium activation parameters, no significant differences were detected between the Na^{+}-K^{+}-ATPases prepared from the active and hibernating animals with respect to the kinetic parameters studied. Since the project was designed to evaluate a probable role for membrane lipid in the fine control of the Na^{+}-K^{+}-ATPase, and given the cyclic hibernating pattern of the hamster ('hibernating sleep' was punctuated by brief periods of activity every 3-4 days), it was necessary to determine whether any membrane lipid changes had taken place under the experimental conditions. This aspect was approached by comparing the lipid composition of the membranes prepared from both active and hibernating animals. This was investigated in terms of the gross lipid composition, the gross phospholipid composition and the fatty acid composition of the main phospholipid fractions.

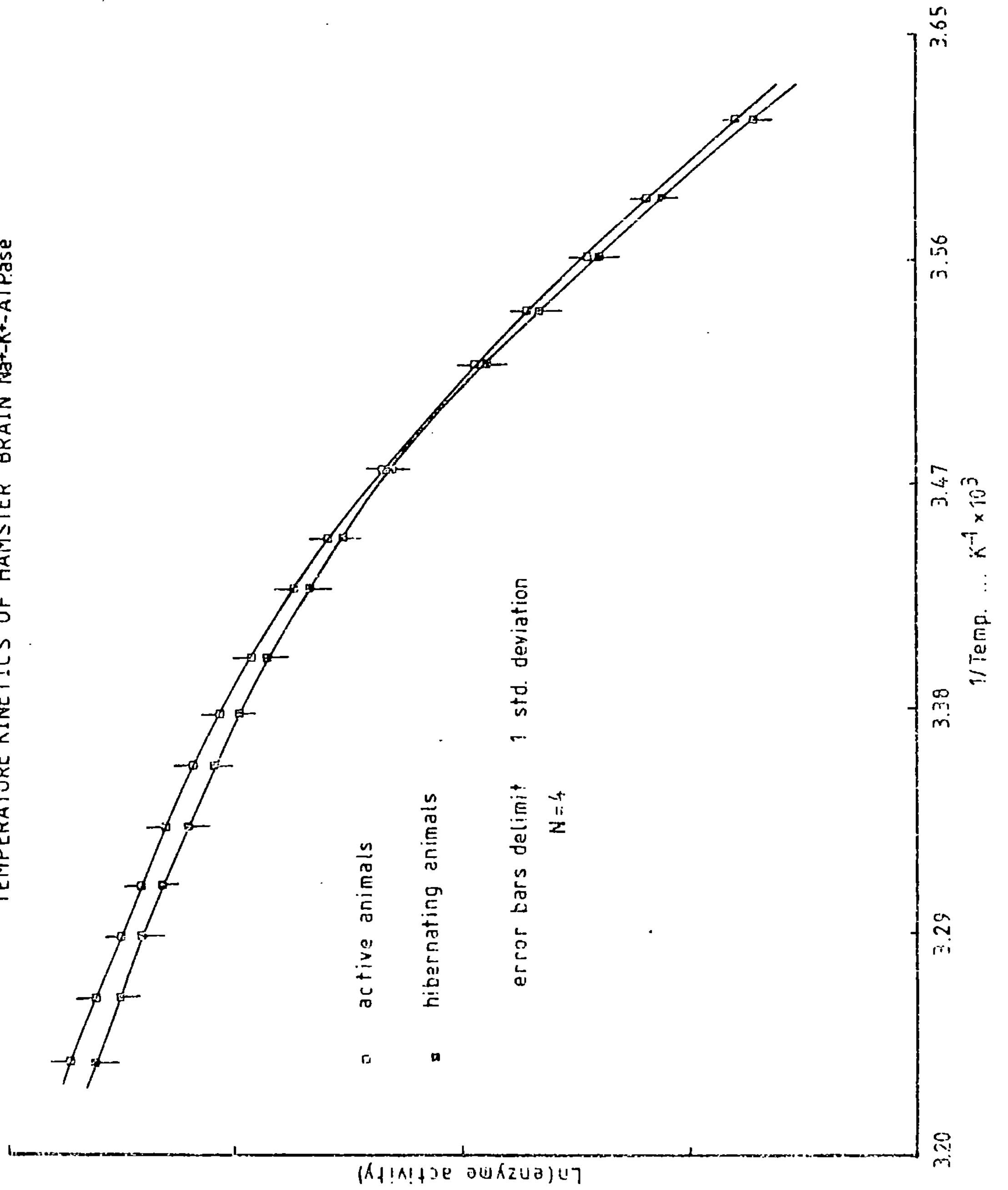


Fig. 8.10

EFFECT OF HIBERNATION ON THE
TEMPERATURE KINETICS OF HAMSTER BRAIN Na+-K+-ATPase

The results of the gross lipid analysis are shown in Table 8.5, where they are expressed relative to the protein content of the respective membranes. Each preparation used for these and other lipid analyses was obtained from brain extracts of 6-8 animals. The membranes prepared from the hibernating animals were found to contain lower molar contents of total phospholipid and cholesterol than those from the active animals. The effect of hibernation also appeared to result in a significant increase in the molar plasmalogen content of the membranes (≃50%) while the sphingolipid content was apparently unaffected. The observed differences with respect to the molar contents of lipid phosphorous, cholesterol and plasmalogen were all found to be significant ($p<0.05$). However, since the results were expressed relative to protein, the observations could have been the result of changes in the protein content either as a result of hibernation or as a result of differences in the efficiency of the SDS extraction procedure. Although there is no data to enable an unambiguous evaluation of the possible increase in the protein content of the membrane samples as a result of hibernation, this possibility seemed unlikely on account of the differences in the relative changes of the various species analysed (i.e. lipid phosphorous ≃-18%; cholesterol ≃-10%; plasmalogen ≃+50% and spingolipids ≃0%). Differences in protein content as a result of a variation in the efficiency of the SDS extraction procedure were also not considered to be significant contributors to the observation since any such variation would have affected the specific activities of the Na^+-K^+-ATPase preparations and as is obvious from the footnotes to Table 8.5, the specific activities of the Na^+-K^+-ATPase in the source membranes are well within the range previously established for SDS

TABLE 8.5

Effect of hibernation on the gross lipid composition of Hamster brain synaptic membranes

Lipid fraction	Active animals	Hibernating animals
Total phospholipid	1.283 ± 0.023	1.01 ± 0.05
Cholesterol	0.861 ± 0.060	0.676 ± 0.009
Plasmalogen	0.173 ± 0.020	0.266 ± 0.013
Sphingolipids	0.05 ± 0.002	0.049 ± 0.002

All analyses done on S.D.S. extracted synaptic membranes containing Na^+-K^+-ATPases of the following specific activities.

(a) Active animals (3 preparations) 348 ± 15 uMoles Pi.mg/protein^{-1}.hr^{-1}.

(b) Hibernating animals (2 preparations) 325 ± 7 uMoles Pi.mg/protein^{-1}.hr^{-1}.

Lipid content expressed in units of uMoles mgprotein^{-1}.

Values are means of 2 or 3 preparations (see (a) and (b) above) ± 1 standard deviation.

extracts of the enzyme from hamster brain synaptic membranes (see Table 6.2). Thus it seemed likely that the observed differences were the result of hibernation induced membrane lipid changes.

In further analyses of the respective membranes, the distribution of the major phospholipid species in lipid extracts of the membranes was compared. Two dimensional thin layer chromatographic separation of the phospholipids showed that both membrane lipid extracts contained relatively large quantities of Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and phosphatidyl serine/phosphatidyl inositol (PS/PI). Note: Phosphatidyl serine and phosphatidyl inositol were not resolved by the T.L.C. system used), along with smaller quantities of sphingolipids, lysophosphatidyl ethanolamine (LPE), cardiolipin and probably traces of glycolipids. Table 8.6 shows the distribution of the major phospholipid classes in the respective lipid extracts, as was estimated by the gross lipid composition (Table 8.5) and the isotope dilution assays. These measurements show that the four/five main phospholipid classes present (P.choline, P. ethanolamine, P.serine/P.inositol and Lyso-P.ethanolamine) account for approximately 80% of the lipid phosphorous present in both extracts. Furthermore, there appeared to be no significant effect on the relative molar proportions of the phospholipid classes assayed (approximately 85% of total lipid phosphorous) as a result of hibernation, since significant differences in the distribution of the phospholipid classes were not detected between the lipids extracted from the active and hibernating animals.

TABLE 8.6

Effect of hibernation on the gross composition of the phospholipids from Hamster brain synaptic membranes

Phospholipid	Relative Composition (Mole %)	
	Active Animals (n=3)	Hibernating animals (n=2)
†P. Choline	37.2 ± 1.4	35.1 ± 2.5
†P.Ethanolamine	22.4 ± 0.7	24.7 ± 1.8
†P. Serine/Inisitol	16.3 ± 0.8	12.4 ± 2.1
†Lyso.P.ethanolamine	6.9 ± 1.2	8.3 ± 1.1
*Sphingolipids	3.8 ± 0.8	4.7 ± 0.9
Others	≃12 - 14	≃13 - 15

Values - mean of n preparations ± 1 Standard deviation and are expressed as a molar percent of total lipid phosphorous in the lipid extracts.

All analyses on lipid extracts of S.D.S. extracted synaptic membranes containing Na^+-K^+-ATPases of the following specific activities:

(a) Active animals (3 preparations) 348±15 uMoles Pi.mg $protein^{-1}.hr^{-1}$.

(b) Hibernating animals (2 preparations) 325± 7 uMoles Pi.mg $protein^{-1}$. hr^{-1}.

† Estimated by isotope dilution analysis

* Estimated from total phospholipid analyses (Table 8.5).

In addition to the above lipid analyses, the fatty acid composition of the four(five) main phospholipid classes in both extracts were investigated by gas liquid chromatography of methanolysates of the phospholipid fractions. The results of such analyses are shown in Tables 8.7 - 8.10. The results show that in both cases, four fatty acids (16:0, 18:0, 18:1:w9 and 22:6:w3) accounted for most of the mass of the fatty acids found in all of the phospholipid classes analysed. In the case of the lipids extracted from the active animals, these four samples formed a larger fraction of the total mass of the fatty acids present (PC≃90%, PE≃78%, PS/PI≃88%, LPE≃60%), when compared with those of the hibernating animals (PC≃42%, PE≃41%, PS/PI≃51%, LPE≃48%). Thus the phospholipids isolated from the hibernating animals contained a more heterogenous population of fatty acids than that isolated from the active animals, and the major changes occurred in the PC, PE and PS/PI fractions. On a closer inspection of the fatty acid composition of the various phospholipids, it was found that the PC, PE and PS/PI fractions isolated from the hibernating animals contained considerably lower proportions of saturated fatty acids (mainly 16:0 and 18:0) than those obtained from the active animals, where the proportion of these fatty acids was some 40-65% greater than that of the hibernators (PC≃62%, PE≃40%, PS/PI≃63%). Similar changes were not found in the LPE fractions in which the unsaturated fatty acids formed only a small fraction of the total (<10%). It was also found that the relative proportions of the fatty acid 22:6:w3 in the phospholipids isolated from the hibernators was lower than that found in the phospholipids isolated from the active animals, especially when the given phospholipid class contained a 'high' proportion of

TABLE 8.7

Effects of hibernation on the fatty acid composition of the choline phosphoglycides of S.D.S. extracted hamster brain synaptic membranes.

Fatty Acid	Relative Composition (Wt %)	
	†Active animals (n=3)	*Hibernating animals (n=2)
16:0	49.37 ± 1.8	17.31 ± 0.9
16:2:w6		1.74 ± 0.2
18:0	8.62 ± 0.8	3.74 ± 0.9
18:1:w9	27.87 ± 1.1	13.09 ± 0.8
18:2:w7	trace	
18:2:w9	trace	trace
18:3:w6		4.12 ± 0.38
18:4:w4		trace
20:1:w9	1.49 ± 0.2	7.58 ± 0.6
20:2:w9		
20:3:w9	trace	
20:4:w3		trace
20:4:w6	5.16 ± 0.9	10.10 ± 0.94
21:0	trace	1.17 ± 0.3
22:1:w9	trace	
22:2:w9	trace	9.13 ± 0.83
22:4:w3		
22:4:w6		9.67 ± 0.95
22:5:w3	trace	trace
22:6:w3	4.47 ± 0.6	7.85 ± 0.6

†Sample contained 3 unidentified peaks each in trace quantities (<1%)

*Sample contained 5 unidentified peaks comprising approximately 12.5% of the total.

Values mean of n preparations ± 1 standard deviation.

TABLE 8.8

The effect of hibernation on the fatty acid composition of the ethanolamine phosphoglycerides of S.D.S. extracted hamster brain synaptic membranes

Fatty acid	Relative composition (Wt%)	
	†Active animals (n=3)	*Hibernating animals (n=2)
16:0	8.40 ± 0.3	3.26 ± 0.4
16:2:w7	trace	3.64 ± 0.6
18:0	25.43 ± 1.7	16.82 ± 1.3
18:1:w9	9.89 ± 0.8	14.17 ± 0.8
18:2:w6	trace	
18:2:w7	trace	trace
18:2:w9		trace
18:3:w6	trace	
20:0	trace	
20:1:w9	trace	4.55 ± 0.6
20:2:w9	trace	
20:3:w9	1.43 ± 0.07	
20:4:w3	trace	trace
20:4:w6		5.61 ± 0.4
20:5:w3	14.8 ± 0.97	
21:0	trace	1.85 ± 0.2
22:9:w9		3.89 ± 0.5
22:3:w6	trace	
22:4:w3	2.81 ± 0.2	2.14 ± 0.3
22:4:w6		1.45 ± 0.1
22:5:w3	2.75 ± 0.4	
22:6:w3	33.85 ± 1.93	16.48 ± 1.1

†Samples contained 2 unidentified peaks each in trace quantities (<1%)

*Samples contained 7 unidentified peaks comprising approximately 14.4% of total.

Values mean of n preparations ± 1 standard deviation.

TABLE 8.9

Effect of hibernation on the fatty acid composition of the Lyso-phosphatidyl ethanolamine phosphoglycerides from SDS extracted hamster brain synaptic membranes.

Fatty acid	Relative composition (Wt.%)	
	†Active animals (n=3)	*Hibernating animals (n=2)
16:0	1.47 ± 0.3	2.36 ± 0.3
16:1:w7		2.31 ± 0.3
16:2:w7	1.06 ± 0.2	
17:2:w9		trace
18:0	2.48 ± 0.5	2.8 ± 0.3
18:1:w9	5.22 ± 0.9	7.42 ± 0.4
18:2:w6	trace	
18:2:w7	trace	trace
18:2:w9	trace	trace
18:3:w6	trace	6.31 ± 0.5
20:0	trace	
20:1:w9	1.69 ± 0.4	9.13 ± 0.5
20:2:w9	trace	
20:4:w3		2.18 ± 0.7
20:4:w6		12.44 ± 0.3
20:5:w3	23.59 ± 1.7	
21:0	trace	2.18 ± 0.9
22:3:w6	9.30 ± 0.9	2.49 ± 0.6
22:4:w6	trace	13.32 ± 0.95
22:6:w3	50.87 ± 1.3	35.56 ± 1.3

†Samples contained 6 unidentified peaks each in trace quantities (<1%)

*Samples contained 5 unidentified peaks each in trace quantities (<1%)

Values mean of n preparations ± 1 standard deviation.

TABLE 8.10

Effect of hibernation on the fatty acid composition of the serine and Inositol phosphoglycerides from S.D.S. extracted hamster brain synaptic membranes.

Fatty Acid	Relative composition (Wt.%)	
	†Active animals (n=3)	*Hibernating animals (n=2)
16:0	2.59 ± 0.3	4.33 ± 0.5
16:1:w7	trace	1.75 ± 0.5
16:2:w6		6.97 ± 0.8
16:2:w7	trace	
17:0		4.00 ± 0.9
18:0	33.99 ± 1.02	9.15 ± 0.7
18:1:w9	8.41 ± 0.8	11.19 ± 0.8
18:2:w6	trace	
18:2:w7	trace	trace
18:2:w9		trace
18:3:w3		1.12 ± 0.08
18:3:w6	trace	trace
20:0	trace	trace
20:1:w9	trace	8.15 ± 0.6
20:2:w9	trace	
20:3:w6		trace
20:4:w3		2.51 ± 0.15
20:4:w6		9.11 ± 0.8
20:5:w3	4.2 ± 0.8	
21:0	trace	2.10 ± 0.7
22:2:w9	trace	7.07 ± 0.9
22:3:w3	2.65 ± 0.2	
22:3:w6		trace
22:4:w3	trace	
22:4:w6		5.43 ± 0.9
22:6:w3	43.12 ± 1.7	16.11 ± 1.1

† Samples contained 4 unidentified peaks each in trace quantities (<1%)

* Sample contained 5 unidentified peaks each in trace quantities.

Values mean of n preparations ± 1 standard deviation.

the fatty acid. Thus the effect was very apparent in the PE, PS/PI and LPE fractions where the reduction in the levels of the fatty acid ranged from 30-63 percent (PE≈51%, PS/I≈63%, LPE≈30%). However the PC fraction isolated from the hibernating animals contained higher levels of this fatty acid (22:6:w3) than that of the active animals though this component formed less than 10% of the total mass of fatty acids present in the fraction. The greater heterogeneity of the fatty acid population of the phospholipids isolated from the hibernating animals seemed to occur mainly by an increase in the relative levels of unsaturated C20 and C22 fatty acids since significant increases in the levels of 20:1:w9, 20:4:w6, 22:1:w9 and 22:4:w6 were generally found in all the phospholipid classes. Thus the evidence provided by these measurements and the other lipid analyses suggest that membrane lipid changes did occur as a result of the induced hibernation under the conditions adopted and that such changes were probably targeted at the relative gross lipid composition of the membranes (Table 8.5) as well as the fatty acid composition of the phospholipids.

(c) The effect of lipid peroxidation on the Na^+-K^+-ATPase and the lipid composition of rat brain synaptic membranes.

The source membranes for this study were the crude synaptic membranes prepared from rat brain. The specific activities of the Na^+-K^+-ATPase in those membranes before and after the peroxidation procedure are shown in Table 8.11. The values shown are all normalised against a control value of 100 on account of the relatively large variations in that of the crude preparation. The mean value obtained

TABLE 8.11

Comparison of the specific activities of the Na^+-K^+-ATPase in crude and lipid peroxidised rat brain synaptic membranes

Sample	Specific Activity (normalised against a control of 100)
Crude membranes	100
Lipid peroxidised membranes	94.3 ± 8

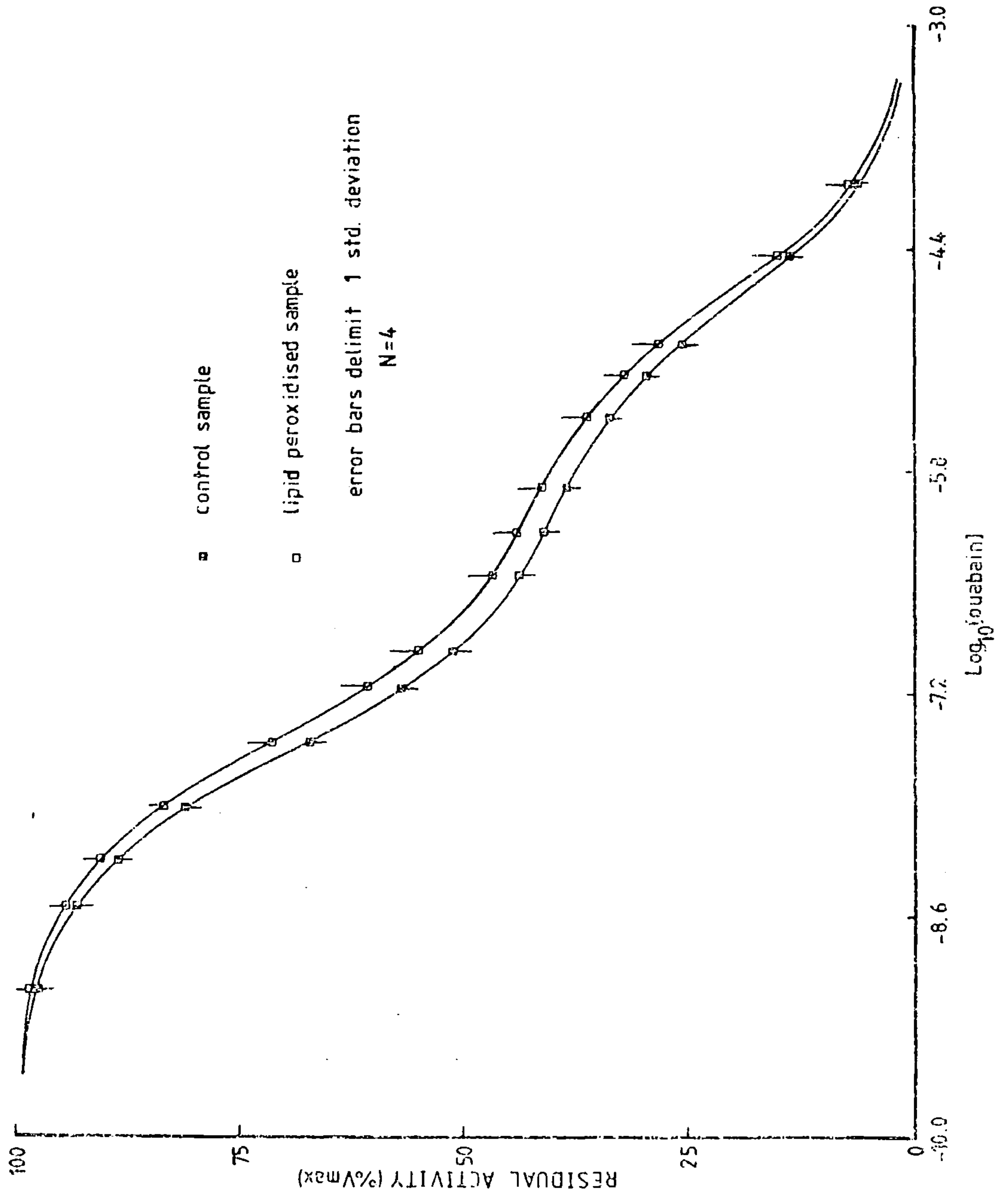
Values mean of 4 preparations $\pm$ 1 Standard deviation

for the lipid peroxidised preparation was slightly lower than that obtained for the crude preparation, but the difference was not significant ($p>0.05$). The observed similarity in the specific activity of the Na^+-K^+-ATPase in the lipid peroxidised membranes was expected on account of some low ionic strength solubilisation of some membrane associated proteins like the acetylcholine esterase (see Beauregard & Roufogalis, 1977). Thus it seemed likely that the probability of a slight inactivation of the enzyme may have to be considered when interpreting the data.

The effect of lipid peroxidation on the ouabain inhibition of the enzyme is shown in Fig. 8.11. The ouabain dose response kinetics of both preparations described a biphasic response to increasing ouabain concentration, and the data obtained from the peroxidised preparations showed a slight bias towards a lower ouabain sensitivity. The data was numerically described according to the model adopted in Chapter 3, and from the list of defining parameters (Table 8.12A), it can be seen that the lipid peroxidised preparation was described by a slightly larger Ki value (control $\simeq 2.32 \times 10^{-8}$ vs lipid peroxidised

Fig. 8.11

EFFECT OF LIPID PEROXIDATION ON THE OUABAIN INHIBITION OF RAT BRAIN Na^{+}-K^{+}-ATP.ase



$\approx 3.16 \times 10^{-8}$), a slightly larger ϕ value (control ≈ 0.8 vs lipid peroxidised ≈ 0.85) and a slightly smaller β value (control ≈ 866 vs lipid peroxidised ≈ 674) when compared with the control. However, the Table 8.12A also shows that the values of these primary parameters (ϕ, β, Ki) of the lipid peroxidised sample are all associated with relatively large standard deviations. However, this was mainly a reflection of variations between preparations rather than inaccuracies in the measurement of individual preparation. Consequently no significance was attributed to the differences between these primary parameters ($p>0.05$) and the difference between the derived parameter (pI_{50} - control ≈ 6.95 vs lipid peroxidised ≈ 6.72) which though statistically significant ($p<0.02$), probably was the cumulative result of the small (insignificant) differences between the primary parameters from which it was calculated. Thus it was concluded that the ouabain inhibition of the enzyme was not significantly responsive to the lipid modulation procedure adopted.

The effect of increasing potassium ion concentrations on the activity of the Na^+-K^+-ATPase in the crude and lipid peroxidised membranes is shown in figure 8.12. The potassium activation of both samples showed a sigmoidal concentration dependence, with the lipid peroxidised sample being biased towards a lower potassium sensitivity (i.e. lower levels of activity relative to V_{max} at non-saturating potassium ion concentrations. This was numerically defined by a stoichiometric sigmoid curve (see Chapter 5), and the list of parameters defining the fitted curves is shown in Table 8.12B. The bias towards a lower potassium sensitivity in the case of the lipid peroxidised sample is reflected in slightly higher values for the Ka parameter (control $\approx 2.25 \times 10^{-4}$ vs lipid peroxidised $\approx 3.98 \times 10^{-4}$)

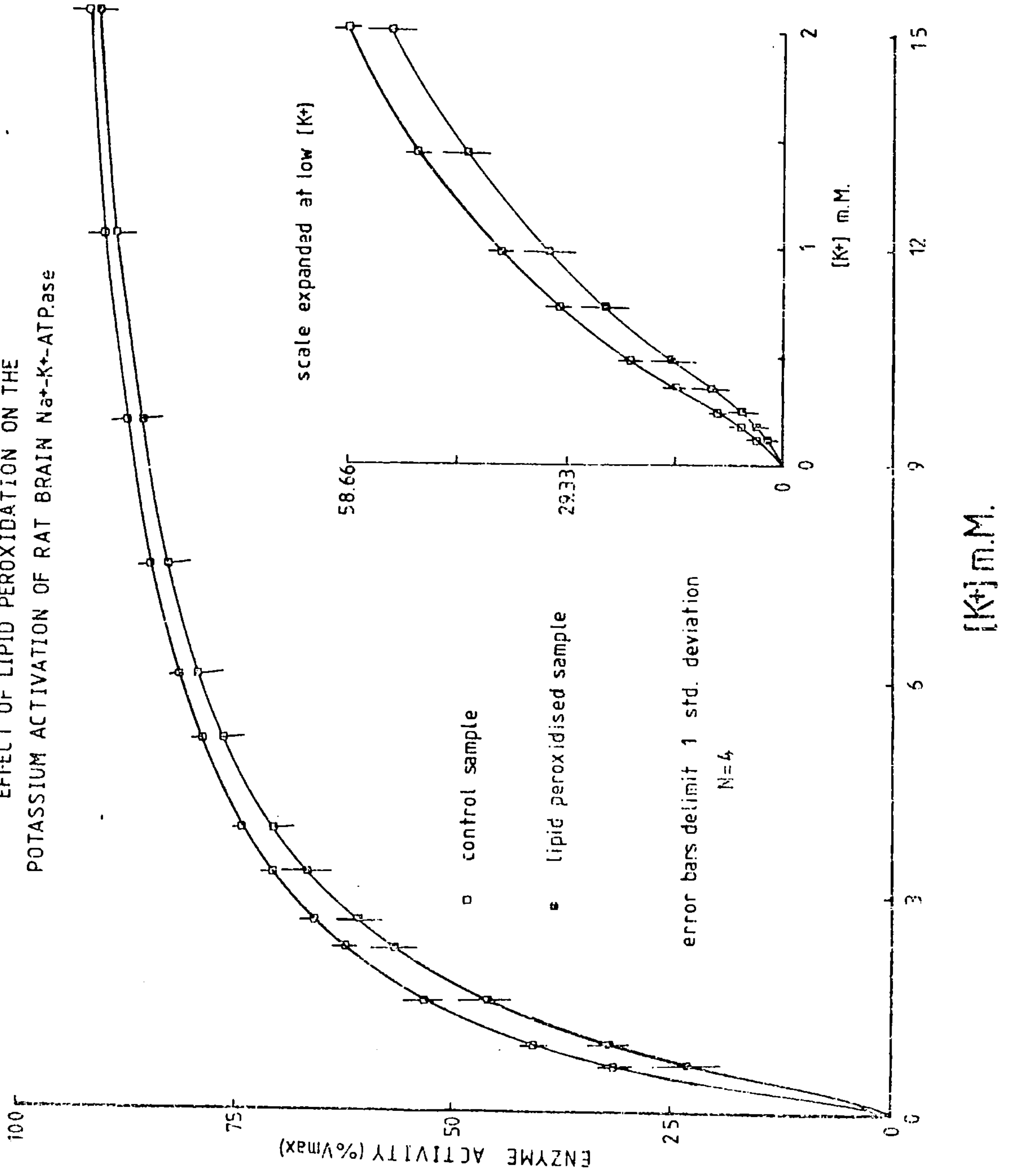


Fig.8.12

EFFECT OF LIPID PEROXIDATION ON THE POTASSIUM ACTIVATION OF RAT BRAIN Na^{+}-K^{+}-ATPase

TABLE 8.12

Effect of lipid peroxidation on the kinetic properties of the Na^{+}-K^{+}-ATPase from rat brain synaptic membranes.

A. OUABAIN INHIBITION

*Parameters	Control	Lipid Peroxidised
Ki (M)	$2.32\times10^{-8} \pm 3.5\times10^{-9}$	$3.16\times10^{-8} \pm 1.25\times10^{-8}$
ϕ	0.799 ± 0.031	0.845 ± 0.07
β	866 ± 25	674 ± 273
apparent pI_{50}	$6.95 \pm .003$	$6.72 \pm .01$

* See Chapter 3.

B. POTASSIUM ACTIVATION

†Parameters	Control	Lipid Peroxidised
Ka	$2.25\times10^{-4} \pm 6.67\times10^{-5}$	$3.98\times10^{-4} \pm 1.64\times10^{-4}$
Kb	$1.26\times10^{-3} \pm 4.97\times10^{-5}$	$1.47\times10^{-3} \pm 2.75\times10^{-4}$
$K_{0.5}$	$1.45\times10^{-3} \pm 1.13\times10^{-5}$	$1.77\times10^{-3} \pm 1\times10^{-4}$

† See Chapter 5.

C. TEMPERATURE KINETICS

▲Parameters	Control	Lipid peroxidised
U $(K.J.mol^{-1})$	67.7 ± 4.4	61.1 ± 6.5
$\Delta H (K.J.mol^{-1})$	-170.7 ± 9.0	-137.2 ± 8.4
$\Delta S (J.K.^{-1}mol^{-1})$	-592 ± 32	-472 ± 33

▲ See Chapter 4.

All values mean of 4 preparations $\pm$ 1 standard deviation.

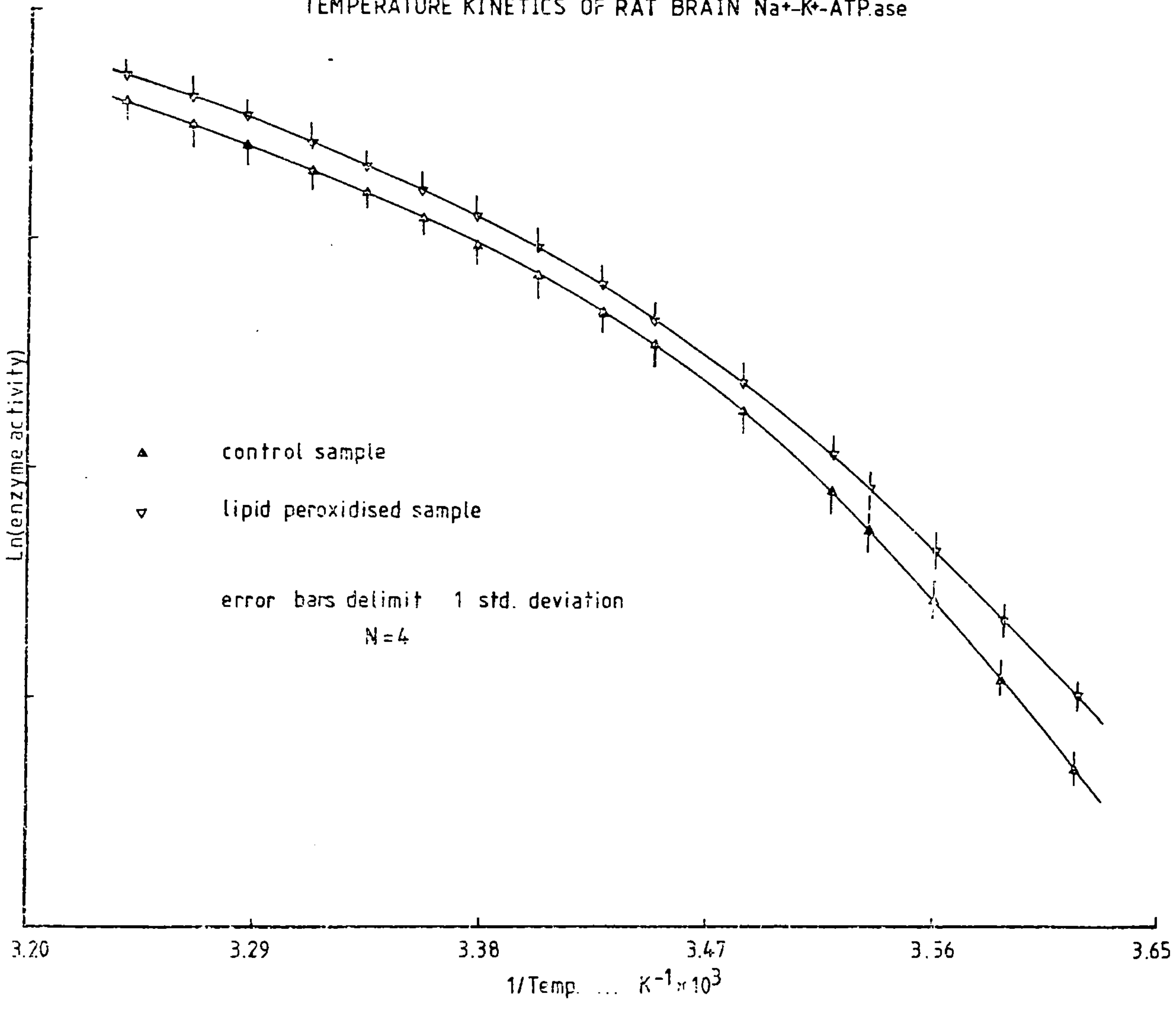
and the Kb parameter (control ≃1.26 x 10^{-3} vs lipid peroxidised ≃1.47 x 10^{-3}) when compared with those of the control sample. This is also reflected in the higher value observed for the derived parameter of the peroxidised sample ($K_{0.5}$ control ≃1.47 x 10^{-3} vs lipid peroxidised ≃1.77 x 10^{-8}). However, as is obvious from Table 8.12B, the primary parameters defining the behaviour of the peroxidised sample, are associated with relatively large standard deviations. This was also a reflection of variations in the prepared samples and not inaccurate measurements, and consequently no significance was attributed to the observed differences ($p>0.05$).

Figure 8.13 shows the effect of temperature on the catalytic activity of the Na^+-K^+-ATPase in both the crude and lipid peroxidised membranes. Both preparations described non-linear Arrhenius plots and the control sample showed signs of a slightly greater deviation from linearity at the lower temperatures. When the data was interpreted within the context of the model described in Chapter 4, the defining parameters (Table 8.12C) suggested that there was no significant difference between the apparent activation energy parameter (U - control ≃67.7 vs lipid peroxidised 61.1 $p>0.05$), but there may be a small but significant difference between the enthalpy (ΔH control ≃170 vs lipid peroxidised ≃137 $p<0.05$) and the entropy parameters (ΔS control ≃592 vs lipid peroxidised ≃472 $p<0.05$) describing the transition to the inactive state (see Table 8.12C). Thus it seemed probable that one aspect of the temperature dependence of the enzyme was responsive to the lipid targeted modulation procedure.

The results of the above kinetic measurements showed that, with the probable exception of some of the temperature kinetic parameters,

Fig.8.13

EFFECT OF LIPID PEROXIDATION ON THE TEMPERATURE KINETICS OF RAT BRAIN Na^+-K^+-ATPase



no significant difference was detected between the kinetic parameters measured for the Na^+-K^+-ATPase in the crude and lipid peroxidised membranes. The interpretation of this data within the context of any involvement of membrane lipid in the fine control of the Na^+-K^+-ATPase required some estimate of the magnitude of the effect of the peroxidation procedure under the conditions adopted. Since lipid peroxidation is obviously targeted at the carbon carbon double bonds of unsaturated fatty acyl chains, this aspect was approached by a study of the fatty acid composition of the main phospholipids present in both membrane preparations.

Thin layer chromatographic separation of the phospholipids extracted from both the crude and peroxidised membrane samples showed that they contained mainly phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine and phosphatidyl inositol (PS/PI - these were not resolved by the solvent systems used), and lyso-phosphatidyl ethanolamine (LPE) along with smaller quantities of sphingolipids, cardiolipin and probably some glycolipids. The gas liquid chromatographic analyses were done on methanolysates of the PC, PE, PS/PI and LPE fractions. The results of these measurements (Tables 8.13 - 8.16) showed that a large fraction of the mass of the fatty acids present in the extracts from the control sample was made up of contributions from only four or five fatty acids (16:0; 18:0; 18:1:w9; 20:5:w3 and 22:6:w3). These same fatty acids formed a large fraction of the total fatty acid mass of the lipid peroxidised samples, but the fraction of the total was considerably smaller and in addition the chromatograms of the respective fractions all showed

TABLE 8.13

Effect of lipid peroxidation on fatty acid composition of the choline phosphoglycerides from rat brain synaptic membranes.

Fatty Acid	Relative Composition (Wt.%)	
	†Control Sample	*Lipid peroxidised sample
16:0	40.2 ± 1.4	28.11 ± 3.3
18:0	11.73 ± 0.8	12.49 ± 1.4
18:1:w9	25.17 ± 1.1	22.44 ± 3.6
18:2:w4	trace	trace
18:2:w7	trace	trace
18:3:w6	1.13 ± 0.3	trace
20:1:w9	trace	trace
20:3:w6	trace	trace
20:5:w3	7.13 ± 0.8	9.81 ± 2.1
22:2:w9	trace	trace
22:4:w6	1.18 ± 0.2	trace
22:5:w3	1.05 ± 0.2	trace
22:6:w3	6.54 ± 0.9	14.45 ± 3.3

†Samples contained 4 unidentified peaks each in trace quantities (<1%)

*Samples contained 9 unidentified peaks comprising approx. 10% of the total.

Values mean of 3 preparations ± 1 standard deviation.

TABLE 8.14

Effect of lipid peroxidation on the fatty acid composition of the serine and inositol phosphoglycerides from rat brain synaptic membranes.

Fatty Acid	Relative Composition (Wt.%)	
	†Control sample	*Lipid peroxidised sample
18:0	35.01 ± 1.2	27.57 ± 2.4
18:1:w9	5.59 ± 0.4	11.73 ± 1.1
18:2:w6	trace	trace
18:2:w9	trace	trace
18:3:w6	1.35 ± 0.3	trace
20:1:w9	1.73 ± 0.5	trace
20:5:w3	3.27 ± 0.6	13.79 ± 1.5
21:0	trace	trace
22:0	trace	trace
22:2:w9	1.01 ± 0.4	trace
22:5:w3	1.67 ± 0.3	4.66 ± 0.3
22:5:w6	3.67 ± 0.7	1.43 ± 0.4
22:6:w3	45.13 ± 2.1	34.25 ± 3.1

†Sample contained 3 unidentified peaks in trace quantities (<1%)

*Sample contained 6 unidentified peaks comprising approx. 6% of the total.

Values mean of 3 preparations ± 1 standard deviation.

TABLE 8.15

Effect of lipid peroxidation on the fatty acid composition of the ethanolamine phosphoglycerides from rat brain synaptic membranes.

Fatty Acid	Relative Composition (Wt.%)	
	†Control Sample	*Lipid Peroxidised sample
16:0	6.77 ± 0.8	1.77 ± 0.5
18:0	27.78 ± 1.2	7.23 ± 0.85
18:1:w9	7.92 ± 0.75	10.94 ± 0.95
18:2:w4	2.41 ± 0.3	trace
18:3:w6	3.10 ± 0.2	1.43 ± 0.4
20:3:w9	3.42 ± 0.15	10.81 ± 1.1
20:4:w6	3.05 ± 0.5	2.02 ± 0.5
20:5:w3	9.35 ± 0.7	9.03 ± 0.6
22:5:w3	2.52 ± 0.4	4.93 ± 0.3
22:5:w4	4.19 ± 0.4	8.44 ± 0.7
22:6:w3	24.68 ± 1.05	5.01 ± 0.5

†Sample contained 4 unidentified peaks each in trace quantities (<1%).

*Sample contained 11 unidentified peaks comprising approx. 34% of the total.

Values mean of 3 preparations ± 1 Standard deviation.

TABLE 8.16

Effect of lipid peroxidation on the fatty acid composition of the lyso-phosphatidyl ethanolamine phosphoglycerides from rat brain synaptic membranes

Fatty Acid	Relative Composition (Wt.%)	
	†Control Sample	*Lipid peroxidised sample
16:1:w7	trace	trace
16:2:w6	trace	trace
16:2:w7	trace	trace
18:0	1.83±0.4	3.17±0.8
18:1:w9	2.45±0.3	1.02±0.4
18:2:w9	trace	trace
18:3:w6	1.46±0.2	trace
18:4:w4	2.37±0.2	trace
20:1:w9	trace	trace
20:5:w3	14.41±0.8	15.06±0.9
21:0	trace	trace
22:0	1.74±0.3	4.37±0.9
22:2:w9	2.01±0.5	4.41±0.4
22:4:w6	1.95±0.3	2.46±0.7
22:5:w6	10.20±1.1	3.40±0.8
22:6:w3	50.61±2.4	44.78±2.4

†Samples contained 6 unidentified peaks comprising approx. 9% of the total

*Samples contained 9 unidentified peaks comprising approx. 20% of the total.

trace quantities <1% of total

Values mean of 3 preparations ± 1 Standard deviation

traces of breakdown products since the proportion of unidentified material was considerably greater in the chromatographs of the peroxidised samples (see footnotes to Tables 8.13 - 8.16). The phospholipid classes containing relatively high proportions of polyunsaturated fatty acids (i.e. PE, PS/PI, LPE) showed evidence of a reduction in the levels of these fatty acids (especially 22:6:w3) as a result of peroxidisation, an observation which is consistent with the olefinic double bonds being the target of peroxidation reactions. However, the proportion of the saturated fatty acids in the extracts from the peroxidised samples was also lower than that found in the control samples, especially in the fractions which contained a large proportion of these acids (i.e. PC and PE). Given that saturated fatty acyl chains are stable to peroxidation under the conditions used and since loss of such acids by cleavage of the ester linkages on the phospholipid molecule is extremely unlikely under the mild conditions used (i.e. 25°C and pH 7.2), this observation seemed surprising. A possible explanation of this 'contradictory' result could be that the changes in the polarity of the phospholipid molecule after peroxidation affected the R^{f} values of those molecules (relative to the thin layer chromatographic procedure used), and that there was a resultant reduction in the recovery of the relevant phospholipid fraction after thin layer chromatography. This seems plausible since peroxidation involves the cleavage of the olefinic double bond and the replacement of an apolar carbon chain by a polar aldehyde group. However, in spite of this uncertainty, it is clear from these lipid analyses that the peroxidation procedure has resulted in substantial changes in the fatty acid moieties of the membrane lipids.

DISCUSSION

The results presented here suggest that the three kinetic properties investigated showed little (if any) sensitivity to the membrane lipid changes associated with the experimental procedures employed. Although no attempt was made to estimate the nature and magnitude of the membrane lipid changes which occurred in the differently acclimated trout, there is ample literature evidence that the acclimation of ectothermic animals like trout to temperature conditions similar to those described here, results in significant changes in membrane lipids (see Johnson & Roots, 1964; Roots, 1968; Kemp & Smith, 1970; Driedzic & Roots, 1975). These changes usually involve a tendency towards an increased unsaturation index and a shorter mean chain length in the cold acclimated state, and are usually interpreted to be adaptive so as to enable the maintenance of a functional membrane fluidity at the lower temperatures (see Cossins, 1977). Thus the apparent insensitivity of the kinetic properties of the Na^{+}-K^{+}-ATPase to the membrane lipid changes expected argues against a role for membrane lipid in the fine control of the Na^{+}-K^{+}-ATPase. In this respect, the kinetic measurements reported here are similar to some ion activation experiments reported by Smith, Colombo & Munn (1967), but is at variance with the results of some experiments on the temperature kinetics of Na^{+}-K^{+}-ATPases extracted from $8^{o}C$ and $30^{o}C$ acclimated goldfish (Smith, 1967). Although a resolution of the differences in the data reported is difficult, (Smith, (1967) reported that the enzyme from the $8^{o}C$ acclimated fish showed ideal Arrhenius temperature kinetics over a wider temperature range than that from the $30^{o}C$ acclimated fish,) there may be some

grounds for suspecting that the differences in the data could be a reflection of the different tissue sources used, since it is recognised that the temperature tolerance of goldfish is considerably more plastic than that of fish like trout (see Driedzic & Roots, 1975). Thus it is likely that the difference between the membrane lipids of the $5^{o}C$ and $20^{o}C$ acclimated trout were too small to have had measurable effects on the kinetic properties of the Na^{+}-K^{+}-ATPase.

The kinetic measurements on the Na^{+}-K^{+}-ATPases extracted from the active and hibernating animals are generally similar in form to those obtained from the trout in that little (if any) differences in the kinetic properties of the enzyme was observed as a result of any in-*vivo* response to environmental temperature. This observation was made in spite of the different temperatures at which the membranes of both groups of animals had to function (active $\approx 37^{o}C$ vs hibernating $\approx 4^{o}C$), and to which the membranes were apparently adapted. The lipid analyses provided evidence that changes had occurred in the composition of the membrane lipids as a result of hibernation. The changes reported are similar in form to those hibernation induced lipid changes reported elsewhere (Platner, Patnayak & Musacchia, 1972; Goldman, 1975; Blaker & Moscatelli, 1978) in that there was a significant bias towards a higher unsaturation index in the lipids extracted from the hibernating animals. The results of the lipid analyses were generally similar to those reported for some hamster brain fractions (Blaker & Moscatelli, 1978; Goldman, 1975) especially with respect to the fatty acid analyses on the active animals. The changes in the fatty acid composition of the various lipid fractions reported here tended to show a greater bias towards a higher unsaturated index than that presented in other analyses

of lipids from hibernating hamster brain (Blaker & Moscatelli, 1978; Goldman, 1975). Given the different membrane fractions used for such analyses, the different *in vitro* histories of the membranes prior to lipid analyses (the membranes used here were SDS extracted synaptic membrane preparations), and the different methods used for methanolysing the said lipids (the method used here was milder than that used by the other authors), a direct comparison of the data presented here and elsewhere is difficult. However, it is apparent that any disagreement between the data sets is only with respect to the magnitude of the changes in membrane lipid induced by hibernation, and thus it is unlikely that there are any fundamental differences between the observations. The kinetic measurements reported here suggest that there is no kinetic difference between the Na^+-K^+-ATPase present in the membranes extracted from the active and hibernating animals. In this respect the data present here agrees with some previously reported kinetic measurements (Goldman & Albers, 1975; Charnock & Simonson, 1978(a), 1978(b)). However, there are some areas of disagreement between the data presented here and other reports with respect to ouabain sensitivity (Charnock & Simonson, 1978(a)), and temperature dependence (Bowler & Duncan, 1969; Goldman & Willis, 1973). Charnock & Simonson (1978a)have suggested that prolonged hibernation ($>$100 days) of ground squirrel results in a decrease in the apparent ouabain sensitivity of the Na^+-K^+-ATPase. However, the conditions under which those measurements were made were such that very little allowance was made for the slow rate of ouabain binding and the effects of potassium on the kinetics of ouabain binding. Consequently, no comparison can be made between that report and the data presented here. The disagreement between the temperature kinetic measurements reported here and those

of Bowler & Duncan, 1969 and Goldman & Willis, 1973 are apparently more fundamental, since the latter authors suggest that the behaviour of Na^+-K^+-ATPase extracted from the hibernating animals is different at low temperatures (5-10^oC). In this respect, the latter reports also disagree with the reported failure to detect similar differences between the enzyme extracted from hibernating and active ground squirrels (Charnock & Simonson, 1978(a), 1978(b)). Since the above reports refer to work done on different sources of the Na^+-K^+-ATPase a direct comparison of all the reports will be very difficult. Furthermore, the different methods used to process the different sets of data and the different assay conditions have made the resolution of the issue even more difficult. However, Charnock & Simonson (1978a) have reported that upon treatment of the data reported by Bowler & Duncan 1969, and Goldman & Willis, 1973, within the context of the phase change effect (using a computer assisted procedure), no significant difference was detected between the temperature kinetics of the Na^+-K^+-ATPases extracted from the active and hibernating animals. Although the validity of the procedure used by Charnock & Simonson, (1978a) has been questioned (see Chapter 4), that report suggests that any differences which may exist between the Na^+-K^+-ATPase in the active and hibernating animals must be small. Given these anomalies it is apparent that the above issue will only be settled when the more fundamental issue of the interpretation of the temperature kinetics of the Na^+-K^+-ATPase is conclusively settled.

The results of measurements made on the crude and lipid peroxidised rat brain synaptic membranes also suggested that the kinetic properties of the Na^+-K^+-ATPase are not sensitive to in *vitro* membrane lipid changes which do not result in any substantial loss of enzymic

activity. This was evident from the apparent insensitivity of most of the measured kinetic parameters to the substantial perturbation of the fatty acid chains as a result of the membrane lipid peroxidation procedure. In this respect the data presented here is at variance with previously reported work (Sun, 1971). However, the data reported by Sun (1971) was done on membranes in which there was a substantial inactivation of the Na^+-K^+-ATPase after enzymic lipid peroxidation, and since there is some evidence suggesting that a partial inactivation of the Na^+-K^+-ATPase is accompanied by a change in some of the kinetic properties of the enzyme (see Chapter 9), it seems unlikely that the data of Sun (1971) and those presented here are comparable.

The data presented above suggests that membrane lipid is probably not involved in the fine control of the Na^+-K^+-ATPase. However, such a conclusion can only be tentative, since the lipid analyses reported here and elsewhere, can only give an estimate of the changes that took place in the bulk membrane lipid phase. Given that the Na^+-K^+-ATPase is only likely to react to perturbations in its tightly bound lipid annulus, the issue concerning a role for membrane lipid in the fine control of this enzyme system can only be conclusively settled when it is possible to prepare and experiment on biologically active Na^+-K^+-ATPase preparations in which the protein moiety is free of all non essential membrane lipids.

CHAPTER 9

The effects of some protein targeted modifiers on the Na^{+}-K^{+}-ATPase

INTRODUCTION

Most mechanistic studies on enzyme systems have been carried out on highly purified biologically active preparations. This approach has not yet been possible in the case of the Na^{+}-K^{+}-ATPase since a pure biologically active sample that is free of membrane lipid is not yet available. The best preparations so far prepared (Jorgensen, 1974 a)have been found to contain a large polypeptide (M.W. 95,000-100,000) and a sialo-glycoprotein (M.W. ≃ 45,000-55,000) which, in the biologically active state, are firmly associated with membrane phospholipid. This has suggested that the biological activity of the Na^{+}-K^{+}-ATPase is heavily dependent on the association of the catalytic species with their membrane surrounds, and this in turn has suggested the possible use of protein targeted and lipid targeted modifiers in obtaining mechanistic information about the enzyme.

The use of modifiers in mechanistic studies on the Na^{+}-K^{+}-ATPase has been very extensive (see Schwartz, Lindenmayer & Allen, 1975). Most of the modifiers used are potent inhibitors of the enzyme, a property which precludes their use in evaluating the respective effects of the protein and lipid components of the enzyme system on the fine control of catalytic activity. In principle, a modifier or modification procedure which interferes with catalytic species without inhibiting the overall reaction should be better suited for such studies. The demonstration of a change in the apparent ouabain sensitivity of the Na^{+}-K^{+}-ATPases from 'heat-treated' rat and trout brain synaptic

membranes (see Chapter 3), and the demonstration of Concanavalin A binding to the glycoprotein component of 'purified' Na^+-K^+-ATPase preparations without any apparent effects on catalytic activity (Marshall, 1976), have suggested that the above procedures may be useful modifiers of this enzyme. Thus the effects of 'heat-treatment' and Concanavalin 'A' binding on some of the kinetic properties of the Na^+-K^+-ATPase have been investigated with the aim of establishing whether such procedures can be useful probes in mechanistic studies on the Na^+-K^+-ATPase.

MATERIALS AND METHODS

(1) Preparation of rat brain synaptic membranes (crude preparation). These were prepared as described in Chapter 2.

(2) Membranes partially enriched in Na^+-K^+-ATPase activity. These were prepared by extracting the crude preparation with sodium dodecyl sulphate as described in Chapter 6.

(3) Heat treatment of rat brain synaptic membranes. The procedure for heat treatment of the membrane preparations was designed to produce a sample in which all significant Na^+-K^+-ATPase activity was attributable to the thermo-stable species defined in Chapter 7. This was done by heating the preparation in a low ionic strength buffer (10mM Imidazole, 1mM E.D.T.A. pH 7.2 at 20°C) for 4 hours at 46.7°C. The heat-treatment was terminated by rapidly cooling the sample on an ice bath. The final sample (heat-treated preparation) was stored on ice at 0-4°C, until required. The kinetic studies described in Chapter 7 showed that under the conditions adopted for heat treatment, the 'thermo-stable' species had a half life of 110-130 minutes, while the 'thermo-labile' species was approximately 12 - 15 minutes. The heat

treatment procedure thus proceeded for more than 10 half lives of the 'thermo-labile' species and two half lives of the 'thermo-stable' species and was considered adequate for producing the desired effect.

(4) Incubation of the synaptic membranes with Concanavalin A.
The membrane preparation was diluted with an equal volume of a low ionic strength buffer (10mM Imidazole, 1mM E.D.T.A., pH 7.2 at room temperature) containing Concanavalin A so as to produce a Concanavalin A concentration of 200 $\mu g\ cm^{-3}$ and a membrane protein concentration 20 - 80 $\mu g\ cm^{-3}$. The mixture obtained was incubated at 0 - 4°C for 2 hours before use.

(5) Assay of Na^{+}-K^{+}-ATPase at varying temperatures.
The procedure adopted here has been described in Chapter 4.

(6) Assay of Na^{+}-K^{+}-ATPase at non saturating ouabain concentrations.
The procedure adopted here has been described in Chapter 3.

(7) Assay of Na^{+}-K^{+}-ATPase activity at non saturating potassium concentrations.
The relevant procedures were the same as described in Chapter 5.

(8) Assay of Na^{+}-K^{+}-ATPase in the presence of Concanavalin A.
The prepared solutions were the same as described for the various assay conditions above. However, Concanavalin A was added to the ionic (or Buffered) media so as to produce a run-time concentration of 200 $\mu g\ cm^{-3}$. The assay was then implemented according to the assay conditions described above.

RESULTS

(a) Effects of the heat treatment procedure.

The relative specific activities of the Na^{+}-K^{+}-ATPase in the control (crude rat brain synaptic membranes) and heat treated preparations are shown in Table 9.1. This shows that the Na^{+}-K^{+}-ATPase activity of the heat treated preparation was about 23% that of the control preparation. This value was greater than expected ($<$ 12%)of a regime in which the decay process proceeded for ten half lives of the 'thermo-labile' species and two half-lives of the 'thermo-stable' species. This was attributed to the practical problems of obtaining the exact pH and temperature conditions such that the decay process would proceed as expected. It was thus necessary to test all heat-treated preparations so as to determine whether the Na^{+}-K^{+}-ATPase activity after heat treatment contained significant contributions from the 'thermo-labile' species.

The testing procedure adopted assumed that the 'thermo-stable' species produced by heat-treatment was a species containing one active ouabain sensitive catalytic site. As mentioned in Chapter 3, the apparent ouabain sensitivity of such a species should be described by simple uncompetitive inhibition kinetics. Since, as also mentioned in Chapter 3, ouabain is believed to bind the phosphorylated intermediate in the reaction mechanism, then the ouabain binding affinity of the intermediate ought to be independent of the method of formation, and thus the apparent ouabain sensitivity of the system ought not to be dependent on the nature of the phosphorylating agent used to form the intermediate. Thus, the dose-response ouabain sensitivities of the

TABLE 9.1

Comparison of the specific activities of the Na^+-K^+-ATPase from the control and 'heat treated' rat brain synaptic membranes

Control	100
Heat treated	23.4 $\pm$ 3.1

Values (mean of 4 samples $\pm$ 1 standard deviation) normalised against a control value of 100.

TABLE 9.2

Comparison of apparent ouabain pI_{50} values of the K^+-PNPase and Na^+-K^+-ATPase from 'heat treated' rat brain synaptic membranes.

Activity	Apparent pI_{50}
Na^+-K^+-ATPase	4.04 $\pm$ 0.008
K^+-PNPase	3.79 $\pm$ 0.01

Na^+-K^+-ATPase and the K^+-PNPase activities of a given heat treated preparation were compared, and the preparation was considered adequate for further kinetic measurements only if the 'ouabain curves' of both the Na^+-K^+-ATPase and the K^+-PNPase were monophasic and not much different from each other (i.e. difference in apparent pI_{50} <0.4 units).

Figure 9.1 shows the 'ouabain curves' of the Na^+-K^+-ATPase and the K^+-PNPase from heat treated preparations of rat brain synaptic membranes. This shows that both curves are monophasic and definable in terms of a simple uncompetitive inhibition system (i.e. decay from 95% V_{max} - 5% V_{max} spans two orders of magnitude of ouabain concentration). Table 9.2 shows the apparent ouabain pI_{50} values for both enzymic activities. The K^+-PNPase (apparent pI_{50} 3.79) was less ouabain sensitive than the Na^+-K^+-ATPase (apparent pI_{50} 4.04). However, the difference in sensitivity ($\Delta pI_{50} \simeq 0.25$) was considerably smaller than that observed for the difference between the similar enzymic activities of the control preparations ($\Delta pI_{50} \simeq 0.7$). The small difference for the heat treated preparation could be explained by the effects of nucleotides and monovalent cations on the K^+-phosphatase (see Rega & Garrahan, 1976) and, as a result, the heat treated preparations were considered adequate for further kinetic measurements.

Figure 9.2 shows the ouabain dose response curve of the control and heat treated preparations. It is clear that heat treatment significantly altered the ouabain sensitivity profile of the enzyme. The shape of the decay curve changed from the biphasic pattern

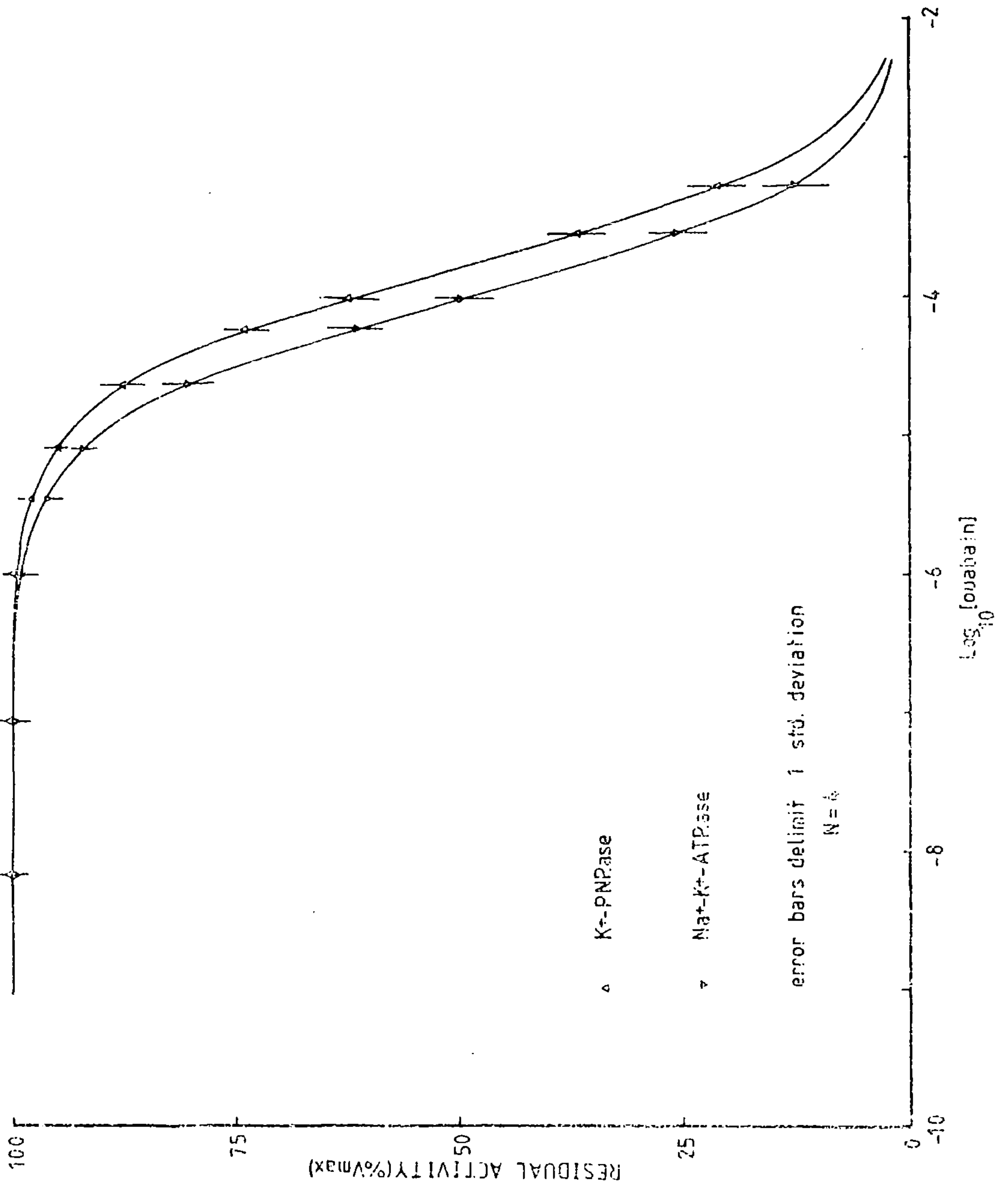


Fig.9.1

OUABAIN INHIBITION OF THE OUABAIN SENSITIVE PHOSPHATASE FROM HEAT TREATED RAT BRAIN SYNAPTIC MEMBRNES

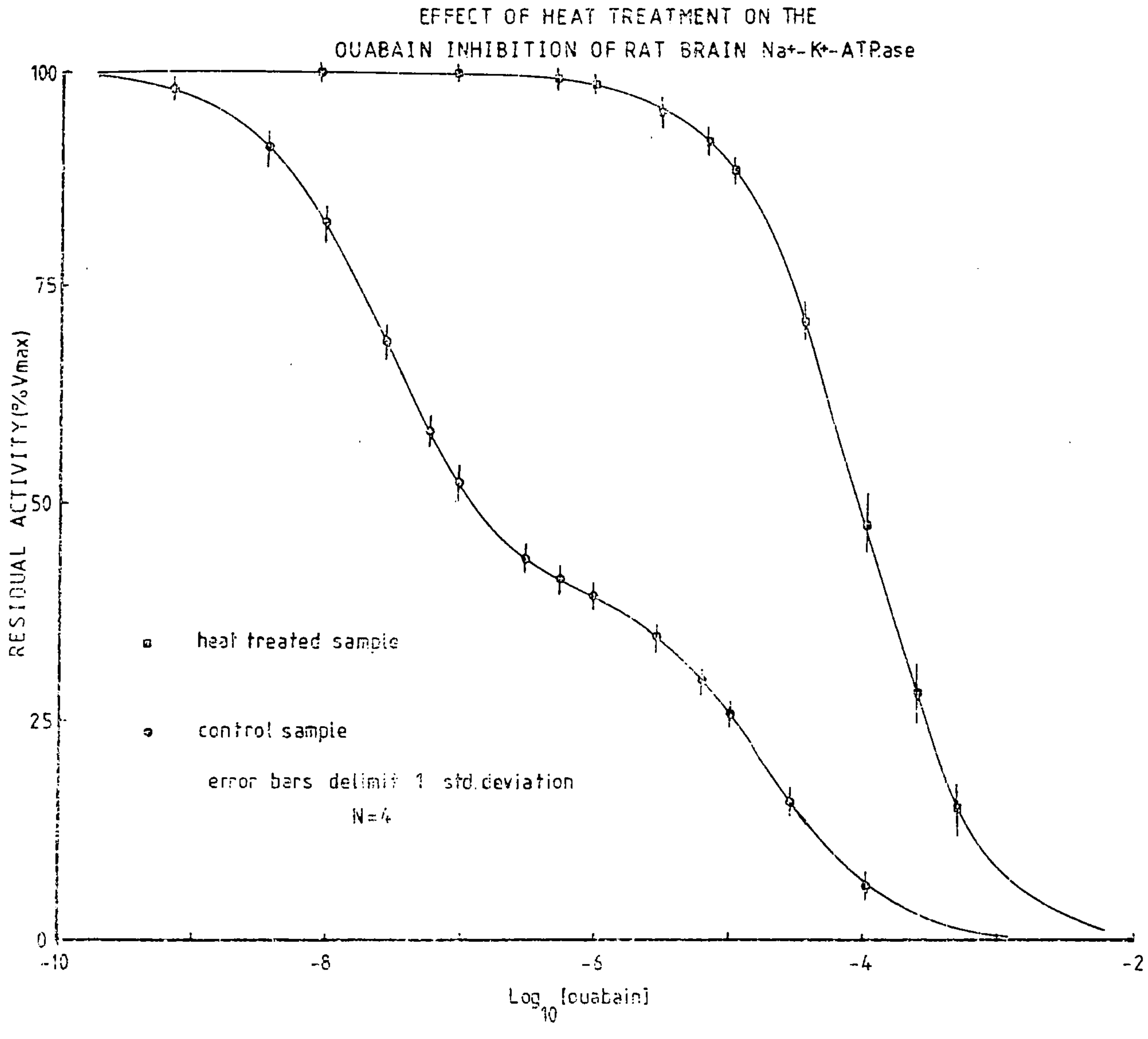


Fig. 9.2

EFFECT OF HEAT TREATMENT ON THE OUABAIN INHIBITION OF RAT BRAIN Na^{+}-K^{+}-ATPase

characteristic of the control to a monophasic uncompetitive form, in the case of the heat treated preparation. Furthermore, there was a significant change in the apparent ouabain sensitivity after heat treatment. This is underlined by the apparent pI_{50} values (see Table 9.3a) which show that the values calculated for the heat treated sample is considerably less than that of the control, indicating an apparent loss of ouabain sensitivity on heat treatment.

Figure 9.3 shows the potassium activation curves of the Na^{+}-K^{+}-ATPase from the control and heat treated preparations. The pattern of potassium activation of both preparations describes a sigmoid curve, with the heat treated preparation being slightly more potassium sensitive than that of the control. The curves drawn in figure 9.3 are the stoichiometric sigmoid curves discussed in Chapter 5. Table 9.3b shows that the defining parameters of these curves are all smaller in the case of the heat treated preparation. The mean value obtained for the half saturation potassium concentration at the high affinity site on the heat treated preparation (1.46×10^{-4}M) though smaller than that of the control (2.25×10^{-4}M) was not considered significant ($P > 0.05$). However, the potassium concentration for half saturation of the low affinity site (K_b), and the potassium concentrations at half maximal activity ($K_{0.5}$) were both significantly lower in the case of the heat treated samples ($P < 0.02$). These results suggested that the heat treatment procedure resulted in a slight increase in the potassium sensitivity of the enzyme.

Figure 9.4 shows the temperature kinetics of the Na^{+}-K^{+}-ATPase from the control and heat treated preparations. This shows that both

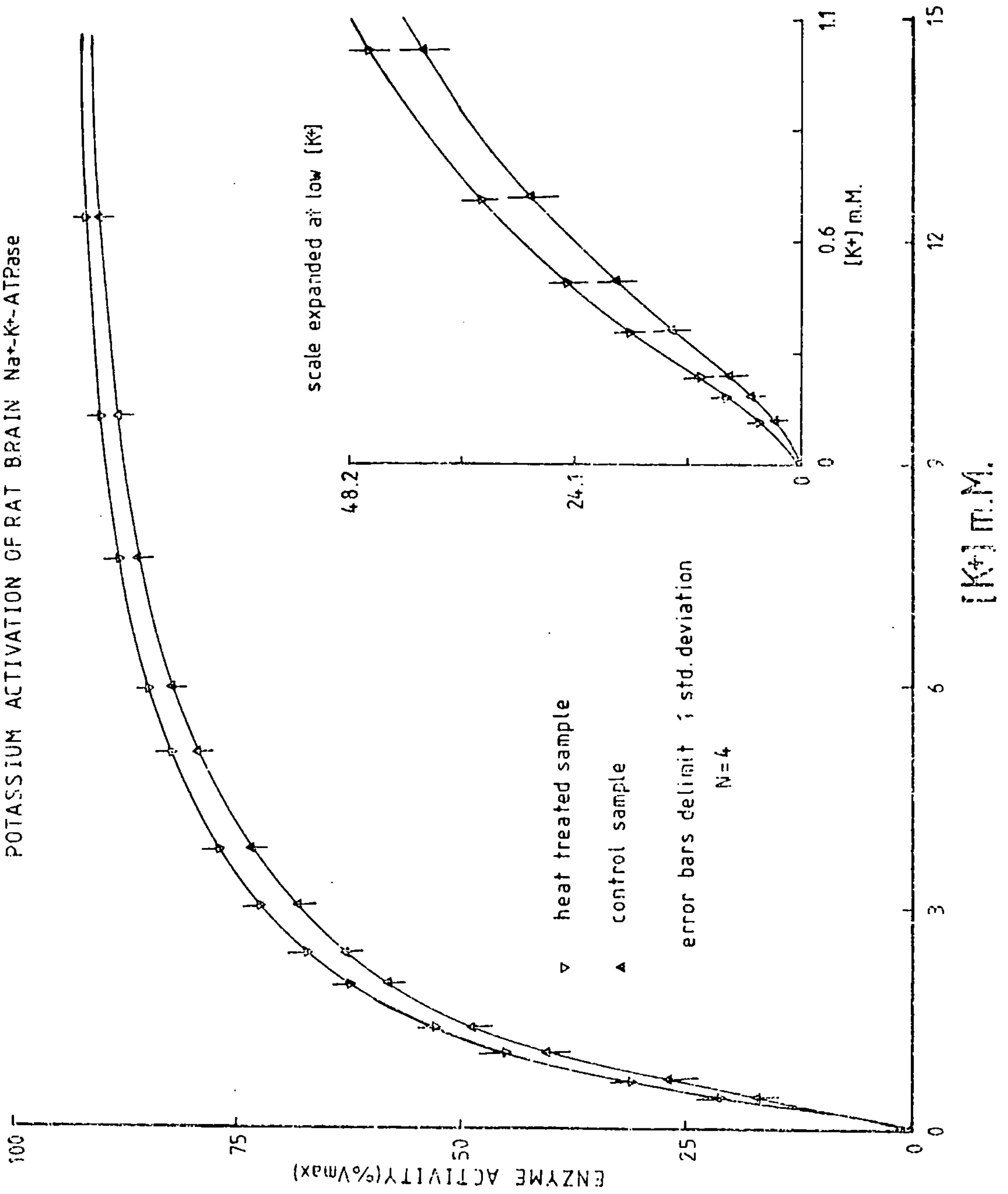


Fig. 9.3

EFFECT OF HEAT TREATMENT ON THE POTASSIUM ACTIVATION OF RAT BRAIN Na+-K+-ATPase

Fig.9.4

EFFECT OF HEAT TREATMENT ON THE
TEMPERATURE KINETICS OF RAT BRAIN Na+-K+-ATPase

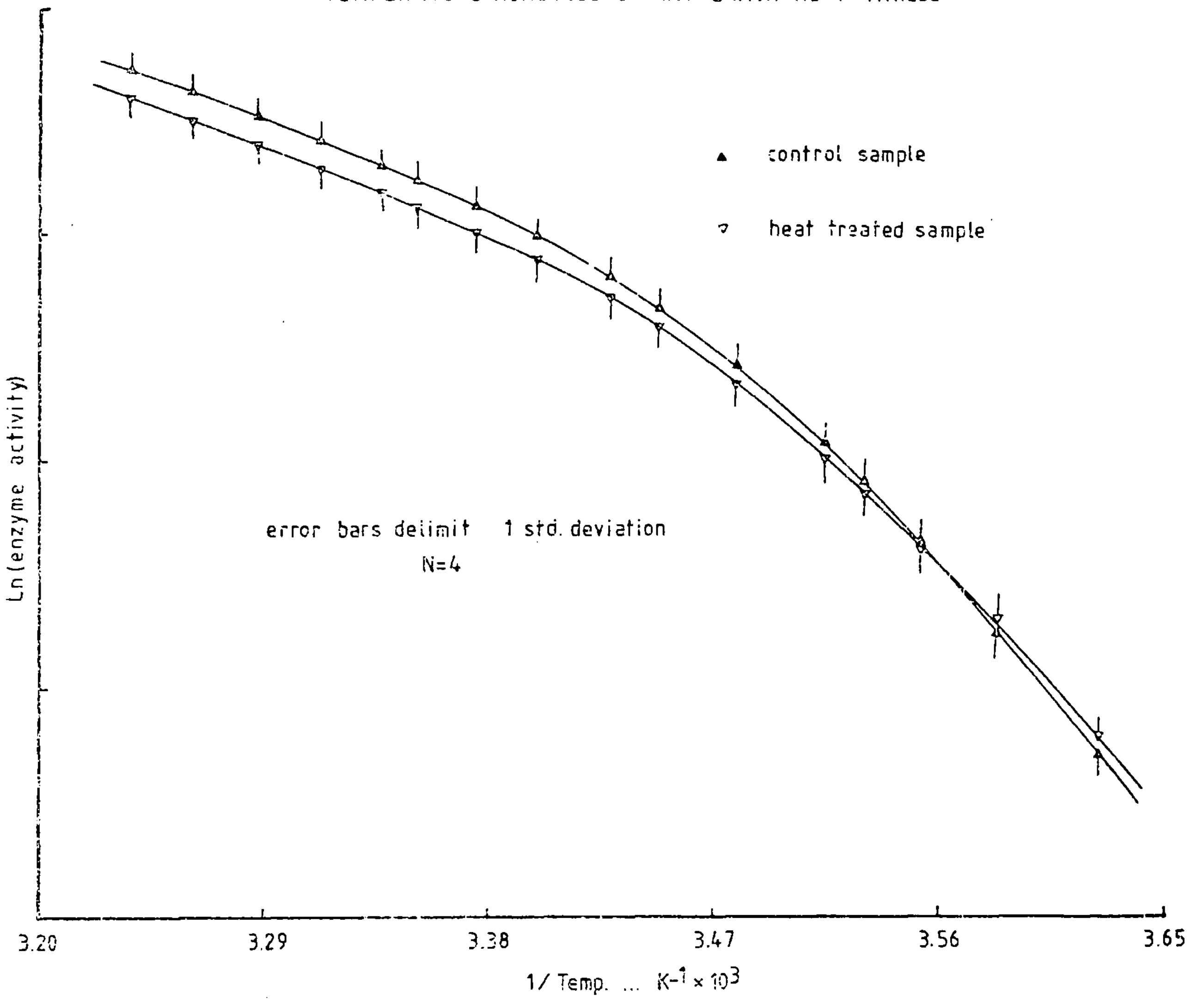


TABLE 9.3

Comparison of the parameters describing the properties of the Na^+-K^+-ATPase activity in control and heat treated rat brain synaptic membranes.

A. OUABAIN INHIBITION

Parameter	Control	Heat treated
▲K_i (M)	$2.32 \times 10^{-8} \pm 3.5 \times 10^{-9}$	*
▲β	866 ± 25	*
▲ϕ	0.799 ± 0.003	*
apparent pI_{50}	6.95 ± 0.003	4.04 ± 0.008

* Values not applicable - system better defined by simple uncompetitive inhibition.

▲ See Chapter 3

B. POTASSIUM ACTIVATION

Parameter	Control	Heat treated
†K_a (M)	$2.25 \times 10^{-4} \pm 6.67 \times 10^{-5}$	$1.46 \times 10^{-4} \pm 6 \times 10^{-5}$
†K_b (M)	$1.26 \times 10^{-3} \pm 4.97 \times 10^{-5}$	$1.07 \times 10^{-3} \pm 6.5 \times 10^{-5}$
†$K_{0.5}$ (M)	$1.45 \times 10^{-3} \pm 1.13 \times 10^{-5}$	$1.26 \times 10^{-3} \pm 5.5 \times 10^{-5}$

† See Chapter 5

C. TEMPERATURE DEPENDENCE

Parameter	Control	Heat treated
+μ (K.J.mol^{-1})	67.7 ± 4.4	66.7 ± 5.3
+ΔH (K.J.mol^{-1})	-170.7 ± 9.0	-150 ± 8.3
+ΔS (J.K.$^{-1}$mol^{-1})	-593 ± 32	-521 ± 44

\+ See Chapter 4

All values mean of 4 preparations $\pm$ 1 standard deviation

preparations described non-ideal Arrhenius plots. The curvature of the Arrhenius plot described by the heat treated prearation was less than that described by the control. The parameters which define the curve drawn (see Chapter 4) are listed in Table 9.3c. This shows that there is apparently no difference between the apparent activation energy terms (μ) describing the relative high temperature states. However the enthalpy (ΔH) and entropy (ΔS) parameters are significantly lower in the case of the heat treated preparation ($P<0.02$). These smaller enthalpy and entropy parameters suggested a broader transition to the inactive state in the case of the heat treated preparation.

(b) Effects of Concanavalin A.

The kinetic measurements of this study were done on the rat brain synaptic membranes that were partially enriched (relative to the crude preparation) in the Na^{+}-K^{+}-ATPase activity by a mild extraction with sodium dodecyl sulphate. This preparation was used in preference to the crude sample since it was considered likely to contain fewer species capable of binding Concanavalin A and thereby interfering with the target reaction. Table 9.4 shows the relative specific activities of the Na^{+}-K^{+}-ATPase from such preparations in the presence of Concanavalin A and when assayed at saturating concentrations of its essential ligands. It is clear that the activity of the Na^{+}-K^{+}-ATPase was not significantly affected by the concentrations of the nitrogenic lectin used (200 $\mu g.cm^{-3}$).

Figure 9.5 shows the ouabain dose response sensitivity curves described by the Na^{+}-K^{+}-ATPase in the presence and absence of Concanavalin A. The response to increasing ouabain concentrations was biphasic

TABLE 9.4

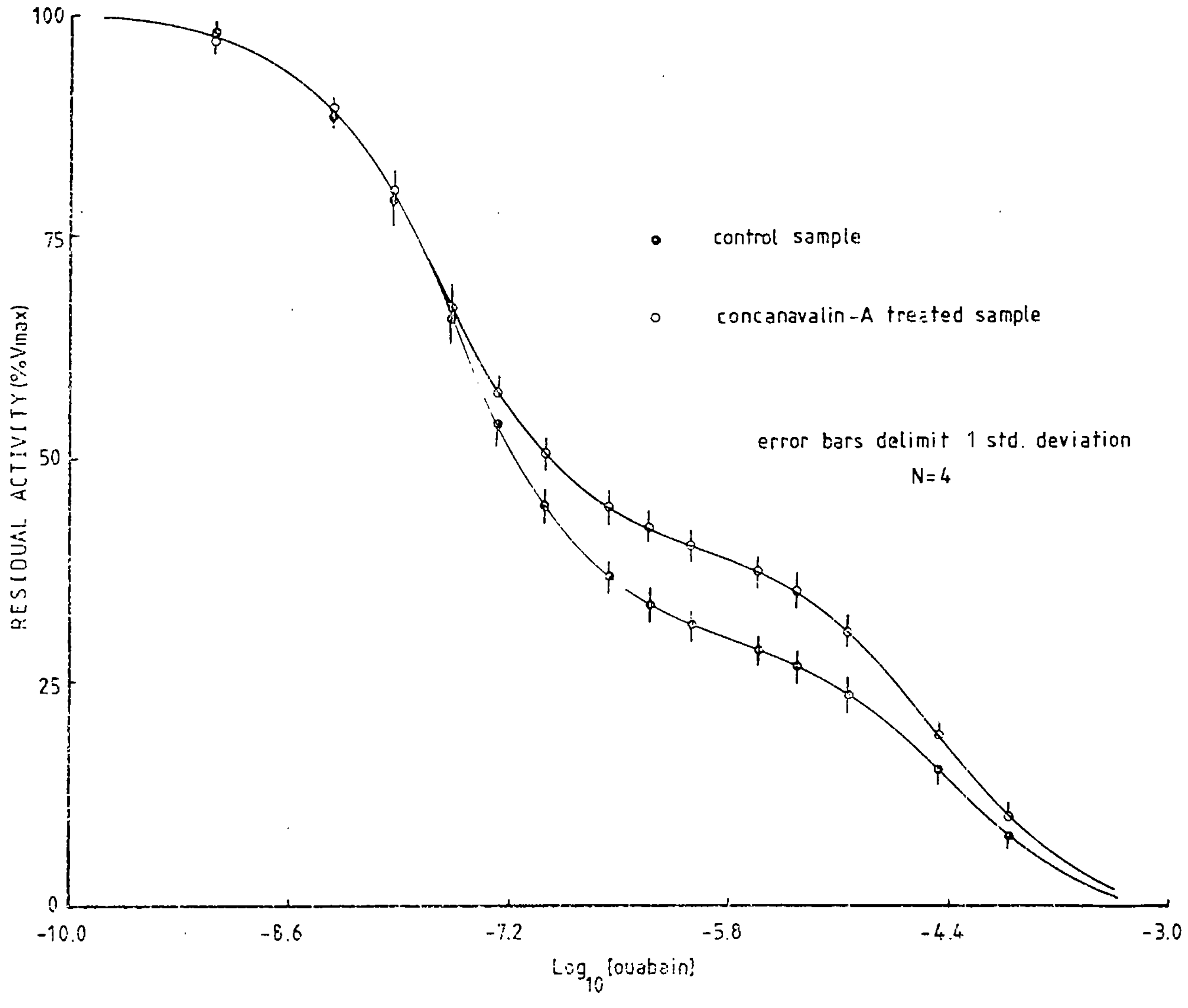
Comparison of the specific activities of the Na^{+}-K^{+}-ATPase from S.D.S. extracted rat brain synaptic membranes in the presence and absence of Concanavalin A.

Control	100
$\bar{c}$ Concanavalin A	98.7 $\pm$ 3.2

Values (mean of 4 preparations $\pm$ 1 standard deviation) are normalised against a control value of 100.

Fig.9.5

EFFECT OF CONCANAVALIN-A ON THE OUABAIN INHIBITION OF RAT BRAIN Na^{+}-K^{+}-ATPase



in the presence and absence of the lectin. The curves tended to converge at the low concentrations ($<10^{-8}$M) and at the higher concentrations ($>10^{-4}$M), but showed a significant divergence from each other at concentrations where the inflection in the curves is apparent (10^{-7}M - 10^{-5}M). This is reflected in the parameters defining the two curves (see Table 9.5a) which shows no significant difference between the K_i and β parameters (P 0.05) that define the apparent ouabain affinities at the two sites. The catalytic co-operativity parameter (ϕ) appears to have been affected by the binding of the lectin as evidenced by greater value (0.81) returned in the presence of the lectin, compared to that of the control (0.6). This effect seems to be responsible for the decreased apparent ouabain sensitivity of the enzyme when the lectin is present (apparent pI_{50} 6.95), compared with that of the control (apparent pI_{50} 7.16).

The effects of Concanavalin A on the potassium activation kinetics of the Na^+-K^+-ATPase are shown in figure 9.6. . The pattern of activation is not apparently affected by the lectin in that sigmoidal activation curves are described both in the presence and absence of Concanavalin A. Figure 9.6 also shows very little difference between the activation curves, a fact underlined by the list of parameters which define the stoichiometric sigmoid curves drawn (see Table 9.5b). This shows that the mean potassium concentrations calculated for half saturation at the low affinity site (K_b), the high affinity site (K_a) and half maximal activity ($K_{0.5}$) to be slightly lower in the presence of Concanavalin A. However, the differences between the respective values were not considered significant (P>0.5).

TABLE 9.5

Comparison of the parameters describing the properties of the Na^+-K^+-ATPase activity of S.D.S. extracted rat brain synaptic membranes in the presence and absence of Concanavalin A.

A. OUABAIN INHIBITION

Parameter	Control	Concanavalin A.
▲K_i (M)	$2.76 \times 10^{-8} \pm 5.3 \times 10^{-9}$	$2.16 \times 10^{-8} \pm 3.4 \times 10^{-9}$
▲β	1282 ± 43	1342 ± 234
▲ϕ	0.60 ± 0.016	0.81 ± 0.048
apparent pI_{50}	7.16 ± 0.008	6.95 ± 0.008

▲ See Chapter 3.

B. POTASSIUM ACTIVATION

Parameter	Control	Concanavalin A
†K_a (M)	$4.09 \times 10^{-4} \pm 1.34 \times 10^{-4}$	$3.71 \times 10^{-4} \pm 4 \times 10^{-5}$
†K_b (M)	$1.16 \times 10^{-3} \pm 1 \times 10^{-4}$	$1.1 \times 10^{-3} \pm 4.3 \times 10^{-5}$
†$K_{0.5}$ (M)	$1.45 \times 10^{-3} \pm 5.65 \times 10^{-5}$	$1.39 \times 10^{-3} \pm 2.3 \times 10^{-5}$

† See Chapter 5

C. TEMPERATURE DEPENDENCE

Parameter	Control	Concanavalin A
*μ ($K.J.mol^{-1}$)	75.21 ± 5.8	54.41 ± 2.7
*ΔH ($K.J.mol^{-1}$)	-176 ± 19	-137 ± 9.2
*ΔS ($J.K.^{-1}mol^{-1}$)	-611 ± 66	-463 ± 13

* See Chapter 4

Values mean of 4 preparations $\pm$ 1 standard deviation

Fig.9.6

EFFECT OF CONCANAVALIN-A ON THE
POTASSIUM ACTIVATION OF RAT BRAIN Na+-K+-ATPase

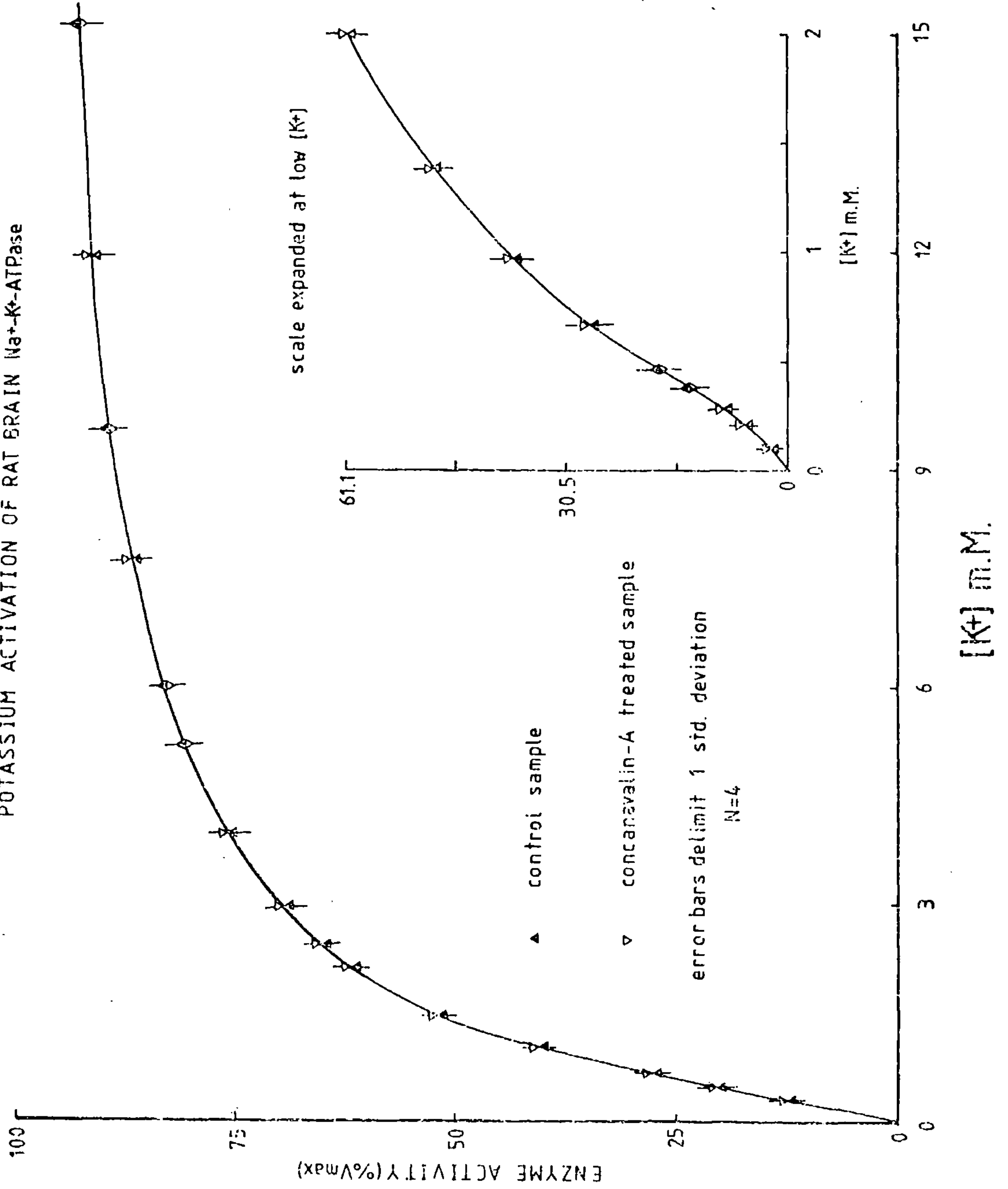


Figure 9.7 compares the Arrhenius plots of the Na^+-K^+-ATPase in the presence and absence of Concanavalin A. This shows that both Arrhenius plots are non-ideal and that the curvature of the control sample is greater than that observed in the presence of Concanavalin A. Such is reflected in the parameter lists defining the curves (see Table 9.5c) which show that the control preparation is characterised by a significantly larger apparent activation energy, and that its transition to the inactive state is characterised by larger enthalpy (ΔH) and entropy (ΔS) values.

DISCUSSION

The above results indicated that the three kinetic properties of the Na^+-K^+-ATPase observed here, were responsive to the protein targeted modification procedures used (i.e. heat-treatment and Concanavalin A binding). Ouabain inhibition appeared to be the most sensitive property to these 'modifiers' as is evident from the magnitude and pattern of the effects shown in figures 9.2 and 9.5. Potassium activation was not as responsive to the effects of these 'modifiers' as was ouabain inhibition since the changes detected were generally small and sometimes insignificant. Similarly the temperature kinetics of the activity of the enzyme were not as responsive to the 'modifiers' as was ouabain inhibition. However the results suggested that these modification procedures affected mainly the transition between the active and inactive states of the enzyme, as evident from the effects of the modifiers on the enthalpy (ΔH) and entropy (ΔS) parameters describing the process.

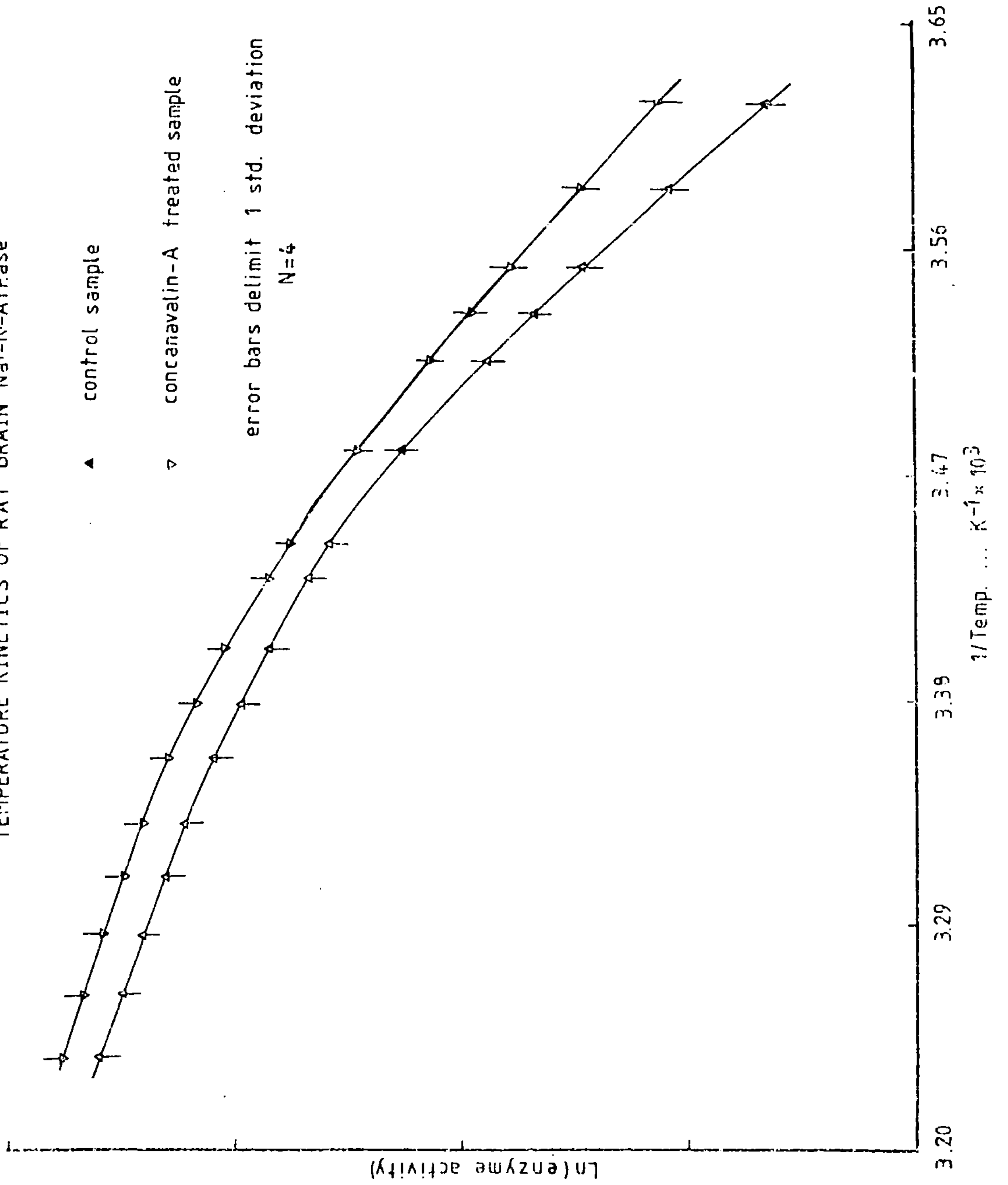


Fig.9.7

EFFECT OF CONCANAVALIN-A ON THE TEMPERATURE KINETICS OF RAT BRAIN Na^+-K^+-ATPase

The failure to demonstrate significant effects on the maximal specific activities of the Na^+-K^+-ATPase by the binding of Concanavalin A is suggestive of a minor role for the sialo-glycoprotein component of the Na^+-K^+-ATPase in the catalytic process. Thus, this result is at variance with the reported inhibition of Na^+-K^+-ATPase activity by the lectin (Swann, Daniel, Albers & Koval, 1975), but is in agreement with more recently reported work (Marshall, 1976; Dornand, Reminak & Mani, 1978). The implicit suggestion of a minor role for the sialo-glycoprotein in catalysis is supported by the reported failure to affect the Na^+-K^+-ATPase activity by the enzymic removal of sialic acids from the sialo-glycoprotein in 'purified' preparations of the enzyme (Perrone, Hackney, Dixon & Hokin, 1975; Marshall, 1976). The kinetic measurements, especially those of ouabain inhibition and temperature kinetics, suggest an indirect role for the sialo-glycoprotein in the regulation of this enzyme system. Given that there is evidence supporting a close association of the sialo-glycoprotein with the 'catalytic' polypeptide in the membrane (Kyte, 1972; Sweadner, 1977), the postulated effects of the glycoprotein would most probably be exerted via inter-subunit co-operativity.

The results of the heat treatment experiments cannot be interpreted unambiguously, since as shown in Chapter 7 , the nature of the 'thermo-stable' species formed by the heat treatment procedure has not been clearly established. However, if the 'preferred description' (see Chapter 7) is assumed, then the thermo-stable species would be dimeric, with one of its subunits being catalytically inactive. Within this context, the observed effects of the heat treatment procedure would be indicative of large co-operative effects to which the observed

kinetic properties are all responsive. Furthermore, the results will also suggest that the sigmoidal pattern of potassium activation is not the product of inter subunit co-operativity, since the sigmoidal pattern was unchanged by the heat treatment procedure. Thus the results of the heat treatment suggest that the procedure may be a useful probe for studying the role of protein-protein interactions and inter subunit co-operativity in the fine control of the catalytic activity of the Na^+-K^+-ATPase.

The effects of the protein targeted modification procedures on the kinetic properties studied, are in broad agreement with the potent effects of other specific protein targeted modifiers, especially antibodies (Sachs, 1974; Askari, 1974; Kyte, 1974; Michael, Wallick & Schwartz, 1977). Unlike the antibodies and other broad spectrum protein modifiers (See Schwartz, Lindenmayer & Allen, 1975), these appear to exert their effects without any obvious inhibition of catalytic activity. However, the nature of their effects is yet to be fully characterised and quantified. In spite of this, the results presented here suggest that when the quantification and characterisation of their effects are done, these procedures should be useful probes in obtaining mechanistic information about the role of protein-protein interactions, and possibly inter subunit co-operativity in the fine control of the Na^+-K^+-ATPase.

CHAPTER 10

Thimerosal as a modifier of the Na^+-K^+-ATPase

INTRODUCTION

A potassium activated phosphatase has invariably been found in preparations containing Na^+-K^+-ATPase activity, and soon after the first reports of its discovery (Judah, Ahmed & McLean, 1962), there were suggestions of a relationship between the two enzymic activities (Tosteston, 1962). A considerable amount of research effort has been employed in the characterisation of the Na^+-K^+-ATPase and the K^+-Phosphatase, with the result that the experimental evidence currently supporting a relationship between both enzymic activities is very strong (See Rega & Garrahan, 1976). However there have been suggestions that a fraction of the potassium dependent phosphatase is not related to the Na^+-K^+-ATPase (Pouchan, Garrahan & Rega, 1969), since some of the potassium dependent phosphatase activity remained insensitive to ouabain at concentrations greater than 10^{-3}M. In spite of this however, it is generally accepted, that the ouabain sensitive potassium dependent phosphatase activity, and the Na^+-K^+-ATPase activity are associated with the same molecular species. This is supported by observations that:

(a) The specific activities defining their maximal activities increase in parallel during purification procedures (Uesegi, Dulak, Dixon, Hexum, Dahl, Perdue & Hokin, 1971).

(b) The phosphorylated oligopeptides formed by enzymic degradation of the phospho-protein intermediates of the Na^+-K^+-ATPase and the K^+-Phosphatase are chromatographically and electrophoretically similar (Bond, Bader & Post, 1971).

(c) The trans-membrane assymetry, which characterises the requirement for the binding of activating ligands and inhibitors is the same for both enzymic activities (Rega, Garrahan & Pouchan, 1970).

The ability to phosphorylate the catalytic units of the Na^+-K^+-ATPase with the so called 'pseudo-substrates' in the absence of sodium ion (Israel & Titus, 1967; Bond et al, 1971) is in marked contrast to the absolute requirement for sodium ion when ATP is the phosphorylating ligand. This suggests that the K^+-Phosphatase is a partial reaction sequence in the overall mechanism of the Na^+-K^+-ATPase, diverging from the latter with respect to the method by which the phosphorylated intermediate is formed. This is supported by the observation that oligomycin, a reagent reported to block sodium dependent ATP phosphorylation of the enzyme (Garrahan & Glynn, 1967), is a potent inhibitor of Na^+-K^+-ATPase activity, while the K^+-Phosphatase is relatively insensitive to this reagent (Askari & Koyal, 1971). However, the exact role of the K^+-Phosphatase in the overall reaction mechanism of the Na^+-K^+-ATPase is yet to be established.

It is known that, at low temperatures ($\approx$ 0-4°C), and saturating concentrations of the relevant ligands, the formation of the phosphorylated intermediate is quick and quantitative (Bond et al, 1971). However, ouabain sensitive phosphatase activity proceeds very slowly at such low temperatures (Charnock, Cook & Casey, 1971). This has suggested that the rate limiting step in the overall reaction mechanism occurs during the steps involved in the potassium dependent discharge of the phosphorylated intermediate and the regeneration of the enzyme in its original form. Hence the K^+-Phosphatase is considered a feasible system for studying the terminal and rate limiting steps of the sodium potassium pump.

In recent work (Henderson & Askari, 1976, 1977), the treatment of membranes containing Na^{+}-K^{+}-ATPase activity with thimerosal was found to inhibit Na^{+}-K^{+}-ATPase activity. This effect was attributed to the interaction of the reagent with sulphydryl groups since the authors found that the effect could be reversed by post treatment of the thimerosal treated membranes with dithiothretol. The authors also reported that the sodium dependent ATP phosphorylation of the enzyme, and the potassium dependent discharge of the phosphorylated intermediate were not affected by the thimerosal treatment. Furthermore, the potassium dependent phosphatase activity was not affected by the concentrations of the reagent used to inhibit Na^{+}-K^{+}-ATPase activity. Given that thimerosal obviously acted via the protein component of the Na^{+}-K^{+}-ATPase, the above reports suggested the reagent to be a useful modifier of the enzyme system, and that it could possibly be used as a probe in the study of the effect of protein-protein interaction on the enzyme system. This approach has been adopted here, and the effects of thimerosal have been studied with emphasis on the properties of the K^{+}-Phosphatase which are considered to be in common with the overall reaction mechanism of the Na^{+}-K^{+}-ATPase.

MATERIALS AND METHODS

(i) Preparation of rat brain synaptic membranes.

These were prepared as previously described in Chapter 2.

(ii) Preparation of thimerosal treated rat brain synaptic membranes.

Prepared solutions

(a) Thimerosal incubation buffer

50mM Tris..... pH 7.4 at 37°C (HCl)

10^{-5}M Thimerosal

(b) Low ionic strength buffer

10mM Imidazole ... pH 7.2 at 20°C (HCl)

1mM E.D.T.A.

The membrane preparation was quickly added to the thimerosal incubation buffer (pre-warmed at 37°C) to give a protein concentration of between 300-600 ug cm^{-3}. The incubation was allowed to proceed for 10 minutes at 37°C after which time, the mixture was diluted five-fold with ice cold low ionic strength buffer. The membranes were then sedimented at 0-4°C, by centrifugation at 100,000 g for 30 minutes (M.S.E. prepspin 50 Ultracentrifuge). The sedimented membranes were then washed by three cycles of resuspension in low ionic strength buffer and sedimentation, before being resuspended in the low ionic strength buffer to a protein concentration of 1-1.5 mg cm^{-3} and stored on ice at 0-4°C until needed.

(iii) Assay of ouabain sensitive potassium dependent p-nitrophenol phosphatase.

The assay conditions with respect to concentrations of essential ligands, pH and temperature (isothermal studies) were the same as discussed for the standard assay conditions in Chapter 2.

(a) Assays at varying temperatures.

The assay procedure was implemented in the manner described for the standard assay conditions (see Chapter 2). However the temperature dependent change in the pH of the buffer system (Tris/HCl) was restricted to about $\pm$ 0.05 pH units from the set value (7.5). The procedure adopted was the same as described for the Na^{+}-K^{+}-ATPase in Chapter 4.

(b) Assays at non-saturating ouabain concentrations.

The assay procedure used was as previously described in Chapter 3.

(c) Assays at non saturating potassium concentrations.

Prepared solutions:

(1) Substrate

4.4 x 5mM p-nitrophenolphosphate pH 7.5 (Tris/HCl) at 20°C.

(2) Ouabain

4.4 x 1mM Ouabain

(3) Buffer medium

4.4 x 50mM Tris pH 7.5 at 37°C (HCl)

4.4 x 5mM Magnesium chloride

(4) Potassium Chloride

These were prepared as was previously described in Chapter 5.

The reaction media were prepared as follows:

(i) Total Phosphatase medium

0.5 cm^3 substrate

0.5 cm^3 distilled water

0.5 cm^3 buffer medium

0.5 cm^3 potassium chloride

(ii) Ouabain insensitive phosphatase medium.

This mixture was the same as that prepared for the total phosphatase except that 0.5 cm^3 of ouabain was substituted for the distilled water.

The reaction was started by the addition of 0.2 cm^3 of an enzyme preparation to the reaction media (thermoequilibriated at 37°C). After a suitable run time, the reaction was quenched and liberated p-nitrophenol determined as previously described in Chapter 2. The ouabain sensitive K^+-phosphatase was determined by the difference between the enzymic release of p-nitrophenol in the presence and absence of 10^{-3}M ouabain.

(d) Assays in the presence of 3uM ATP.

The prepared solutions were the same as described for the various assay conditions above. However a small volume of a concentrated solution of ATP was added to the ionic (or buffer) media (approximately 50 ul ATP per 50 cm^3 medium) so as to produce a run-time concentration of 3uM ATP. The assay was then implemented according to the conditions described above.

RESULTS

Table 10.1 shows the specific activities of the K^+-PNPase and Na^+-K^+-ATPase from the control and thimerosal treated rat brain synaptic membranes. This shows the procedure adopted for thimerosal treatment completely removed the Na^+-K^+-ATPase activity, but some 25% of the initial K^+-PNPase activity remained intact. Thus the sensitivity of this preparation to thimerosal was considerably greater than that reported for guinea-pig kidney microsomes (Henderson & Askari, 1977). In spite of the procedures used here being milder than those described by the above authors, the effect on the brain preparation was relatively more severe. However, since the thimerosal treated preparation was required for the characterisation of some of the kinetic properties of the K^+-PNPase, the complete removal of the Na^+-K^+-ATPase activity was necessary, if the data obtained were to be free of ambiguity from this source. The thimerosal treatment procedure adopted, was the mildest consistent with the above requirements.

Figure 10.1 shows the response of the K^+PNPase from the control and thimerosal treated membranes, to increasing ouabain concentrations.

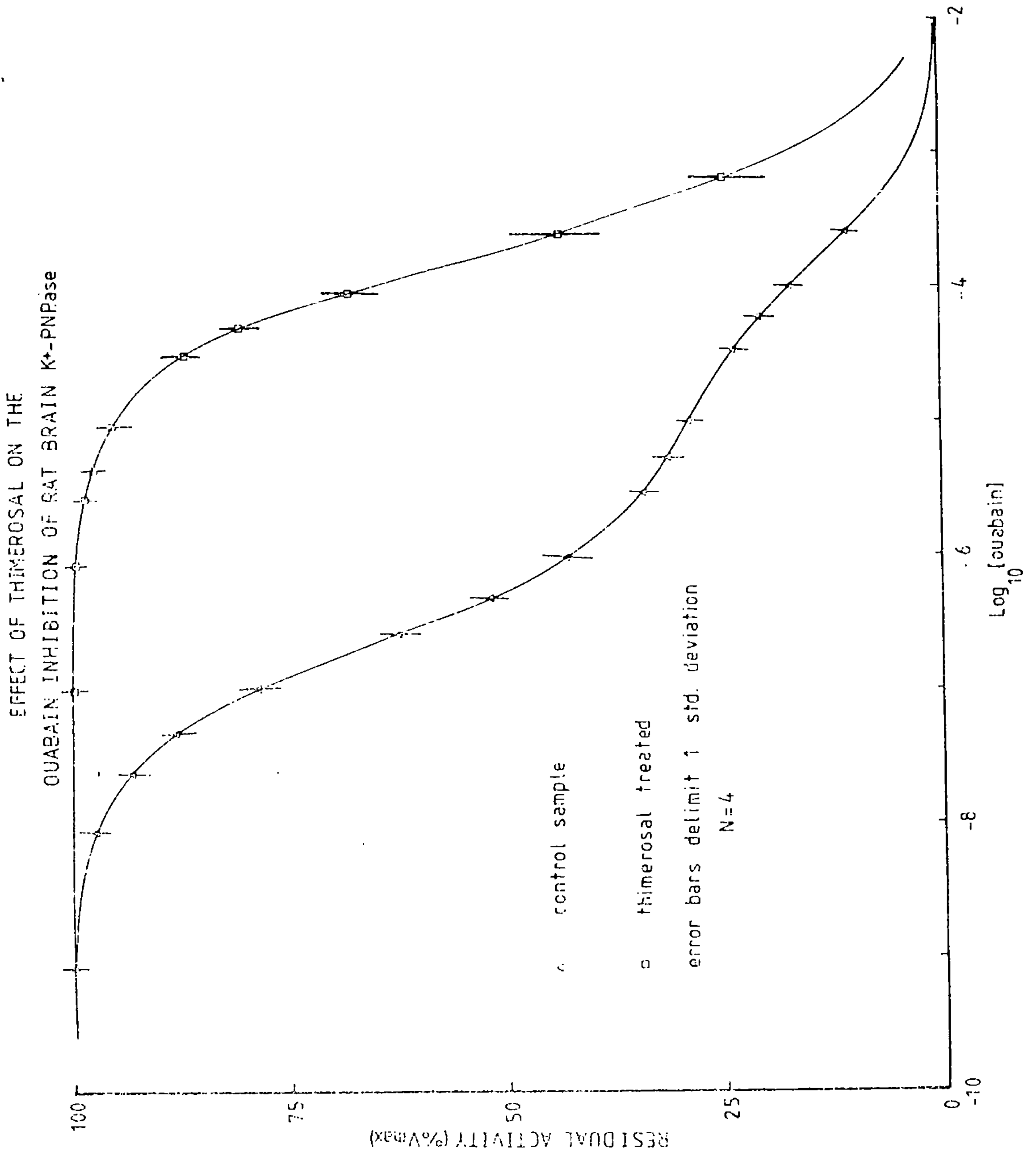


Fig.10.1

EFFECT OF THIMEROSAL ON THE QUABAIN INHIBITION OF RAT BRAIN K+-PNPase

TABLE 10.1

Comparison of maximal activities of the ouabain sensitive phosphatase activities from control and thimerosal treated rat brain synaptic membranes.

Preparation	Na^+-K^+-ATPase	K^+-PNPase
Control	85.7 ± 9.3	15.7 ± 2.3
Thimerosal	0.4 ± 0.3	3.8 ± 0.9

Values - mean of 4 preparations ± 1 standard deviation.

Enzyme activity in units of μMoles of product $mg.protein^{-1}.hr^{-1}$.

This shows that the response of the control preparation described a biphasic decay curve of similar shape to that described by the Na^{+}-K^{+}-ATPase (see Chapter 3). In contrast, the response of the thimerosal treated preparation apparently described a monophasic decay curve. Table 10.2 lists the parameters defining the respective curves. This clearly shows that the thimerosal treated preparation is less ouabain sensitive than the control preparation as suggested by the apparent pI_{50} values (3.66 thimerosal, 6.24 control).

The response of the preparations to increasing potassium concentrations is shown in figure 10.2. In both cases, the potassium activation of the K^{+}-PNPase describes a complex curve which could not be accurately fitted to a Michaelis-Menten rectangular hyperbola, a Hill sigmoid curve nor a stoichiometric sigmoid curve (see Chapter 5). In spite of this, figure 10.2 clearly shows that the thimerosal treated preparation was more potassium sensitive than the control, as evidenced by the lower potassium concentrations needed for half-maximal activity (≈0.75mM.... thimerosal vs ≈ 4mM control) of the thimerosal treated preparation. Furthermore, the response of the thimerosal treated preparation, relative to maximal activity, was greater than that of the control at all non saturating potassium concentrations. These results are thus similar to those reported for guinea pig kidney microsomes (Henderson & Askari, 1977).

The temperature kinetics of the K^{+}-PNPase from the two preparations are shown as Arrhenius plots in figure 10.3. Like the Na^{+}-K^{+}-ATPase (see Chapter 4), the temperature dependence of the K^{+}-PNPase from the control sample deviated from the ideal Arrhenius

TABLE 10.2

Comparison of ouabain inhibition parameters of the K^+-PNPase from the control and thimerosal treated rat brain synaptic membranes.

Parameter	Control	Thimerosal
▲K_i	$2.42 \times 10^{-7} \pm 3.1 \times 10^{-8}$	* -
▲ϕ	0.58 ± 0.059	* -
▲β	507 ± 59	* -
apparent pI_{50}	6.24 ± 0.03	3.66 ± 0.05

* Values not applicable. The thimerosal treated preparation was better described by a monophasic uncompetitive inhibition curve.

▲ Parameters are as defined in Chapter 3.

All values mean of 3 preparations $\pm$ 1 standard deviation.

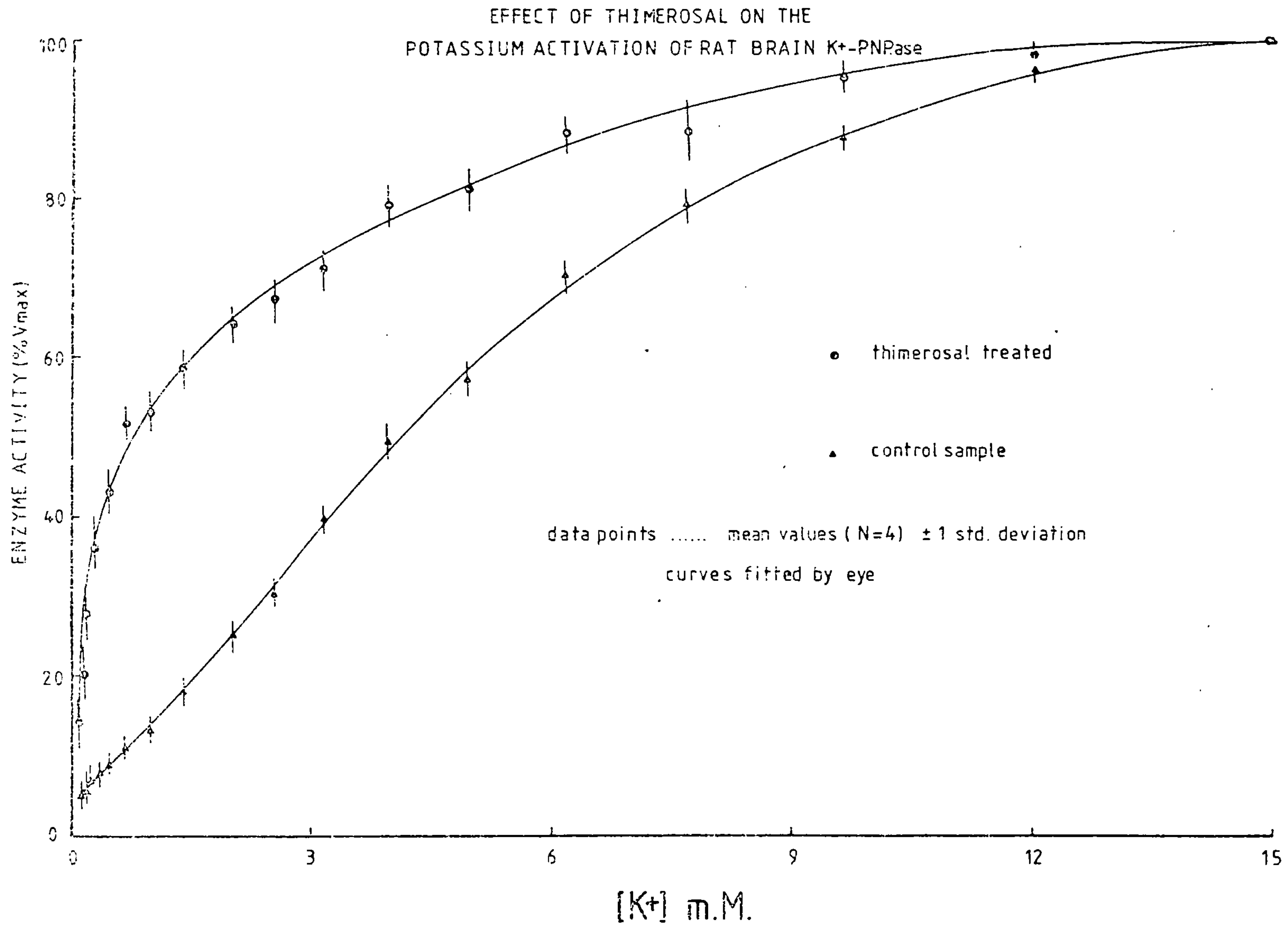


Fig.10.2
EFFECT OF THIMEROSAL ON THE
POTASSIUM ACTIVATION OF RAT BRAIN K+-PNPase
ENZYME ACTIVITY (% Vmax)
100
80
60
40
20
0
0
3
6
9
12
15
[K+] m.M.
thimerosal treated
control sample
data points ...... mean values (N=4) ± 1 std. deviation
curves fitted by eye

description at low temperatures. The curve fitted to the data points in figure 10.3 was drawn according to the model adopted in Chapter 4. This defines the system with an apparent Arrhenius 'up of 54.3 $K.J.mol^{-1}$, an enthalpy of transition of 167 $K.J.mol^{-1}$ and an entropy of transition of 586 $J.K.^{-1}\ mol^{-1}$ (see Table 10.3). In contrast, the temperature dependence of the K^+-PNPase from the thimerosal treated preparation could be adequately described by an Arrhenius straight line of apparent Arrhenius 'u' of 80.3 $K.J.mol^{-1}$.

It has been established that the observed kinetic properties of the K^+-phosphatase is significantly altered by low ($\approx 10^{-4}M$) concentrations of ATP (Israel & Titus, 1967; Bader & Sen, 1966). Given that there is evidence supporting the existence of a high affinity, non-catalytic ATP binding site essential to the mechanism of the Na^+-K^+-ATPase (Post, Hegyvary & Kume, 1972), the effect of low ATP concentrations on the observed kinetic properties of the K^+-phosphatase can be attributed to the effects of ATP binding to this high affinity site. The previous reports of thimerosal effects on the Na^+-K^+-ATPase (Henderson & Askari, 1976, 1977), are in line with this suggestion and they also suggest that the effects of thimerosal may be the result of a direct or indirect blockading of the ATP binding to this high affinity site. This was investigated by comparing the kinetic measurement on the K^+-PNPase in the presence and absence of low ATP concentrations.

The effects of 3uM ATP on the maximal activities of the K^+-PNPase from the control and thimerosal treated preparations are shown in Table 10.4 This shows that the maximal activities of the given preparations were not significantly affected by the low levels of ATP used. These results appear to conflict with the previously reported inhibition of the K^+-phosphatase by ATP (Israel & Titus, 1967; Garrahan, Pouchan

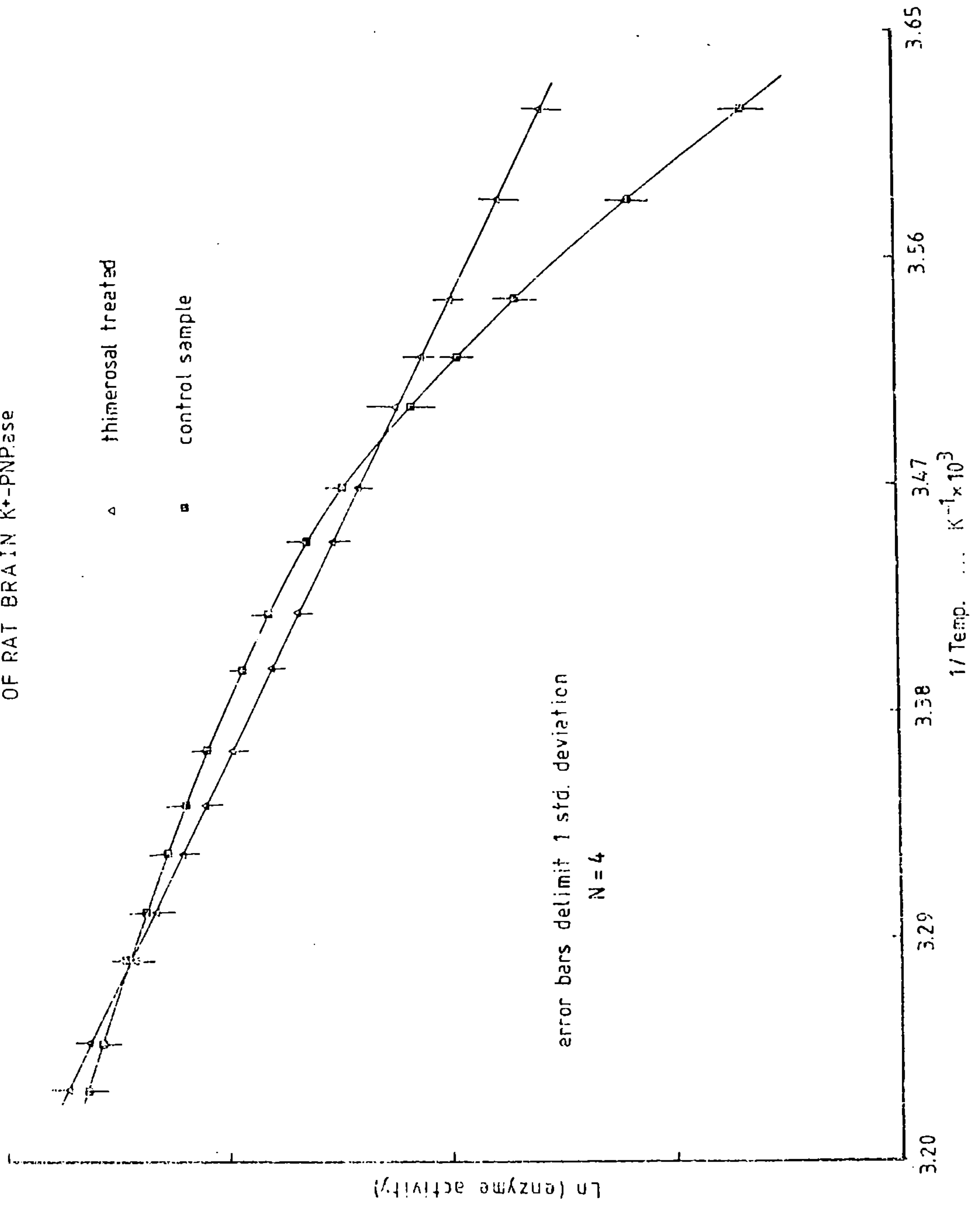


Fig.10.3
EFFECT OF THIMEROSAL ON THE TEMPERATURE KINETICS
OF RAT BRAIN K+-PNPase
thimerosal treated
control sample
error bars delimit 1 std. deviation
N = 4
Ln (enzyme activity)
1/Temp. ... K^{-1}x 10^{3}
3.20
3.29
3.38
3.47
3.56
3.65

TABLE 10.3

Comparison of Arrhenius temperature parameters of the K^+-PNPase from the control and thimerosal treated rat brain synaptic membranes.

Parameter	Control	Thimerosal
μ	54.3 $\pm$ 2.3	80.3 $\pm$ 5.4
ΔH	-167.6 $\pm$ 16.9	* -
ΔS	- 58.6 $\pm$ 57	* -

* Values not applicable - system better described by a linear Arrhenius plot in the range 4 - 37°C.

μ Apparent activation energy of the active state (K.J.Mol^{-1})

ΔH Enthalpy of transition (K.J.Mol^{-1}) - see Chapter 4.

ΔH Entropy of transition (J.K.Mol^{-1}) - see Chapter 4.

Values mean of 3 preparations $\pm$ 1 standard deviation

TABLE 10.4

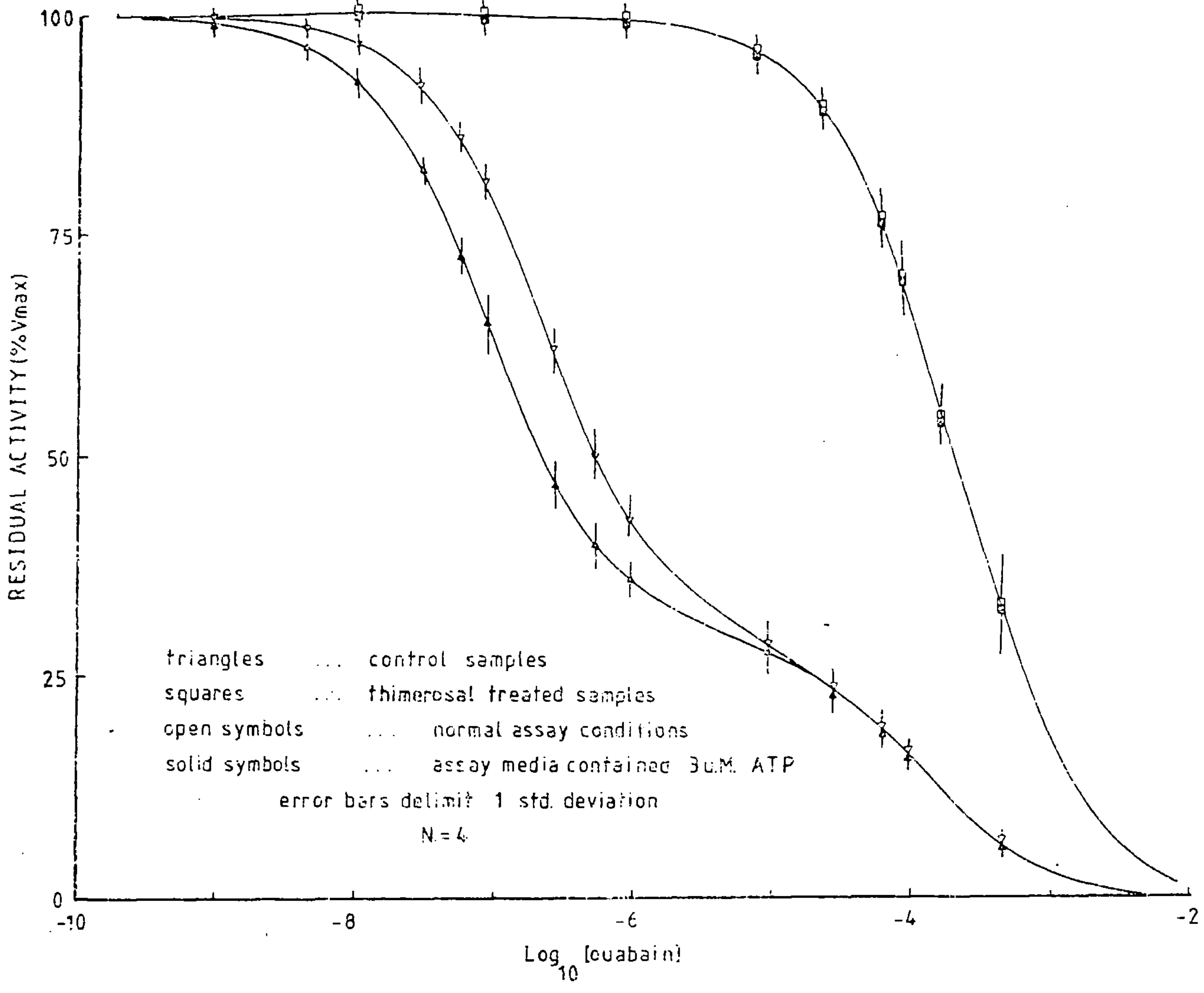
Comparison of maximal activities of K^+-PNPase from control and thimerosal treated preparations ($\bar{c}$ 3μM ATP).

Preparation	No. ATP	3μM ATP
Control	15.7 ± 2.3	14.9 ± 2.6
Thimerosal	3.8 ± 0.9	4.1 ± 0.8

Values mean of 4 preparations ± 1 standard deviation
Enzyme activity μMoles of product $mg.protein^{-1}.hr^{-1}$.

Fig.10.4

EFFECT OF ATP ON THE OUABAIN INHIBITION OF THE K+-PNPase FROM RAT BRAIN SYNAPTC MEMBRANES



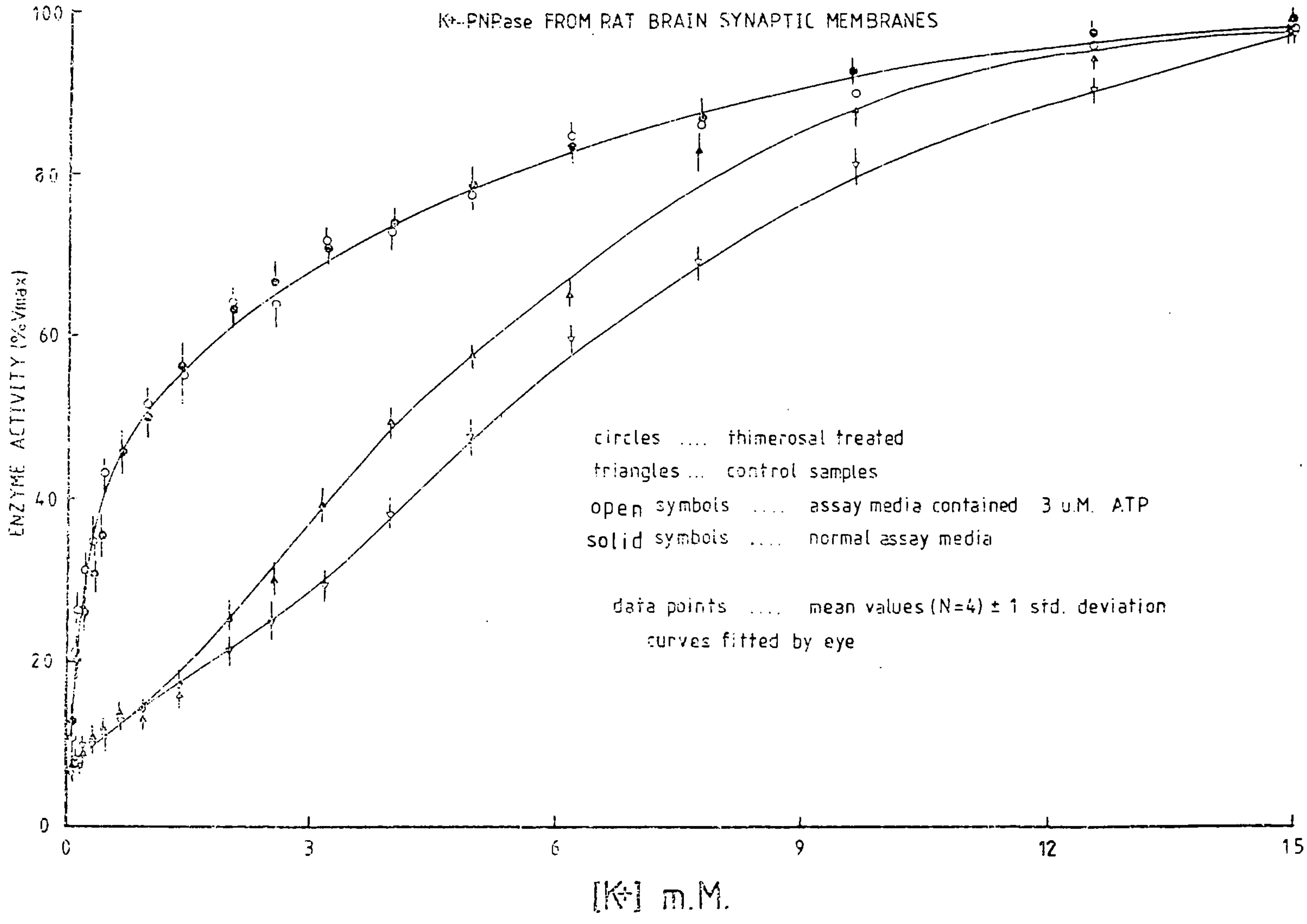
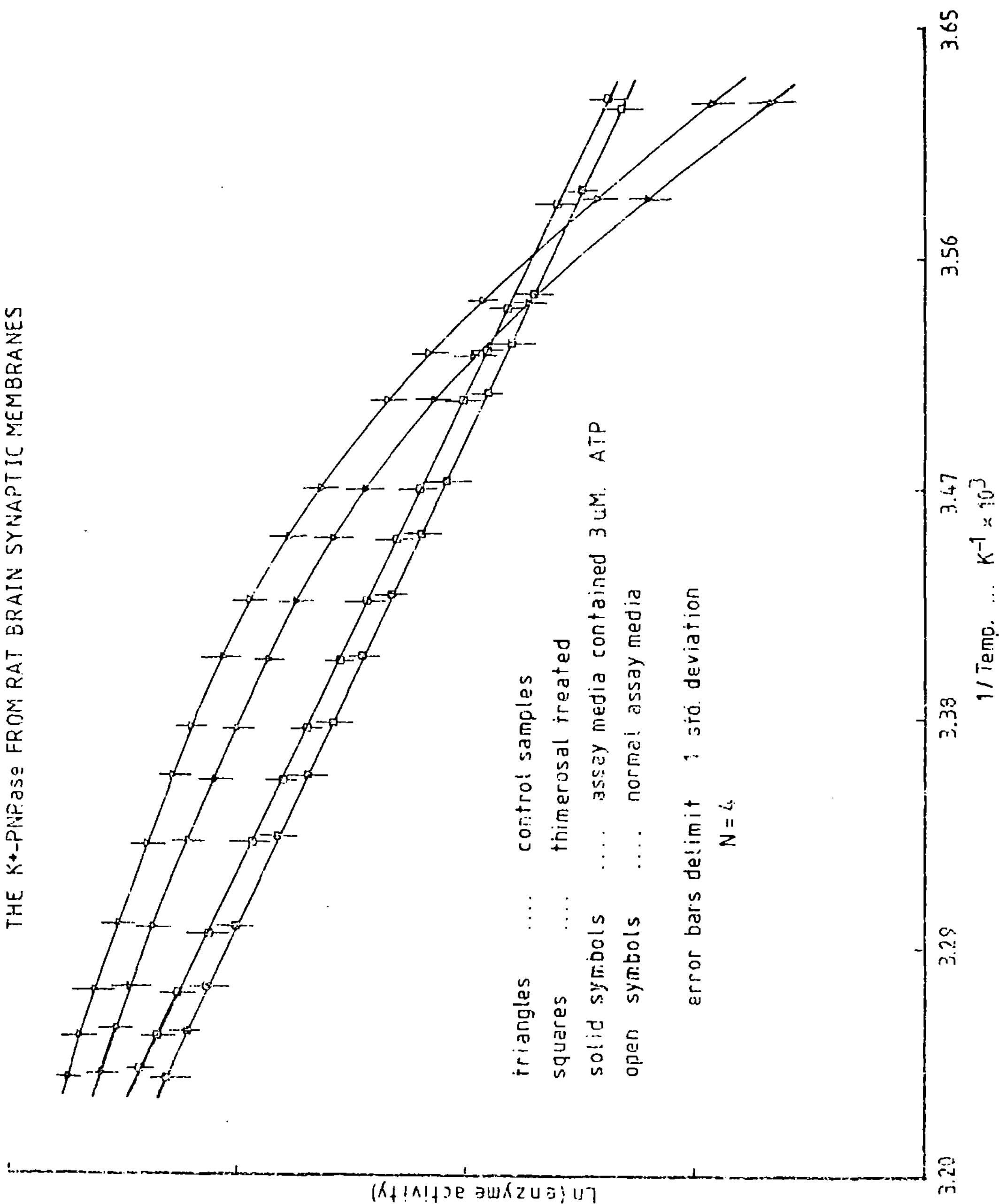


Fig.10.5
EFFECT OF ATP ON THE POTASSIUM ACTIVATION OF THE
K+-PNPase FROM RAT BRAIN SYNAPTIC MEMBRANES
ENZYME ACTIVITY (%Vmax)
100
80
60
40
20
0
3
6
9
12
15
circles .... thimerosal treated
triangles ... control samples
open symbols .... assay media contained 3 u.M. ATP
solid symbols .... normal assay media
data points .... mean values (N=4) ± 1 std. deviation
curves fitted by eye
[K+] m.M.

Fig.10.6

EFFECT OF ATP ON THE TEMPERATURE KINETICS OF THE K+-PNPase FROM RAT BRAIN SYNAPTIC MEMBRANES



& Rega, 1970). However, such inhibition is usually observed at higher ATP concentrations ($\simeq 10^{-4}$M), at which the inhibition has been reported to fit competition kinetics (Rega & Garrahan, 1970). Given that there is evidence that ATP concentrations in the range 1×10^{-7}M-5×10^{-7}M are required for half saturation of the high affinity ATP binding sites (Pitts, 1974), the concentration of ATP used (3×10^{-6}M) was considered adequate for the saturation of the high affinity ATP binding sites, while avoiding the ambiguity attributable to competitive inhibition of the K^+-phosphatase by higher ATP concentrations.

Figures 10.4,5 and 10.6 compare the observed kinetic properties of the K^+-PNPase from both control and thimerosal treated preparations in the presence and absence of ATP. These figures clearly show that the behaviour of the given preparations in the presence of ATP could be described by similar curve types to those observed in the absence of ATP. Furthermore, it is also apparent that the observed kinetic properties of the thimerosal treated preparation was not significantly affected by the presence of 3uM ATP. This is further emphasised by the parameters describing the relevant curves (see Tables 10.5a & 10.5b) which show that there is no significant difference between the parameters describing the kinetic properties of the thimerosal treated preparation in the presence and absence of 3uM ATP. In contrast, figures 10.4,10.5 & 10.6 show that the behaviour of the K^+-PNPase from the control preparations was significantly affected by the presence of ATP. The apparent sensitivity to potassium ion was decreased by the inclusion of the ATP, as evidenced by the lower levels of potassium ion needed to produce half maximal response in the absence of ATP (no ATP $\simeq$4mM vs 3uM ATP$\simeq$ 5mM).

TABLE 10.5

Effects of 3μM ATP on the K^+-PNPase from control and thimerosal treated rat brain synaptic membranes

A. OUABAIN INHIBITION

Parameters	Control		Thimerosal	
	No ATP	3μM ATP	No ATP	3μM ATP
▲K_i (M)	$2.42\times10^{-7} \pm 3.1\times10^{-8}$	$8.69\times10^{-8} \pm 6.1\times10^{-9}$	* -	* -
▲ϕ	0.58 ± 0.059	0.59 ± 0.023	* -	* -
▲β	507 ± 59	1259 ± 33	* -	* -
apparent pI_{50}	6.24 ± 0.05	6.67 ± 0.04	3.66 ± 0.05	3.68 ± 0.04

* Values not applicable - system better described by an uncompetitive inhibition curve

▲ See Chapter 3

B. TEMPERATURE DEPENDENCE

Parameters	Control		Thimerosal	
	No ATP	3μM ATP	No ATP	3μM ATP
+ μ (K.J.Mol^{-1})	54.3 ± 2.3	58.2 ± 6.1	80.3 ± 5.4	78.9 ± 6.2
Φ ΔH (K.J.Mol^{-1})	-167.6 ± 16.9	-173.2 ± 18.1	† -	† -
Φ ΔS (J.$K.^{-1}mol^{-1}$)	-586 ± 57	-606 ± 43	† -	† -

+ Apparent activation energy
Φ See Chapter 4
† Not applicable - system better defined by an Arrhenius straight line.

All values mean of 3 preparations ± one standard deviation.

Furthermore, the presence of ATP resulted in an increase in the apparent sensitivity to ouabain, especially at the lower ouabain concentrations. This is evident from Table10.5a which shows that the parameters relating to ouabain binding and ouabain binding co-operativity effects (K_i & β) were significantly altered by the presence of 3uM ATP. However the catalytic co-operativity parameter (ϕ), was not affected by the ATP, a result that is in accord with the absence of any observable effect of 3uM ATP on the maximal activities of the K^+-PNPase. However, the concentration of ouabain needed for half saturation of the second ouabain binding site (see Chapter 3) was not significantly affected by ATP. This is evident from figure 10.4, and by the observation that the product βK_i (concentration needed for half saturation of the second ouabain binding site) was not significantly altered by the presence of ATP. Finally, the temperature dependence of the K^+-PNPase from the control preparation appeared to be unaffected by the presence of ATP. This is further emphasised by the list of defining parameters in Table 10.5b which show no significant difference between those calculated in the presence and absence of ATP. This result was also consistent with the absence of any observable effect of 3uM ATP on the maximal activities.

DISCUSSION

The above results underline some important points concerning the kinetic properties of the K^+-phosphatase. It is obvious from the data that the kinetic parameters measured for the K^+-phosphatase are significantly different from those measured for the Na^+-K^+-ATPase (see Table10.6 and figure 10.7). The differences are greater in the

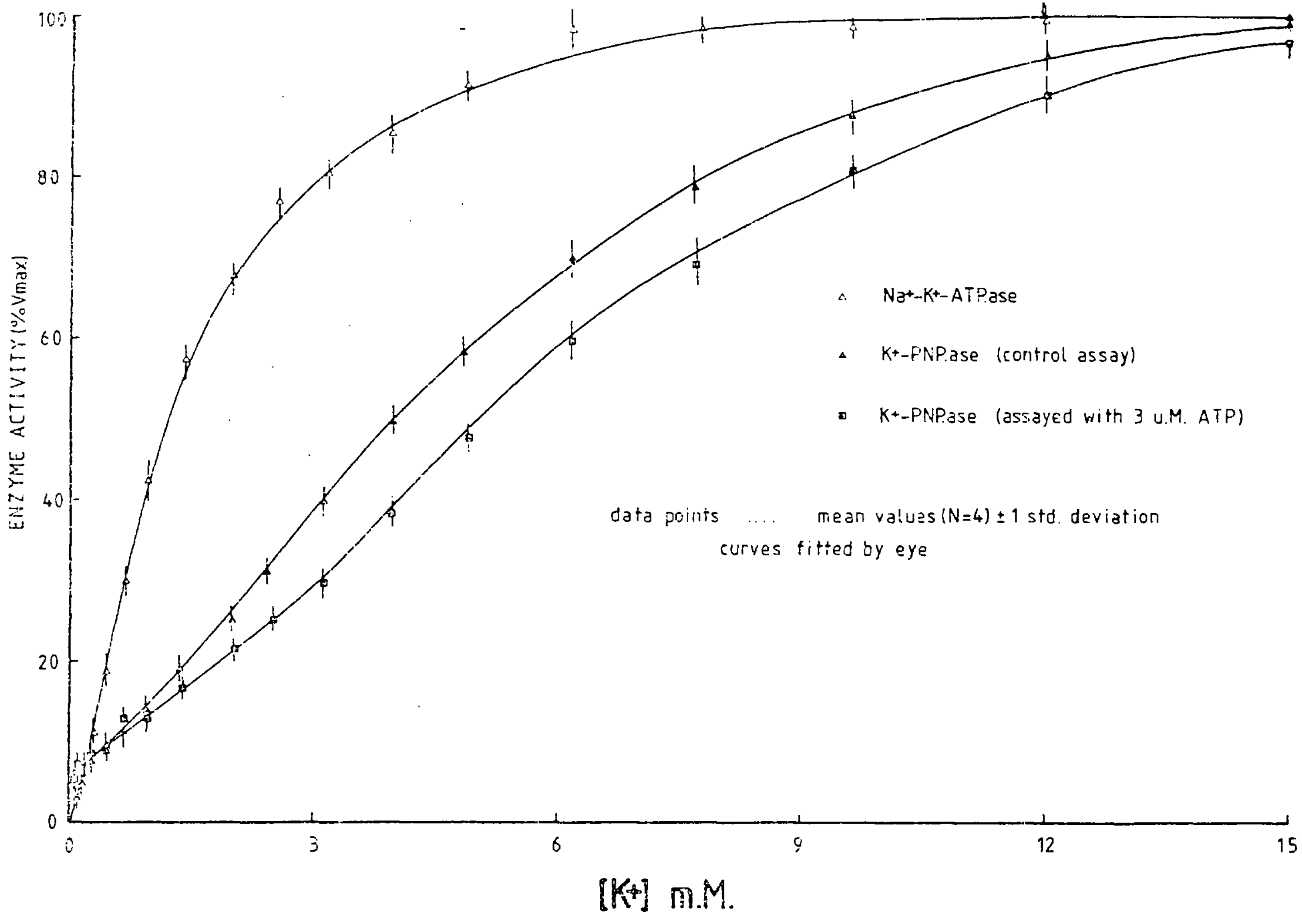


Fig.10.7
POTASSIUM ACTIVATION OF THE OUABAIN SENSITIVE
PHOSPHATASE FROM RAT BRAIN SYNAPTIC MEMBRANES
ENZYME ACTIVITY (%Vmax)
100
80
60
40
20
0
0
3
6
9
12
15
[K+] m.M.
Na+-K+-ATPase
K+-PNPase (control assay)
K+-PNPase (assayed with 3 u.M. ATP)
data points .... mean values (N=4) ± 1 std. deviation
curves fitted by eye

TABLE 10.6

Comparison of the kinetic properties of the ouabain sensitive phosphatases from rat brain synaptic membranes

A. OUABAIN INHIBITION

Parameters	Na^+-K^+-ATPase	K^+-PNPase	
		No ATP	3μM ATP
$^*K_i$ (M)	$2.32 \times 10^{-8} \pm 3.5 \times 10^{-9}$	$2.42 \times 10^{-7} \pm 3.1 \times 10^{-8}$	$8.69 \times 10^{-8} \pm 6.1 \times 10^{-9}$
$^*\phi$	0.79 ± 0.031	0.58 ± 0.059	0.59 ± 0.023
$^*\beta$	866 ± 25	507 ± 59	1295 ± 33
apparent pI_{50}	6.95 ± 0.003	6.24 ± 0.05	6.67 ± 0.04

* See Chapter 3

B. TEMPERATURE DEPENDENCE

Parameter	Na^+-K^+-ATPase	K^+-PNPase	
		No ATP	3μM ATP
$^\dagger\mu$ ($K.J.Mol^{-1}$)	67.7 ± 4.4	54.3 ± 2.3	58.2 ± 3.1
$^\dagger\Delta H$ ($K.J.Mol^{-1}$)	-170.7 ± 9.0	-167.6 ± 16.9	-173.2 ± 18.1
$^\dagger\Delta S$ ($J.K.Mol^{-1}$)	-593 ± 32	-586 ± 57	-606 ± 43

† See Chapter 4.

Values mean of 3 preparations ± 1 standard deviation

in the cases of the apparent sensitivities to potassium and to ouabain. In these cases, the data suggest a complex concentration dependence of the relevant kinetic property, as evidenced by the biphasic response to ouabain, and the failure to obtain an accurate fit of the potassium activation to a sigmoid curve. Though the latter is at variance with previous reports (Robinson, 1969; Gache, Rossi & Lazdunski, 1977; Tashima & Hasegawa, 1975), it can be resolved by the observation that the experiments conducted here covered a wider concentration range than those generally reported, and that the spacing of the experimental points was closer than that generally reported. Thus, the experiments were more likely to detect any 'anomalies'. In spite of this, the magnitude of the differences observed between the Na^+-K^+-ATPase and the K^+-PNPase with respect to potassium and ouabain sensitivities, are similar to those previously reported (Yoshida, Izumi & Nagai, 1966; Israel & Titus, 1967; Gache, Rossi & Lazdunski, 1977). The observed differences between the K^+PNPase and the Na^+-K^+-ATPase are smaller in the case of the temperature kinetics measurements. Here, the data suggest that the differences can be solely attributable to the differences in the kinetic properties of the active state, since a significant difference was found between the apparent Arrhenius 'u' values ($P < 0.01$), while no such differences were found between the parameters defining the transition to the inactive state. This result, while being consistent with the other measurements, is at variance with a previous report of the temperature dependence of the K^+-PNPase from lamb kidney microsomes (Barnett & Parlazzotto, 1974). This earlier report described the temperature kinetics of the K^+-PNPase with a linear Arrhenius plot of apparent Arrhenius 'u' 37-42 $K.J.mol^{-1}$. It is difficult to resolve these conflicting results; they must reflect either the different tissues source of the enzyme or the different

methodologies of the two laboratories. It is obvious, however, that the linear Arrhenius plot reported by Barnett & Parlazzotto, 1974, is not consistent with the generally accepted view that the K^+-phosphatase is involved in the dephosphorylation and regeneration reactions of the Na^+-K^+-ATPase, reactions which are inclusive of the rate limiting step.

The results presented here also indicate that thimerosal is a potent modifier of the K^+-phosphatase activity of the Na^+-K^+-ATPase system. The effect of the reagent on the apparent sensitivity to potassium ion, observed here, is similar in form and magnitude to that previously reported (Henderson & Askari, 1977). In the case of the ouabain inhibition studies, the results show that the thimerosal treatment results in a decrease in the apparent sensitivity to ouabain. Furthermore, the form of the response to increasing ouabain concentrations was changed to a monophasic one as opposed to the biphasic one observed in the control samples. This observation is difficult to interpret unambiguously. The ouabain inhibition of the Na^+-K^+-ATPase (see Chapter 3) and the control K^+-PNPase are suggestive of two ouabain binding sites, while the response of the thimerosal treated sample suggests the presence of one ouabain binding site. Thus the data may be interpreted as the direct or indirect blockading of one of the ouabain binding sites on the enzyme. However, given that the ouabain inhibition studies (see Chapter 3) suggested that the functioning of the Na^+-K^+-ATPase is associated with large co-operativity effects, it cannot be ruled out that the thimerosal acts by altering the co-operativity factors (especially ϕ), rather than simply blocking one of the postulated ouabain binding sites.

The modifying effects of thimerosal on the K^+-PNPase are further underlined by the observations of the effects of the reagents on the temperature kinetics of the enzyme. The results show that, in addition to an increase in the apparent activation energy of the active state, the behaviour of the enzyme is also radically altered by the thimerosal treatment. This is obvious because the treated sample can be described by a linear Arrhenius plot as opposed to the non-linear Arrhenius plot which characterised the control samples. Given that the non-ideal Arrhenius behaviour normally observed in the Na^+-K^+-ATPase system, has been traditionally attributed to lipid effects, this result seems surprising, especially since the target of thimerosal action is an accessible sulphydryl group on a protein component. These results therefore suggest that the protein component of the Na^+-K^+-ATPase may be playing a bigger role in describing the temperature kinetics of the enzyme than that commonly envisaged.

The results presented here also indicate that the relationship between the K^+-phosphatase and the Na^+-K^+-ATPase is not a simple one. This is evidenced by the potent effects of ATP on the properties of the K^+-PNPase of the control preparations. Effects, similar in form and magnitude to those presented here, have been widely reported (see Garrahan & Rega, 1976), however most workers have concentrated on quantifying such effects rather than defining them mechanistically Although insufficient data have been collected in the study to attempt such a description, the fact that the effects of low ($\approx$uM) concentrations of ATP on the K^+PNPase can be abolished by the blockading of a sulphydryl group by thimerosal, suggests that a sulphydryl group (or groups) may be involved in ATP binding to the enzyme at a site remote from the catalytic site. This has also been suggested in the previous reports of thimerosal effects on this enzyme system (Henderson & Askari, 1976,

1977) in which the ATP binding site was considered to be of high affinity and not directly involved in the phosphorylation of the catalytic site. The involvement of sulphydryl groups at such a level has also been suggested in recent reports (Schoot, Schoots, DePont, Schuurmans-Stekhoven & Bonting, 1977; DePont & Bonting, 1977).

The results presented here suggest that thimerosal can be used in mechanistic studies on the Na^{+}-K^{+}-ATPase since it obviously is a potent modifier of the enzyme. However, the full effects of this reagent on the Na^{+}-K^{+}-ATPase enzyme system are yet to be quantified and characterised. As a result, there cannot be an unambiguous interpretation of data similar to those presented here, until the effects of thimerosal on this enzyme are fully understood.

CHAPTER 11

GENERAL DISCUSSION

The work presented in this thesis was generally aimed at an evaluation of the parts played by the protein and lipid components of the Na^+-K^+-ATPase in the fine control of its catalytic activity. The approach adopted here assumed that fine control of this enzyme system may be mediated via subtle modulations in its kinetic properties. In consequence the experiments were designed to test the sensitivity of some of the kinetic properties of the Na^+-K^+-ATPase to some protein and lipid targeted modulators, which were expected to exert their effects without gross inactivation of the enzyme. Given the uncertainty surrounding considerable aspects of the functioning of this enzyme and the complexity of its known kinetics (see review by Whittam & Chipperfield, 1975; Schwartz, Lindenmayer & Allen, 1975), such an approach first required a rationalisation of some aspects of the known kinetic properties of this enzyme, such that the data obtained may be more clearly interpreted.

The data presented in Chapter 3 on ouabain inhibition, in Chapter 4 on temperature dependence and in Chapter 5 on potassium activation, generally agree with work already presented in the literature which have already been discussed. However, the framework within which the data is interpreted here differs from that usually adopted especially with respect to the studies on the temperature dependence of its catalytic activity. The data presented here and elsewhere agree in that a deviation from linear Arrhenius temperature kinetics is clearly indicated for the Na^+-K^+-ATPase. However, most workers interpret such data in terms of two intersecting Arrhenius straight

lines (see Charnock, Cook & Opit, 1971; Grisham & Barnett, 1973; Barnett & Parlazzotto, 1974). This interpretation was proposed, for a variety of enzyme systems that yielded non linear Arrhenius kinetics, by Kumamoto, Raison & Lyons (1971), who suggested that a phase change may be responsible for this type of non ideal Arrhenius temperature kinetics. Many other workers have suggested that lipid phase changes may be responsible for such observed behaviour, and this has been supported by some spin labelling studies (Grisham & Barnett, 1973). However the analysis of the data presented in Chapter 4 questions the validity of the phase change model and an alternative description based on a thermodynamic equilibrium between active and inactive states of the system (see Kavanau, 1950) is suggested. The argument developed in Chapter 4 proposed a model whereby the enzyme system undergoes a thermally induced change of state, accompanied by large enthalpy and entropy changes. However, despite the good fit of the experimental data to the model, the absence of any physical process(es) to which such large energy changes can be ascribed with any certainty, raises doubts as to how useful this model may be; but as discussed more fully in Chapter 4, the energy changes associated with lipid-lipid and protein-lipid interactions may be large enough to satisfy the predictions of the model. Furthermore, some calorimetric measurements (see Kuriki, Halsey, Biltonen & Racker, 1976) have suggested that the conformational changes involved in the functioning of the Na^{+}-K^{+}-ATPase are accompanied by large enthalpy ($\approx$200 $K.J.Mol^{-1}$) and entropy ($\approx$580 $J.K.^{-1}Mol^{-1}$) changes. These are of the same order of magnitude as those determined experimentally from the processing of the data according to the model proposed here

(see Chapter 4). Thus, although no attempt has been made to assign the proposed energy changes to any physical process(es), there is evidence that the energy changes associated with conformational change in the Na^{+}-K^{+}-ATPase and the probable interactions of the enzyme with membrane lipids, are great enough to suggest these processes as feasible areas for further investigations into the thermal changes which obviously occur in this enzyme system.

The data presented in the ouabain inhibition studies agree with that generally reported in that a complex relationship between enzyme activity and ouabain concentration is clearly shown. However, very few workers have attempted to define this concentration dependence kinetically. The model adopted here suggests that the behaviour of the enzyme is consistent with its being a co-operative kinetic dimer. In this respect, the description of the Na^{+}-K^{+}-ATPase proposed by the adopted model is convergent with current kinetic and structural evidence (see Chapter 1) especially the 'rocker model' (Kyte, 1975) which suggest that the functional Na^{+}-K^{+}-ATPase is oligomeric and exhibiting some form of half-the-sites reactivity. Furthermore this co-operative kinetic dimer is of particular relevance to the declared aims of this work, since it suggests that fine control of this enzyme system could be easily accomplished by subtle alterations in the interactions between the kinetic subunits.

The description of the potassium activation of the enzyme presented in Chapter 5, is generally in line with most known kinetic evidence pertinent to the activation of this enzyme by potassium ion. Here, the concentration dependence of the potassium activation of this enzyme is described in terms of a sigmoid curve based on the known stoichiometric

requirement of the Na^+-K^+-ATPase for potassium ion. As mentioned in Chapter 5, this description is compatible with more detailed kinetic studies which have presented strong evidence supporting the existence of two potassium sites on the enzyme. Given the relative simplicity of this description and the quasi-mechanistic interpretation of the data it offers, it is surprising that similar descriptions have not been reported. However, the description as proposed here makes no attempt to define the complex ion activation kinetics of this enzyme especially with respect to interactions between the various cation binding sites. Thus in its present form, it should be regarded as an approximation which enables a quasi mechanistic interpretation of the data, as opposed to a simple numerical description of it as is characteristic of some empirical equations (e.g. the Hill equation).

The rest of the data presented was mainly concerned with monitoring the effect of lipid-targeted and protein-targeted modulators on the above three kinetic properties. The lipid targeted procedures used (detergent extraction of the membranes, lipid peroxidation, temperature induced in-vivo changes in membrane lipid composition) were used since they were not expected to have any major deleterious effects on the activity of the enzyme. The kinetic studies pertinent to the effects of these lipid targeted modulators provided little (if any) evidence that the three kinetic properties monitored were sensitive to the procedures. In the instance where some sensitivity to the modulators was detected (i.e. after detergent extraction of the membranes), there was some uncertainty as to whether the effect was attributable to lipid effects on an indirect disruption of the enzyme protein by the modulator. The results of the other kinetic measurements

especially those on the Na^+-K^+-ATPases from the differently acclimated fish and the effect of hibernation on the kinetic properties of the Na^+-K^+-ATPase from hamster brain, were less ambiguous. In those instances, there was very little doubt that the modulator was lipid targeted and in some cases substantial alterations in the lipid composition of the membrane containing the enzyme were demonstrated. However no significant effect on the observed kinetic properties was detected. These observations are generally in line with most similar studies which suggest that the Na^+-K^+-ATPase is tolerant to significant alterations in the membrane lipid composition (see DePont, Van Prooiten-Van Eeden & Bonting, 1973; 1978), and that a modulation of its kinetic properties by a lipid targeted agent is usually demonstrable only after a severe disruption of the membrane with a coincident loss of Na^+-K^+-ATPase activity (see Taniguchi & Iida, 1972b;Charnock, Cook, Almeida & To, 1973; Sun, 1971). Although the failure to demonstrate any significant modulation of the properties of the Na^+-K^+-ATPase by the lipid targeted procedures used could argue against a role for membrane lipid in the fine control of the Na^+-K^+-ATPase, the issue is not that simple owing to two important factors pertinent to the observations. First, there is no conclusive evidence to suggest that the three kinetic properties monitored were capable of detecting any lipid induced changes in the enzyme system. Since there are large areas of uncertainty and ignorance concerning the reaction mechanism of the Na^+-K^+-ATPase, there is no valid reason to suppose that the effect of lipid mediated modulators must affect the three kinetic properties monitored. Obviously, a clear, unambiguous answer to this question must await a complete 'debugging' of the reaction mechanism of the enzyme system. Secondly,

there is no evidence that the experimentally induced membrane lipid changes were 'seen' by the Na^{+}-K^{+}-ATPase. The membrane lipid changes demonstrated (here and elsewhere) are bulk lipid changes and it is very likely that the enzyme reacts only to changes in those lipids which form an annulus around it and are presumably tightly bound to it. Such lipids will obviously form a very small fraction of the total and it would be extremely difficult to determine whether they were responsive to alteration by the experimental procedures used. The clarification of this issue will obviously require experimentation on biologically active Na^{+}-K^{+}-ATPase samples in which the enzyme is only associated with those lipids which are essential for its activity.

The results describing the effect of the protein targeted modulators (i.e. Concanavalin A binding, heat treatment, thimerosal treatment) are generally in line with the effects reported from several other protein targeted modulators (see review by Schwartz, Lindenmayer & Allen, 1975), in that considerable sensitivity of the kinetic properties were demonstrated especially when the procedure resulted in significant loss of enzyme activity. There was evidence that the three kinetic properties monitored were responsive to perturbations in the protein component of the enzyme and the dose-response sensitivity with respect to ouabain appeared to be the most sensitive of those monitored. The demonstration of a modulation of some of the kinetic properties of the enzyme by a glycoprotein targeted agent (Concanavalin A) is of particular interest in that a probable regulatory role is suggested for the sialoglycoprotein found in all 'purified' samples of this enzyme that have been prepared.

Given that the sialoglycoprotein is distinct from the polypeptide species involved in the phosphorylation reactions (see Kyte, 1971a) those results also suggest that a possible mechanism for fine control of this enzyme could involve allosteric interactions between 'regulatory' and 'catalytic' proteins of this enzyme system.

The kinetic measurements on the heat treated samples were subject to a more ambiguous interpretation owing to the substantial loss in enzymic activity coincident with heating. However, the demonstration of a change in the kinetic properties of the enzyme as a result of a partial thermal denaturation, and the biphasic pattern of thermal inactivation were shown to be consistent with the functional enzyme being a co-operative kinetic dimer. Since the heat treatment procedure was only likely to affect the interactions between the various protein components of the enzyme system, those results suggest that the kinetic properties of the Na^{+}-K^{+}-ATPase is extremely sensitive to such interactions. Thus on the basis of these results inter-subunit co-operativity would appear to be a probable mechanism for the fine control of this enzyme system. However, the validity of such a conclusion is largely dependent on the assumed description of the Na^{+}-K^{+}-ATPase as a co-operative kinetic dimer. The evidence presented in support of such a description is largely based on the ouabain inhibition studies and combined thermal inactivation, ouabain inhibition studies (see Chapter 3) and as shown in Chapter 3, the currently available data is not enough to exclude other possibilities. The resolution of this issue will obviously require more detailed investigations into the processes involved in the thermal inactivation of this enzyme, as well as the consideration of the possible existence of isozymic forms of the Na^{+}-K^{+}-ATPase.

The investigations into the effects of thimerosal on the enzyme suggested that the deleterious effect of the reagent on the enzyme may introduce some problems into the intepretation of any such data. However, the fact that the reagent could block the Na^+-K^+-ATPase activity while leaving a substantial part of the K^+-phosphatase activity of the sodium pump intact suggested that this reagent may be a powerful tool in the 'debugging' of the reaction mechanism of the sodium pump. The characterisation of the effects of the reagent on the kinetic properties of the K^+-phosphatase activity of the Na^+-K^+-ATPase clearly indicated that the kinetic properties of the Na^+-K^+-ATPase is extremely sensitive to the effect of protein targeted modulators, since three kinetic properties monitored were significantly altered. The change in the temperature kinetics of the system after thimerosal treatment was particularly interesting since it also suggests that the non-ideal Arrhenius behaviour of the Na^+-K^+-ATPase may not be a property of the membrane lipids, and that the major causative factors may be an integral part of the protein moiety of this enzyme system. However, in the absence of a complete characterisation of the effects of thimerosal on the Na^+-K^+-ATPase such a conclusion can only be tentative. The data described in Chapter 10 is of added interest because the evidence supports the existence of a high affinity ATP binding site on the enzyme. The evidence presented here and by Henderson & Askari, 1976, 1977, suggests that the high affinity ATP binding site is distinct from the site of phosphorylation and that a sulphydryl group is directly involved in the binding of ATP to the high affinity site. Since the removal of Na^+-K^+-ATPase activity, and the absence of any modulation of the kinetic properties of the K^+-phosphatase by low ($\simeq 10^{-6}$M) levels

of ATP are both coincident with an apparent blockading of the sulphydryl group(s) at the high affinity site by thimerosal, it seems likely that the binding of ATP to this site may be playing an important regulatory role in the normal functioning of the Na pump. Thus these observations suggest another mechanism for the fine control of this enzyme system (i.e. modulation in the properties of the high affinity ATP binding site).

The study of the thermal inactivation of the Na^{+}-K^{+}-ATPase, provided some evidence pertinent to the factors involved in the maintenance of the biological activity of this enzyme. Since the enzyme was inactivated at lower temperatures than that required for the inactivation of many soluble proteins (see Joly, 1965; Bull & Breese, 1973(a), 1973(b)), the conclusion that the limiting forces which restrict the Na^{+}-K^{+}-ATPase to its biologically active conformational states are different from those operating in soluble proteins seems justified. Thus, given the known lipid requirement of this enzyme, and the deleterious effects of membrane 'fluidisers' like octanol on its stability (see Chapter 7; Grisham & Barnett, 1973(b)), it would seem likely that such limiting forces may be the hydrophobic interactions between the protein and membrane lipids. The demonstration of a relationship between the thermal stability of the enzyme and the temperature to which its source species has been naturally adapted (see Chapter 7) is compatible with such a suggestion and can be correlated with earlier observations of a significant bias towards a greater fluidity of the membrane lipids extracted from animals that are normally adapted to low temperature habitats (see Cossins, 1977; Cossins & Prosser, 1978). However, it is unlikely thatthe situation can be that simple for in spite of the clear demonstration of a relationship between the thermal stability

of the Na^+-K^+-ATPase and the temperature to which its source species is naturally adapted, the stability of the enzyme was apparently unaffected by the changes coincident with the acclimation of a species to markedly different temperatures. Given that there is ample evidence that membrane lipid changes do occur as a result of hibernation (see Platner, Patnayak & Musacchia, 1972; Goldman, 1975; Blaker & Moscatelli, 1978), and acclimation of ectothermic species to different temperatures (see Johnson & Roots, 1964; Kemp & Smith, 1970), the uncertainty as to whether such membrane lipid changes are 'seen' by the Na^+-K^+-ATPase is also pertinent here. In addition, the probability of there being significant species variation in the Na^+-K^+-ATPase protein must be considered. Although the currently available data (Hokin, 1974; Hopkins, Wagner & Smith, 1976) suggests that there has been little change in the Na^+-K^+-ATPase protein(s) during the evolution of the various groups, the evidence is certainly not conclusive since, to date, only the total amino acid analyses have been reported. Thus it is possible that the results of the comparative thermal inactivation study may simply reflect the fact that the protein components of the Na^+-K^+-ATPases from the various groups have been adapted during evolution to function at different physiological temperature ranges. If such is the case, then it may also account for the failure to demonstrate kinetic changes in the Na^+-K^+-ATPase as a result of temperature acclimation, even though kinetic differences between the Na^+-K^+-ATPases of ectotherms (e.g. trout) and homeotherms (e.g. rat) have been clearly demonstrated (see Table 8.4 and Table 6.3). Another interesting possibility is that the changes in the lipid annulus, that is presumably an integral part of the enzyme system, takes place on the evolutionary time scale.

This would certainly account for the failure to detect changes in the kinetic properties and thermal stability of the enzyme, as a result of temperature acclimation, and at the same time suggest a major role for lipids in the structure and regulation of the Na^{+}-K^{+}-ATPase. Clearly, the resolution of the issues raised here will require more detailed studies on the structure of this enzyme.

The work presented here has raised several points pertinent to aspects of the kinetics, mechanism and lipid dependence of the Na^{+}-K^{+}-ATPase, and given the large areas of uncertainty concerning the mechanism by which this enzyme works, it is difficult to arrive at a firm conclusion. Tentatively, however, it would seem that membrane lipids play little (if any) role in the fine control of the Na^{+}-K^{+}-ATPase, and that their most probable function is in the defining of a potential energy well within which the enzyme is restricted to its biologically active conformational states. It also seems probable that those lipids may define some aspects of the properties of that potential energy well, with respect to the height and elasticity of its walls. The data is biased in favour of the protein component of the enzyme being the target of any 'fine-control-aimed-modulation', and the co-operative kinetic dimer suggested by the ouabain inhibition/thermal inactivation studies is susceptible to such modulation. The data also suggests that fine control of the Na^{+}-K^{+}-ATPase can be mediated by co-operative interactions between the catalytic subunits and or the glycoprotein component, and that there can also be allosteric modulation of this enzyme by ligands binding to sites distant from the primary catalytic centre.

BIBLIOGRAPHY

ACKMAN, R. G. (1963a) Structural correlation of unsaturated fatty acid esters through graphical comparison of gas liquid chromatographic retention times on a polyester substrate. J. American Oil Chemists Society 40: 558-564.

ACKMAN, R. G. (1963b) An analysis of separation factors applicable in the gas liquid chromatograph of unsaturated fatty acid methyl esters on a polyester substrate. J. American Oil Chemists Society 40: 564-467.

AKERA, T. (1971) Quantitative aspects of the interaction between ouabain and the Na^{+}-K^{+}-ATPase. Biochimica et Biophysica Acta 245: 53-62

AKERA, T., BRODY, T. M., SO, R. H. M., TOBIN, T. & BASKIN, S. I. (1974) Factors and agents that influence cardiac glycoside Na^{+}-K^{+}-ATPase interaction. Annals of the N.Y. Academy of Sciences 242: 617-634.

ALBERS, R. W., FAHN, S. & KOVAL, G. J. (1963) The role of sodium now in the activation of electrophorus electric organ adenosine triphosphatase. Proceedings of the National Academy of Sciences U.S.A. 50, 474-481.

ALBERS, R. W. (1967) Biochemical aspects of active transport Annual Review of Biochemistry 36: 727-756.

ARRHENIUS, S. A. (1889) Uber die Reaktionsgeschurindig Keit bie der Inversion von Rohizucher durch Sauren. Z Physik Chem. 4: 226-248.

ASKARI, A. (1974) The effects of antibodies to Na^{+}-K^{+}-ATPase on the reactions catalysed by the enzyme. Annals of the N.Y. Academy of Sciences 242: 372-388.

ASKARI, A. & KOYAL, D. (1971) Studies on the partial reactions of the Na^{+}+K^{+}-activated ATPase. II. Effects of oligomycin and other inhibitors of the ATPase on the p-nitrophenol phosphatase. Biochimica et Biophysica Acta 225: 20-25.

ATKINS, G. L. (1973) A simple digital-computer program for estimating the parameters of the Hill equation. European Journal of Biochemistry 33: 175-180.

ATKINSON, A., GATENBY, A. D. & LOWE, A. G. (1971) Subunit structure of the Na^{+}-K^{+}-dependent transport ATPase. Nature New Biology 233: 145-146.

ATKINSON, A., GATENBY, A. D. & LOWE, A. G. (1973) The determination of inorganic phosphate in biological systems. Biochimica et Biophysica Acta. 320: 195-204.

BADER, H. & SEN, A. K. (1966) K^{+}-dependent acylphosphatase as part of the Na^{+}-K^{+}-ATPase of cell membranes. Biochimica et Biophysica Acta 118: 116-123.

BALLENTINE, R (1957) Determination of total Nitrogen and Ammonia Methods in Enzymology III, 984-995, publ. Academic Press.

BAKER, P. F. (1972) The sodium pump in animal tissues and its role in the control of cellular metabolism and function. Metabolic Pathways VI: 243-268. Ed. Hokin, L. E. Publ. Academic Press.

BAKKEREN, J. A. J. M. & BONTING, S. L. (1968) Studies on (Na^{+}-K^{+}) activated ATPase. Biochimica et Biophysica Acta. 150: 460-466.

BANERJEE, S. P., DOWISH, J. L., KHANNA, V. K. & SEN, A. K. (1970) Solubilisation of guinea-pig kidney in Na^{+}-K^{+}ATPase with Lubrol-W and Triton X-100. Biochimica et Biophysica Acta. 211: 345-355.

BANNISTER, W. H., ANASTASI, A. & BANNISTER, J. V. (1976) Least squares estimation of Michaelis-Menton parameters by a fibonacci search method. F.E.B.S. Letters 64: 290-292.

BARNETT, R. F.,& PARLAZZOTTO, J. (1974) Mechanism of the effects of lipid phase transitions on the Na^{+}-K^{+}-ATPase, and the role of protein conformational changes. Annals of the N.Y. Academy of Sciences 242: 69-76.

BASKIN, L. S. & LESLIE, R. B. (1968) Effects of substrates and products on beef brain microsomal (Na^{+}-K^{+}-Mg^{2+}) ATPase activity. Biochimica et Biophysica Acta. 159: 509-513.

BARTLET, J. C. & SMITH, D. M. (1960) The determination of the areas of resolved and partially resolved chromatography peaks. Canadian Journal of Chemistry 38: 2057-2065.

BEAUGE, L. A. & GLYNN, I. M. (1977) A modifier of (Na^+-K^+) ATPase in commercial ATP. Nature 268: 355-356.

BEAUREGARD, G. & ROUFOGALIS, B. D. (1977) The role of tightly bound phospholipid in the activity of erthrocyte acetyl cholinesterase. Biochemical and Biophysical Research Communication 72: 211-219.

BLAKER, W. D. & MOSCATELLI, E. A. (1978) The effect of hibernation of the lipids of brain myelin and microsomes in the Syrian Hamster. J. Neurochemistry 31: 1513-1518.

BOGARTZ, R. S. (1968) A least squares method for fitting intersecting line segments to a set of data points. Psychological Bulletin 70: 749-755.

BOLDYREV, A., RUGGE, E., SMIRNARA, I. & TABAK, M. (1977) Na-K-ATPase. The role of state of lipids and my ions in activity regulation. F.E.B.S. Letters 80: 303-307.

BOLSTEIN, R. (1966) Evidence for a phosphorylated intermediate of red cell membranes adenosine triphosphatase. Biochemical and Biophysical Research Communications 22: 598-602.

BOLSTEIN, R. & CHU, L. (1977) Sidedness of (Sodium, Potassium). Adenaline triphosphatase of inside our red cell membrane vessicles. J. Biological Chem. 252, 3035-3053.

BOND, G. H., BADER, H. & POST, R. L. (1971) Acetyl phosphate as a substitute for ATP in (Na^+-K^+) dependent ATPase. Biochimica et Biophysica Acta. 241: 57-67.

BONTING, S. L. (1966) Studies on the sodium-potassium-activated adenosinetriphosphatase XV. The nectal gland of the elasmobranchs. Comparative Biochemistry and Physiology 17: 953-966.

BOWLER, K. & DUNCAN, C. J. (1969) The temperature characteristics of Brain microsomal ATPases of the Hedgehog: Changes associated with hibernation. Physiological Zoology 42: 211-219.

BRAZHNIKOV, E. Y., CHETYERIN, A. B. & CHIRGADZE, Y. N. (1978) Secondary structure of the Na^{+}-K^{+}-ATPase dependent adenosine triphosphatase. F.E.B.S. Letters 93: 125-128.

BULL, H. B. & BREESE, K. (1973a) Thermal transitions of proteins. Archives of Biochemistry and Biophysics 156: 604-612.

BULL, H. B. & BREESE, K. (1973b) Thermal stability of proteins. Archives of Biochemistry and Biophysics 158: 681-686.

CALDWELL, P. C. & KEYNES, R. D. (1959) The effect of ouabain on the efflux for sodium from a squid axon. J. Physiology 143: 8P-9P.

CANTLEY, L. C., GELLES, J. & JOSEPHSON, L. (1978) Reaction of (Na-K) ATPase with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole: Evidence for an essential tyrosine at the active centre. Biochemistry 17: 418-425.

CANTLEY, L. C. & JOSEPHSON, L. (1976) A slow interconversion between active and inactive states of the Na^{+}-K^{+} ATPase. Biochemistry 15: 5280-5287.

CANTLEY, L. C., JOSEPHSON, L., WARNER, R., YAMAGISAWA, M., LECHENE, C. & GUIDOTTI, G. (1977) Vanadate is a potent (Na-K) ATPase inhibitor in ATP derived from muscle. J. Biological Chemistry 252: 7421-7423.

CARROLL, K. K. (1961) Quantitative estimation of peak areas in gas liquid chromatography. Nature 191: 377-378.

CAVIERES, J. D. & ELLORY, J. C. (1975) Allosteric inhibition of the sodium pump by external sodium. Nature 255: 338-340.

CHAN, P. C. (1967) Reversible effect of sodium dodecyl sulphate on human erythrocyte membrane adensoine triphosphatase. Biochimica et Biophysica Acta 135: 53-60.

CHAPMAN, D., PEEL, W. E., KINGSTON, B. & LILLEY, T. H. (1977) Lipid phase transitions in model Biomembranes. The effect of ions on phosphatidylcholine bilayers. Biochimica et Biophysica Acta 464: 260-275.

CHAPMAN, D. & URBINA, J. (1971) Phase transitions and bilayer structure of mycoplasma liedlawii. F.E.B.S. Letters 12: 169-173.

CHARNOCK, J. S. & POTTER, H. A. (1969) The effect of Mg^{2+} and ouabain in the incorporation of P^{32} from $yATP^{32}$ into Na^{+}- and K^{+}-activated adenosine triphosphatase. Archives of Biochemistry and Biophysics 134: 42-47.

CHARNOCK, J. S. & SIMONSON, L. P. (1978a) Variations in $(Na^{+}-K^{+})$ ATPase and Mg^{2+} ATPase activity of the ground squirrel brain during hibernation. Comparative Biochemistry and Physiology 59B: 223-229.

CHARNOCK, J. S. & SIMONSON, L. P. (1978b) Seasonal variations in the renal cortical $(Na^{+}-K^{+})$ ATPase and Mg^{2+} ATPase of a hibernator. The ground squirrel (Spermophilus richardsonii). Comparative Biochemistry and Physiology 60B: 433-439.

CHARNOCK, J. S., ROSENTHAL, A. S. & POST, R. L. (1963) A phosphorylated intermediate compound in ATP dependent sodium and potassium transport. Federation Proceedings 22: 212.

CHARNOCK, J. S., COOK, D. A. & CASEY, R. (1971) The role of cations and other factors on the apparent energy of activation of $(Na^{+}-K^{+})$-ATPase. Archives of Biochemistry and Biophysics 147: 323-329.

CHARNOCK, J. S., COOK, D. A. & OPIT, L. J. (1971) Role of energised states of $(Na^{+}-K^{+})$ ATPase in the sodium pump. Nature New Biology 233: 171-172.

CHARNOCK, J. S., COOK, D. A., ALMEIDA, A. F. & TO, R. (1973) Activation energy and phospholipid requirements of membrane bound adenosine triphosphatases. Archives of Biochemistry and Biophysics 159: 393-399.

COSSINS, A. R. (1977) Adaptation of biological membranes to temperature. Biochimica et Biophysica Acta 470: 395-411.

COSSINS, A. R. & PROSSER, C. L. (1978) Evolutionary adaptation of membranes to temperature. Proceedings of the National Academy of Sciences of the U.S.A. 75: 2040-2043.

COSSINS, A. R., CHRISTANSEN, J. A., PROSSER, C. L. (1978) Adaptation of biological membranes to temperature. Biochimica et Biophysica Acta 511: 442-454.

CROZIER, W. J. (1924) On biological oxidations as function of temperature. J. Gen. Physiol. 7: 189-216.

DEGUCHI, N., JORGENSEN, P. L. & MAUNSBACH, A. B. (1977) Ultrastructure of the sodium pump. J. Cell Biology 75: 619-634.

DE KRUYFF, B., DEMEL, R. A., VAN DEENEN, L. L. M. (1973) The effect of cholesterol and epicholesterol incorporation on the permeability and on the phase transition of intact acholeplasma laidlamii cell membranes and derived liposomes. Biochimica et Biophysica Acta 307: 1-19.

DE PONT, J. J. H. H. M., VAN PROOIJEM-VAN EEDEN, A. & BONTING, S. L. (1973) Studies on (Na^+-K^+)-activated ATPase. XXXIV. Phosphatidylserine not essential for (Na^+-K^+) ATPase activity. Biochimica et Biophysica Acta 323: 487-494.

DE PONT, J. J. H. H. M., VAN PROOIJEM-VAN EEDEN, A. & BONTING, S. L. (1978) Role of negatively charged phospholipids in highly purified (Na^+-K^+) ATPase from rabbit outer medulla. Biochimica et Biophysica Acta 508: 464-477.

DE PONT, J. J. H. H. M., SCHOOT, B. M., VAN PROOIJEN-VAN EEDEN, A. & BONTING, S. L. (1977) An essential auginine in the ATP binding centre of (Na^+-K^+) ATPase. Biochimica et Biophysica Acta 432: 213-227.

DIXON, J. F. & HOKIN, L. E. (1974) Studies on the characterisation of the sodium-potassium transport adenosine triphosphatase. Archives of Biochemistry and Biophysics 163: 749-758.

DOBRETSOV, G. E., BORSCHEVSKAYA, T. A., PETROV, V. A. & VLADIMIROV, Y. A. (1977) The increase in phospholipid rapidity after peroxidation. F.E.B.S. Letters 84: 125-128.

DORNAND, J., REMINJAC, C. & MANI, J. (1978) Studies of (Na^+-K^+)-sensitive ATPase activity of pig lymphocytes: Effects of concanavilin A. Biochimica et Biophysica Acta 509: 194-200.

DRIEDZIC, W. & ROOTS, B. J. (1975) Glycerophosphatide content and composition of Trout (Salvelinus fontinalis M.) brain. J. Thermal Biology 1: 7-10.

DUNHAM, E. T. & GLYNN, I. M. (1961) Adenosine triphosphatase activity and the active movements of alkali metal ions. J. Physiology 156: 274-293.

ELETR, S. & INESI, G. (1972) Phase changes in the lipid moieties of sarcoplasmic reticulum membranes induced by temperature and protein conformational changes. Biochimica et Biophysica Acta 290: 178-185.

FAGAN, J. B. & RACKER, E. (1977) Reversible inhibition of $(Na^{+}-K^{+})$ ATPase by Mg^{2+}, Adenosine triphosphate and K^{+}. Biochemistry 16: 152-158.

FAHN, S., KOVAL, G. J. & ALBERS, R. W. (1966) Sodium-potassium activated adenosine triphosphatase of electrophorus electric organ. I. An associated sodium activated transphosphorylation. J. Biological Chemistry 241: 1882-1889.

FAHN, S., KOVAL, G. J. & ALBERS, R. W. (1968) Sodium potassium activated adenosine triphosphatase of electrophorus electric organ. V. Phosphorylation by adenosine triphosphate ^{32}P. J. Biological Chemistry 243: 1993-2002.

FARQUHAR, J. W., INSOLL, W., ROSEN, P., STOFFEL, W. & AHREMS, E. H. (1959) The analysis of fatty acid mixtures by gas-liquid chromatography: Construction of an ionisation chamber. Nutrition Review (Supplement) 17: 1-30.

FEINSTEIN, M. B., FERNANDEZ, S. M. & SHA'AFI, R. I. (1975) Fluidity of natural membranes and phosphatidyl senine and ganglioside dispersions effects of local anaesthetics, cholesterol protein. Biochimica et Biophysica Acta 413: 354-370.

FENSTER, L. J. & COPENHAVER, J. H. (1967) Phosphatidyl serine requirement of $(Na^{+}-K^{+})$ activated adenosine triphosphatase from rat kidney and brain. Biochimica et Biophysica Acta 137: 406-408.

FIRPO, J. L., DUPIN, J. J., ALBINET, G., BOIS, A., CASALTI, L. & BARET, J. F. (1978) A theoretical study of phase transitions in bidimensional monolayers. J. Chemical Physics 68: 1369-1374.

FISCHER, H. F. (1964) A limiting law relating the size and shape of protein molecules to their composition. Proceedings of the National Academy of Sciences of the U.S.A. 51: 1285-1291.

FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. S. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biological Chemistry 226: 497-509.

FROEHLICH, J. P., ALBERS, R. W., KOVAL, G. J., GOEBEL, R. & BERMAN, M. (1976) Evidence for a new intermediate state in the mechanism of (Na^+-K^+) Adenosine triphosphatase. J. Biological Chemistry 251: 2186-2188.

FUKUSHIMA, Y. & TONOMURA, Y. (1975) Effects of sodium and potassium ions on the elementary steps in the reaction of Na^+-K^+-dependent ATPase. J. Biochemistry 77: 521-531.

GACHE, C., ROSSI, B. & LAZDUNSKY, M. (1977) Mechanistic analysis of the (Na^+-K^+) ATPase using new pseudosubstrates. Biochemistry 16: 2957-2965.

GARAY, R. P. & GARRAHAM, P. J. (1973) The interaction of sodium and potassium with the sodium pump in red cells. J. Physiology 231: 297-325.

GARRAHAN, P. J. & GLYNN, I. M. (1967) The stoichiometry of the sodium pump. J. Physiology 192: 217-237.

GAZZOTTI, P. & PETERSON, S. W. (1977) Lipid requirement by membrane bound enzymes. J. Bioenergetics and Biomembranes 9: 373-386.

GEORGE, P., PHILLIPS, R. C. & RUTMAN, R. J. (1963) Estimates of thermodynamic data for the formation of the Mg^{++} complexes of ATP and ADP at zero ionic strength. Biochemistry 2: 508-512.

GIBSON, K. D. (1953) True and apparent activation energies of enzymic reactions. Biochimica et Biophysica Acta 10: 221-229.

GIOTTA, G. J. (1976) Quaternary structure of (Na^{+}-K^{+}) dependent adenosine triphosphatase. J. Biological Chemistry 251: 1247-1252.

GIOTTA, G. J. (1977) The effect of $(O\text{-Phenanthroline})_2$ cupric sulphate on several properties associated with (Na^{+}-K^{+}) dependent adenosine triphosphatase. Archives of Biochemistry and Biophysics 180: 504-508.

GITLER, C. (1972) Use of ANS to detect phospholipids and a polar molecules in chromatograms. Analytical Biochemistry 50: 324-325.

GLADWELL, R. T. (1975) Heat death in the crayfish Austropotamobius pallipes: Thermal inactivation of muscle membrane-bound ATPases in warm and cold adapted animals. J. Thermal Biology 1: 95-100.

GOLDBERG, M. L. (1973) Quantitative assay of submicrogram amounts of protein. Analytical Biochemistry 51: 240-246.

GOLDMAN, S. S. (1975) Cold resistance of the brain during hibernation: Evidence of a lipid adaption. American J. Physiology 228: 834-838.

GOLDMAN, S. S. & ALBERS, R. W. (1975) Cold resistance of the brain during hibernation: Temperature sensitivity of the partial reactions of the Na^{+}-K^{+} ATPase. Archives of Biochemistry and Biophysics 169: 540-544.

GOLDMAN, S. S. & WILLIS, J. S. (1973) Cold resistance of the brain during hibernation. II. Na-K-activated ATPase. Cryobiology 10: 218-224.

GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949) Determination of serum proteins by means of the biunit reaction. J. Biological Chemistry 177: 751-766.

GRASSO, A. (1967) A sodium and potassium stimulated adenosine triphosphatase in the cockroach nerve cord. Life Sciences 6: 1911-1918.

GRINNA, L. S. (1975) Multiple thermal discontinuities in glucose-6-phosphatase activity. Biochimica et Biophys Acta 403: 388-392.

GRISAR, T., FRERE, J. M., GRISAR-CHARLIER, J. FRANCK, G. & SCHOFFENJELS, E. (1978) Synaptosomal Na^{+}-K^{+} ATPase in an hysteretic enzyme. F.E.B.S. Letters 89: 173-176.

GRISHAM, C. M. & BARNETT, R. E. (1972) The interrelationship of membrane structure and protein structure in the functioning of the (Na^{+}-K^{+}) activated ATPase. Biochimica et Biophysica Acta 266: 613-624.

GRISHAM, C. M. & BARNETT, R. E. (1973a) Role of lipid-phase transitions in the regulation of the (Sodium-potassium) adenosine triphosphatase. Biochemistry 12: 2635-2637.

GRISHAM, C. M. & BARNETT, R. E. (1973b) The effects of long chain alcohols on membrane lipids and the (Na^{+}-K^{+}) activated ATPase. Biochimica et Biophysica Acta 311: 417-422.

GRISHAM, C. M. & MILDVAM, A. S. (1975) Magnetic resonance and kinetic studies of the mechanism of membrane bound sodium and potassium ion activated adenosine triphosphatase. J. Supramolecular Structure 3: 304-313.

GROVES, W. E., DAVIS, F. C. & SELLS, B. H. (1968) Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. Analytical Biochemistry 22: 195-210.

GRUENER, N. & AVI-DOR, Y. (1966) Temperature-dependence of activation and inhibition of rat brain adenosine triphosphatase activated by sodium and potassium. Biochemical Journal 100: 762-767.

GUIDOTTI, G. (1972) Membrane proteins. Annual Review of Biochemistry 41: 731-752.

HAN, M. H. (1972) Non-linear Arrhenius plots in temperature-dependent kinetic studies of enzyme reactions. J. Theor. Biol. 35: 543-568.

HANSEN, D. (1976) Non-uniform populations of g-strophanthin binding sites of $(Na^{+}-K^{+})$ activated ATPase. Biochimica et Biophysica Acta 433: 383-392.

HART, W. B. & TITUS, E. O. (1973) Sulfhydryl groups of sodium-potassium transport adenosine triphosphatase. J. Biological Chemistry 248: 4674-4681.

HAZEL, J. R. & PROSSER, C. L. (1974) Molecular mechanisms of temperature compensation in poikilotherms. Physiological Reviews 54: 620-670.

HENDERSON, G. R. & ASKARI, A. (1976) Transport ATPase: Thimerosal inhibits the $Na^{+}-K^{+}$-dependent ATPase activity without diminishing the Na^{+}-dependent ATPase activity. Biochemical and Biophysical Research Communications 69: 499-505.

HENDERSON, G. R. & ASKARI, A. (1977) Transport ATPase: Further studies on the properties of the thimerosal treated enzyme. Archives of Biochemistry and Biophysics 132: 221-226.

HEXUM, T., SAMSON, F. E. & HIMMES, R. H. (1970) Kinetic studies of $(Na^{+}-K^{+}-Mg^{++})$ ATPase. Biochimica et Biophysica Acta 212: 322-331.

HILL, A. V. (1910) The possible effects of the aggregation of the molecules haemoglobin on its dissociation curves. J. Physiology 40: iv - vii

HOFFMAN, J. F. (1966) The red cell membrane and the transport of sodium and potassium. American Journal of Medicine 41: 666-668.

HOKIN, L. E. (1974) Purification and properties of the (sodium + potassium)-activated adenosine triphosphatase and reconstitution of sodium transport. Annals of the N.Y. Academy of Sciences 242: 12-23.

HOKIN, L. E. & DAHL, J. (1972) The sodium potassium adenosine triphosphatase. Metabolic Pathways. VI. 270-316. Ed. Hokin, L. E. Publ. Academic Press.

HOKIN, L. E., SASTRY, P. S., GALSWORTHY, P. R. & YODA, A. (1965) Evidence that a phosphorylated intermediate in a brain transport adenosine triphosphatase is an acyl phosphate. Proceedings of the National Academy of Sciences U.S.A. 54: 177-184.

HOKIN, L. E., DAHL, J. L., DEUPREE, J. D., DIXON, J. F., HACKNEY, J. F. & PERDUE, J. F. (1973) Studies on the characterisation of the sodium potassium transport adenosine triphosphatase. J. Biological Chemistry 248: 2593-2605.

HOPKINS, B. E., WAGNER, H. & SMITH, T. W. (1976) Sodium and potassium activated adenosine triphosphatase of the nasal salt gland of the Duck (Anas platyrhyrichos). J. Biological Chemistry 251: 4365-4371.

HUANG, W. & ASKARI, A. (1978) Na^{+}-K^{+}-ATPase: On the nature of the "cross-linked" subunits obtained in the presence of o-phenanthrolene and cupric ion. Biochemical and Biophysical Research Communications 82: 1314-1319.

HUBBELL, W. L. & McCONNELL, H. M. (1969) Motion of steroid spin labels in membranes. Proc. National Acad. Sci. U.S.A. 63: 16-22.

ISRAEL, Y. & TITUS, E. (1967) A comparison of Microsomal (Na^{+}-K^{+}) ATPase with K^{+} acetyl phosphatase. Biochimica et Biophysica Acta 139: 450-759.

JARDETZKY, O. (1966) Simple allosteric models for membrane pumps. Nature 211: 969-970.

JARNEFELT, J. (1972) Lipid requirements of functional membrane structures as indicated by the reversible inactivation of (Na^{+}-K^{+}) ATPase. Biochimica et Biophysica Acta 266: 91-96.

JENSEN, J. & OTTOLENGHI, P. (1976) Adenosine diphosphate bending to sodium-plus-potassium ion-dependent adenosine triphosphatase. The Biochemical Journal 159: 815-817.

JOHNSON, F. H., EYRING, H. & WILLIAMS, R. W. (1942) The nature of the enzyme inhibitions in bacterial luminescence: sulfamilamide urethane temperature and pressure. J. Cell Comp. Physiol. 20: 247-265.

JOHNSON, P. V. & ROOTS, B. I. (1964) Brain fatty acids and temperature acclimation. Comparative Biochemistry and Physiology 11: 303-309.

JOLY, M. (1965) A physico-chemical approach to the denaturation of proteins. Publ. Academic Press.

JORGENSEN, P. L. (1974a) Purification of (Na^+-K^+) ATPase active site determinations and criteria of purity. Annals of the N.Y. Academy of Sciences 242: 36-52.

JORGENSEN, P. L. (1974b) Purification and characterisation of the (Na^+-K^+) ATPase III. Biochimica et Biophysica Acta. 356: 36-52.

JORGENSEN, P. L. (1974c) Isolation of the (Na^+-K^+) ATPase. Methods in Enzymology XXXII. 277-290. Publ. Academic Press.

JORGENSEN, P. L. (1975) Purification and characterisation of (Na^+-K^+) ATPase. V. Conformational changes in the enzyme. Transitions between the Na-form and the K-form studied with tryptic digestion as a tool. Biochimica et Biophysica Acta 401: 399-415.

JORGENSEN, P. L. (1977) Purification and characterisation of (Na^+-K^+) ATPase. VI. Differential tryptic modification of catalytic functions of the purified enzyme in presence of NaCl and KCl. Biochimica et Biophysica Acta 466: 97-108.

JORGENSEN, P. L. & KLODOS, I. (1978) Purification and characterisation of (Na^+-K^+) ATPase. VII. Tryptic degradation of the Na^+-form of the enzyme resulting in selective modification of dephosphorylation reactions of the Na^+-K^+-ATPase. Biochimica et Biophysica Acta 507: 8-16.

JORGENSEN, P. L. & SKOU, J. C. (1971) Purification and characterisation of the (Na^{+}-K^{+}) ATPase I. Biochimica et Biophysica Acta 233: 366-380.

JOSEPHSON, L. & CANTLEY, L. C. (1977) Isolation of a potent (Na-K) ATPase inhibitor from striated muscle. Biochemistry 16: 4572-4578.

JUDAH, J. D., AHMED, K. & McLEAN, A. E. M. (1962) Ion transport and phosphoproteins of human red cell. Biochimica et Biophysica Acta 65: 472-480.

KAVANAU, J. L. (1950) Enzyme kinetics and the rate of biological processes. J. General Physiology 34: 193-209.

KAWAI, K., NAKAO, M., NAKAO, T. & FUJIATA, M. (1973) Purification and some properties of Na-K-Transport ATPase. J. Biochemistry 73: 979-991.

KEMP, P. & SMITH, M. W. (1970) Effect of temperature acclimation on the fatty acid composition of goldfish intestinal lipids. Biochemical J. 117: 9-15.

KIHARA, H. K. & KONO, H. (1968) Micro-assay of protein with micro-cellulose membrane filters. Analytical Biochemistry 24: 96-105.

KING, T. P. & SPENCER, M. (1970) Structural studies and organic ligand-binding properties of bovine plasma albumine. J. Biological Chemistry 245: 6134-6148.

KISTIAKOWSKY, G. B. & LUMRY, R. (1949) Anomalous temperature effects in the hydrolysis of urea by urease. J. American Chemical Society 71: 2006-2013.

KLINE, M. H., HEXUM, T. D., DAHL, J. L. & HOKIN, L. E. (1971) Studies on the characterisation of the sodium-potassium transport adenosine triphosphatase. Archives of Biochemistry and Biophysics 142: 781-787.

KRISHENBAUM, D. M. (1971) A compilation of amino acid analyses of proteins I. Analytical Biochemistry 44: 159-173.

KRISHENBAUM, D. M. (1972) A compilation of amino acid analyses of proteins II. Analytical Biochemistry 49: 248-266.

KRISHENBAUM, D. M. (1973) A compilation of amino acid analyses of proteins III. Analytical Biochemistry 52: 234-254.

KRISCHUMANN, C., LEVEY, I. & DE VRIES, A. (1973) Stabilisation by cations of microsomal ATPase against heat inactivation. Biochimica et Biophysica Acta 330: 167-172.

KUMAMOTO, J., RAISON, J. K. & LYONS, J. M. (1971) Temperature "breaks" in Arrhenius Plots: A thermodynamic consequence of phase change. J. Theoretical Biol. 31: 47-51.

KURIKI, Y. & RACKER, E. (1976) Inhibition of (Na^+-K^+) Adenosine triphosphatase and its partial reactions by quercetin. Biochemistry 15: 4951-4956.

KURIKI, Y., HALSEY, J., BILTONEN, R. & RACKER, E. (1977) Calorimetric studies of the interaction of magnesium and phosphate with (Na^{+}-K^{+}) ATPase: Evidence for a ligand induced conformational change. Biochemistry 15: 4956-4961.

KYTE, J. (1971a) Phosphorylation of a purified (Na^{+}-K^{+}) adenosine triphosphatase. Biochemical and Biophysical Research Communications 43: 1259-1265.

KYTE, J. (1971b) Purification of the sodium and potassium dependent adenosine triphosphatase from canine renal medulla. J. Biological Chemistry 246: 4157-4165.

KYTE, J. (1972) Properties of the true polypeptides of sodium and potassium dependent adenosine triphosphatase. J. Biological Chemistry 247: 7642-7649.

KYTE, J. (1974) The reactions of sodium and potassium ion activated adenosine triphosphatase with specific antibodies. Implications for the mechanism of active transport. J. Biological Chemistry 249: 3652-3660.

KYTE, J. (1975) Structural studies of sodium and potassium ion activated adenosine triphosphatase. J. Biological Chemistry 250: 7443-7449.

LADBROOKE, B.D., CHAPMAN, D. (1969) Thermal analysis of lipids, proteins and biological membranes. A review and summary of some recent studies. Chem. Phys. Lipids 3: 304-356.

LANE, L. K., COPENHAVER, J. H., LINDENMAYER, G. E. & SCHWARTZ, A. (1973) Purification and characterisation of and (^3H) binding to the transport adenosine triphosphatase from outer medulla of canine kidney. J. Biological Chemistry 248: 7197-7200.

LAUTER, C. J. & TRAMS, E. G. (1962) A spectrophotometric determination of sphingosine. J. Lipid Research 3: 136-138.

LAYNE, E. (1957) Spectrophotometric and turbidimetric methods for measuring proteins. Methods in Enzymology III. 447-454. publ. Academic Press.

LELIEVRE, L., CHARLEMAGNE, D. & PARAF, A. (1976a) Plasma membrane studies on drug sensitive and resistant cell lines. II. Ouabain sensitivity of (Na^+-K^+) stimulated Mg^{2+}-ATPase. Biochimica et Biophysica Acta 455: 277-286.

LELIEVRE, L., CHARLEMAGNE, D. & PARAF, A. (1976b) Role de constituents membranaines distincts de l'enzyme dans la sensibilite' de l'ATPase Na^+/K^+ a l'ouabaine. C.R. Acad. Sci. Paris 283: 539-542.

LEVEY, H. M., SHARON, N. & KOSHLAND, D. E. (1959) Purified muscle proteins and the walking rate of ants. Proc. National Acad. Sci. U.S.A. 45: 785-791.

LEWIS, W. E. (1974) Purification and molecular properties of a water soluble transport ATPase. Ph.D. thesis, University of Manchester.

LIANG, S. & WINTER, C. G. (1977) Digitonin-induced changes in subunit arrangement in relation to some in vitro activities of $(Na^{+}-K^{+})$ ATPase. J. Biological Chemistry 252: 8278-8284.

LINDENMAYER, G. E., SCHWARTZ, A. & THOMPSON, H. K. (1974) A kinetic description for sodium and potassium effects on $(Na^{+}-K^{+})$ adenosine triphosphatase. J. Physiology 236: 1-28.

LO, T. N. & TITUS, E. O. (1978) Effects of ligands on conformationally dependent trypsinolysis of (Sodium plus potassium) activated adenosine triphosphatase. J. Biological Chemistry 253: 4432-4438.

LOWE, A. G. & SMART, J. W. (1977) The presteady state hydrolysis of ATP by porcine brain $(Na^{+}-K^{+})$ dependent ATPase. Biochimica et Biophysica Acta 481: 695-705.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biological Chemistry 193: 265-275.

LYONS, J. M., RAISON, J. K. (1970a) A temperature-induced transition in mitochondial oxidation: contrasts between cold and warm-blooded animals. Comp. Biochem. Physiol. 37: 405-411.

LYONS, J. M. & RAISON, J. K. (1970b) Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiology 85: 386-389.

MAHLER, H. R. & CORDES, E. H. (1966) Biological Chemistry. Publ. Harper & Row.

MAIER, V.P., TAPPEL, A.L. & VOLMAN, D.H. (1955) Reversible inactivation of enzymes at low temperatures. Studies of the temperature dependence of phosphatase and peroxidase catalysed reactions. J. American Chemical Soc. 77, 1278-1280.

MANDERSLOOT, J.G., ROELOFSEN, B. & DE GIER, J. (1978) Phosphatidyl inisitol as the endogenous activator of the $(Na^{+}-K^{+})$-ATPase in microsomes of rabbit kidney. Biochimica et Biophysica Acta 508: 478-485.

MARDH, S. (1975a) Bovine Brain Na^{+}, K^{+}-stimulated ATP phosphohydrolase studied by a rapid mixing technique: K^{+} stimulated liberation of ^{32}P phosphoenzyme and resolution of the dephosphorylation into two phases. Biochimica et Biophysica Acta 391: 448-463.

MARDH, S. (1975b) Bovine Brain Na^{+}, K^{+}-stimulated ATP phosphohydrolase studied by a rapid mixing technique: Detection of a transient ^{32}P phosphoenzyme formed in the presence of potassium ions. Biochimica et Biophysica Acta 391: 464-473.

MARDH, S. & LINDAHL, S. (1977) On the mechanism of sodium-and potassium-activated Adenosine triphosphatase Time course of intermediary steps examined by computer simulation of transient kinetics. J. Biological Chemistry 253: 8058-8061.

MARDH, S. & ZETTERQVIST, O. (1974) Phosphorylation and dephosphorylation reactions of bovine brain $(Na^{+}-K^{+})$ stimulated ATP phosphohydrolase studied by a rapid mixing technique. Biochimica et Biophysica Acta 350: 473-483.

MARSHALL, M.O. (1976) Studies on the glycoprotein component of $(Na^{+}-K^{+})$ATPase from dogfish salt gland: Binding of Concanavalin A and removal of sialic acid by neuramimidase. Biochimica et Biophysica Acta 455: 837-848.

MASSEY, V., CURTI, B. & GANTHER, H. (1966) A temperature dependent conformational change in D-amino acid Oxidase and its effects on Catalysis. J. Biological Chemistry 241: 2347-57.

McGRATH, R. (1972) Protein measurement by Ninhydrin determination of amino acids released by alkaline hydrolysis. Analytical Biochemistry 49: 95-102.

MICHAEL, L., WALLICK, E.J. & SCHWARTZ, A. (1977) Modification of the Na^{+}-K^{+}-ATPase function by purified antibodies to the Holoenzyme. J. Biological Chemistry 252: 8476-8480.

NAGLE, J.F. (1973) Theory of Biomembrane phase transitions. J. Chemical Physics 58: 252-264.

NAKAO, M., NAKAO, T., HARA, Y., NAGAI, F., YAGASAKI, S., KOI, M., NAKAGAWA, A & KAWAI, K. (1974) Purification and properties of Na^{+}-K^{+}-ATPase from pig brain. Annals of the New York Academy of Sciences 242: 24-35.

NAKAO, T., NAKAO, M & NAGAI, F. (1973) Microdetermination of protein not affected by the presence of various buffers, sucrose, ATP, and eluates from poly saccharide derivatives. Analytical Biochemistry 55: 358-367.

NAKAO, T., NAKAO, M., MIZUMO, N., KOMATSU, Y. & FUJIATA, M. (1973) Purification and some properties of Na^{+}-K- transport ATPase. J. Biochemistry (Tokyo) 73: 609-619.

NEUFELD, A.H. & LEVEY, H.V. (1970) The steady rate level of Phosphorylated intermediate in relation to the two sodium dependent adenosine Triphosphatase of calf brain microsomes. J. Biological Chemistry 245: 4962-4970.

NISHIGAKI, F., CHEN, T. & HOKIN, L.E. (1974) Studies on the characterisation of the Na^{+}-K^{+}-transport ATPase: XV Direct chemical characterisation of the acyl phosphate in the enzyme as an aspartyl-β-phosphate residue. J. Biological Chemistry. 249: 4911-4916.

NOGUCHI, T. & FREED, S. (1971) Dissociation of lipid components and reconstitution at -75°C of Mg^{2+} dependent, Na^{+} and K^{+} stimulated, adenosine triphosphatase in Rat Brain. Nature New Biology 230: 148-150.

NOZAWA, Y., IIDA, H., FUKUSHIMA, H., OHKI, K. & OHNISHI, S. (1974) Studies on Tetrahymena membranes. Biochimica et Biophysica Acta 367: 134-147.

OPIT, L.J. & CHARNOCK, J.S. (1965) A molecular model for a sodium pump. Nature 203: 471-474.

PAGANO, R.E., CHERRY, R.J. & CHAPMAN, D. (1973) Phase transitions and heterogeneity in lipid bilayers. Science 181: 557-558.

PAGE, E., GOERKE, R.J., STORM, S.R. (1964) Cat heart muscle in Vitro IV. Inhibition of transport in Quiescent Muscles. J. Gen. Physiol. 47: 531-543.

PALATINI, P., DABBENI-SALA, F., PITOTTI, A., BRUNI, A. & MANDERSLOOT, J.C. (1977) Activation of (Na^{+}+K^{+})dependent ATPase by lipid vesicles of negative phospholipids. Biochimica et Biophysica Acta 466: 1-9.

PECKHAM, G. (1970). A new method for minimising a sum of squares without calculating gradients. Computer J. 13: 418-420.

PATZELI-WENCZLER, R., PAULS, H., ERDMANN, E. & SCHONER, W. (1975) Evidences for a sulfhydryl group in the ATP binding site of ($Na^{+}+K^{+}$) activated ATPase. European Journal of Biochemistry 53: 301-311.

PERRONE, J.R., HACKNEY, J.F., DIXON, J.F. & HOKIN, L.E. (1975) Molecular properties of purified (Sodium + potassium) activated Adenosine Triphosphatases and their subunits from the rectal gland of Squalus Acanthias and the electric organ of electrophorus J. Biological Chemistry 250: 4178-4184.

PETER, H.W. & WOLF, H.U. (1972) Kinetics of (Na^{+}, K^{+})-ATPase of human erythrocytes. I. Activation by Na^{+} and K^{+}. Biochimica et Biophysica Acta 290: 300-309.

PITTS, B.J.R. (1974). The relationship of the K^{+}-activated phosphatase to the Na^{+}, K^{+}-ATPase. Annals of the N.Y. Acad. of Sciences 242: 293-304.

PLATNER, W.S., PATNAYAK, B.C. & MUSACCHIA, X.J. (1972) Seasonal changes in the fatty acid spectrum in the hibernating and non hibernating ground squirrel Citellus Tridecemlineatus. Comparative Biochemistry & Physiology 42A: 927-938.

POST, R.L. & KUME, S. (1973) Evidence for an aspartyl phosphate residue at the active site of sodium and potassium ion transport adenosine triphosphatase. J. Biological Chemistry 248: 6993-7000.

POST, R.L., HEGYVARY, C. & KUME, S. (1972) Activation by Adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine Triphosphatase. J. Biological Chemistry 247: 6530-6540.

POST, R.L., SEN, A.K. & ROSENTHAL, A.S. (1965) A phosphorylated intermediate in the ATP dependent sodium and potassium transport across kidney membranes. J. Biological Chemistry 240: 1437-1445.

POST, R.L., TODA, G. & ROGERS, F.N. (1975) Phosphorylation by inorganic phosphate of sodium plus potassium ion transport Adenosine triphosphatase. J. Biological Chemistry 250: 691-701.

POST, R.L., MERRIT, C.R., KINSOLVING, C.R. & ALBRIGHT, C.D. (1960). Membrane adenosine triphosphatases as a participant in the active transport of sodium and potassium in the human erythrocyte. J. Biological Chemistry 235: 1796.

POUCHAN, M.I., GARRAHAN, P.J. & REGA, A.F. (1969) Effects of ATP and Ca^{2+} on a K^{+}-activated phosphatase from red blood cell membranes. Biochimica et Biophysica Acta 173: 151-154.

QUIST, E.E. & HOKIN, L.E. (1978) The presence of two (Na^{+}+K^{+}) ATPase inhibitors in equine muscle ATP. Vanadate and a dithioerythritol dependent inhibitor. Biochimica et Biophysica Acta 511: 202-212.

RACUSEN, D. (1973) Stoichiometry of the Amido black reaction with proteins. Analytical Biochemistry 52: 96-101.

RAHEJA, R.K., KAUR, C., SINGH, A. & BHATIA, I.S. (1973). New colorimetric method for the quantitative estimation of phospholipids without acid digestion. J. Lipid Research 14: 695-697.

RATANABANANGKOON, K., DIXON, J.F., & HOKIN, L.E. (1973) Studies on the characterisation of the sodium-potassium transport adenosine triphosphatase. Archives of Biochemistry & Biophysics 156: 342-349.

REGA, A.F. & GARRAHAN, P.J. (1976) Potassium activated phosphatase. The enzymes of Biological Membranes III Ch. 12: 303-314. Ed. Martonosi, A. Plenum Press.

REGA, A.F., GARRAHAN, P.J. & POUCHAN, M.I. (1970). Potassium activated phosphatase from human red blood cells: The assymetrical effects of K^+, Na^+, Mg^{2+} and Adenosine Triphosphate. J. Membrane Biology 3: 14-20.

ROBB, R., HAMMOND, R. & BIEBER, I.L. (1972) Temperature dependent changes in sphingosine composition and composition of fatty acids of glycrophosphatides from musea domestica. Insect Biochemistry 2: 131-136.

RIENERT, J.C. & STEIN, J.M. (1970) Calorimetric detection of membrane phase transitions in Living Cells. Science 168: 1580-1582.

ROBBINS, A.R., & BAKER, R.M. (1977) (Na,K) ATPase activity in membrane preparations of ouabain resistant Hela cells. Biochemistry 16: 5163-5168.

ROBINSON, J.D. (1967) Kinetic studies on a brain microsomal adenosine triphosphatase. Evidence suggesting conformational changes. Biochemistry 6: 3250-3258.

ROBINSON, J.D. (1969) Kinetic studies on a brain microsomal ATPase II K. dependent phosphatase. Biochemistry 8: 3348-3355.

ROBINSON, J.D. (1970) Phosphatase activity stimulated by Na^+ plus K^+: Implications for the (Na^+ plus K^+)- dependent Adenosine Triphosphatase. Archives of Biochemistry & Biophysics 139: 169-171.

ROBINSON, J.D. (1972) Divalent cations as Allosteric modifiers of the (Na^++K^+)-dependent ATPase. Biochimica et Biophysica Acta 266: 97-102.

ROBINSON, J.D. (1973) Cation sites on the (Na^+-K^+) dependent ATPase: Mechanisms for Na^+ induced changes in K^+ affinity and the phosphatase activity. Biochimica et Biophysica Acta 321: 662-670.

ROBINSON, J.D. (1974a). Nucleotide and divalent cation interactions with (Na^++K^+)-dependent ATPase. Biochimica et Biophysica Acta 341: 232-247.

ROBINSON, J.D. (1974b). Cation interactions with different functional states of the Na^+ K^+-ATPase. Annals of the N.Y. Acad. of Sci. 242, 185.

ROBINSON, J.D. (1975) Functionally distinct classes of K^+ sites on the (Na^++K^+)-dependent ATPase. Biochimica et Biophysica Acta 384: 250-264.

ROBINSON, J.D., FLASHNER, M.S. & MAREN, G.J. (1978) Inhibition of the (Na^++K^+)-dependent ATPases by inorganic phosphate. Biochimica et Biophysica Acta 509: 419-428.

RODNIGHT, R., HEMS, D.A. & LAVIN, B.E. (1966) Phosphate binding by cerebral microsomes in relation to adenosine triphosphatase activity. Biochemical J. 101: 502-515.

ROOTS, B.I. (1968) Phospholipids of the goldfish (Carassius Auratus L) brain: The influence of environmental temperature. Comparative Biochemistry & Physiology 25: 457-466.

ROSEN, H. (1957) A modified ninhydrin calorimetric analysis for amino acids. Archives of Biochemistry & Biophysics 67: 10-15.

ROTHMAN, J.F. (1973) The molecular bias of mesomorphic phase transitions in phospholipid systems. J. Theoretical Biol. 38; 1-16.

ROUSER, G., KRITCHEVSKY, G. & YAMAMOTO, A. (1967) Lipid chromatographic analysis 2, Ed. Marinetti, G.V. Decker, New York.

RU$_0$HO, A. & KYTE, J. (1974) Photoaffinity labelling of the Ouabain binding site on (Na^+-K^+) Adenosine triphosphatase. Proc. Nat. Acad. Sciences, U.S.A. 71: 2352-2356.

RUSSELL, J.C. & PEACH, D.M. (1974) The temperature dependence of (Na^++K^+)-ATPase. Physiol. Chem. & Physics 6: 225-238.

SACHS, J.R. (1974) Kinetics of the Na^+-K^+-Pump in goat red blood cells and the effect of an antibody. Annals of the N.Y. Acad. of Sciences 242: 343-359.

SANDERMANN, H. (1978) Regulation of membrane enzymes by lipids. Biochimica et Biophysica Acta 515: 209-237.

SCHATZMANN, H.J. (1953) Herzglycoside als hemmstoffe fur den aktiven kallium and natrium transport durch die erythrocytenmembran. Helvatia, Physiologica Pharmacologica Acta 11: 346-354.

SCHATZMANN, H.J. (1962) Lipoprotein nature of red cell adenosine triphosphatase. Nature 196: 677.

SCHOOT, B.M., DePONT, J.J.H.H.M., & BONTING, S.L. (1978) Studies on (Na^++K^+) activated ATPase. XLII. Evidence for two classes of sulfhydryl groups. Biochimica et Biophysica Acta. 522: 602-613.

SCHOOT, B.M., SCHOOTS, A.F.M., DePONT, J.J.H.H.M., SCHUURMANS-STEKHOVEN, F.M.A.H., & BONTING, S.L. (1977) Studies on (Na^++K^+) activated ATPase XLI. Effects of N-ethylmaleimide on overall and partial reactions. Biochimica et Biophysica Acta 483: 181-192.

SCHWARTZ, A., LINDENMAYER, G.E. & ALLEN, J.C. (1975) The sodium potassium adenosine triphosphatase: Pharmalogical Physiological and Biochemical Aspects. Pharmacological Reviews 27: 3.134.

SEN, A.K., & POST, R.L. (1964) Stoichiometry and localisation of adenosine triphosphate dependent sodium and potassium transport in the erythrocyte. J. Biological Chemistry 239: 345-352.

SJODIN, R.A. & BEAUGE, L.A. (1968) The kinetics of sodium extrusion in striated muscle as functions of external sodium and potassium ion concentrations. J. General Physiology 52: 389-407.

SHAMOO, A.E. (1974) Isolation of a Sodium-dependent-ionophore from $(Na^{+}+K^{+})$-ATPase preparations. Annals of the New York Academy of Sciences 242: 389-405.

SHAMOO, A.E. & MEYERS, M. (1974) Na^{+}-dependent ionophore as part of the small polypeptide of the $(Na^{+}+K^{+})$-ATPase from eel electroplax membrane. J. Membrane Biology 19: 163-178.

SILVIUS, J.R., READ, B.D. & McELHANEY, R.N. (1978) Membrane enzymes: Artefacts in Arrhenius plots due to Temperature dependence of Substrate binding affinity. Science 199: 902-904.

SINGER, S.J. & NICHOLSON, G.L. (1972) The fluid mosaic model of the strcture of cell membranes. Science 175: 720-731.

SKOU, J.C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochimica et Biophysica Acta 23: 394-401.

SKOU, J.C. (1960) Further investigations on a $(Mg^{++}+Na^{+})$-activated adenosine triphosphatase possibly related to the active linked transport of Na^{+} and K^{+} across the nerve membrane. Biochimica et Biophysica Acta 42: 6.

SKOU, J.C. (1965) Enzymatic Basis for Active transport of Na^{+} and K^{+} across cell membrane. Physiological Review 45: 596-617.

SKOU, J.C. (1975) The $(Na^{+}+K^{+})$ activated enzyme system and its relationship to transport of Sodium and Potassium. Quarterly Reviews of Biophysics 7: 401-434.

SKOU, J.C., BUTLER, K.W. & HANSEN, O. (1971) The effect of magnesium, ATP, Pi and sodium ion on the inhibition of the Na^{+}-K^{+}-ATPase by g-strophanium. Biochimica et Biophysica Acta 241: 443-461.

SMITH, M.W. (1967) Influence of temperature Acclimation on the temperature-dependence and Ouabain sensitivity of goldfish intestinal adenosine triphosphatase. Biochem. J. 105: 65-7.

SMITH, M.W., COLOMBO, V.E. & MUNN, E.A. (1967) Influence of temperature acclimation on the ionic activation of goldfish intestinal Adenosine Triphosphatase. Biochem. J. 107: 691-698.

SOLOMONSON, L.P., LIEPKALNS, V.A. & SPECTOR, A.A. (1976) Changes in (Na^{+}+K^{+})-ATPase activity of Ehrlich Ascites Tumour cells produced by alterations of membrane fatty acid composition. Biochemistry 15: 892-897.

STAUFFER, C.E. (1975) A Linear standard error for the Folin Lowry determination of protein. Analytical Biochemistry 69: 646-648.

STEIN, W.D., LIEB, W.R., KARLISH, S.J.D., EILAM, Y. (1973) A model for active transport of Sodium and potassium ions as mediated by a tetrameric enzyme. Proceedings of the National Academy of Sciences, U.S.A. 70: 275-278.

STRICKLAND, R.D., FREEMAN, M.L. & GURUL, F, T. (1961) Copper Binding by proteins in alkaline solution. Analytical Chemistry 33: 545.

SUN, A.Y. (1971) The effect of lipoxidation on synaptosomal (Na^{+}+K^{+})-ATPase isolated from the cerebral cortex of squirrel monkey. Biochimica et Biophysica Acta 266: 350-360.

SUN, A.Y. & SAMORAJSKI, T. (1970) Effects of ethanol on the activity of Adenosine triphosphatase and acetyl cholinesterase in synaptosomes isolated from guinea pig brain. J. Neurochemistry 17: 1365-1372.

SUN, A.Y., SUN, G.Y. & SAMORAJSKI, T. (1971) The effect of phospholipase C on the activity of Adenosine triphosphatase and acetyl cholinesterase in synaptic membranes isolated from the cerebral cortex of the squirrel monkey. J. Neurochemistry 18: 1711-1718.

SWANN, A.C., DANIEL, A., ALBERS, R.W. & KOVAL, G.J. (1975) Interactions of Lectins with (Na^+-K^+)ATPase of eel electric organ. Biochimica et Biophysica Acta 401: 299-306.

SWEADNER, K.J. (1977) Cross linking and modification of Na-K-ATPase by ethyl acterimidate. Biochemical & Biophysical Research Communication 78: 962-969.

SWEADNER, K.J. (1978) Purification from brain of an intrinsic membrane protein fraction enriched in (Na^++K^+)-ATPase. Biochimica et Biophysica 508: 486-499.

TALEKAR, S.V. (1975) The nature of metal-ligand bonding in the primary hydration shell and in ionophores with alkali metal ions. Biochimica et Biophysica Acta 375: 157-164.

TANIGUCHI, K. & IIDA, S (1972a) Two apparently different binding sites of Na^+, K^+-ATPase. Biochimica et Biophysica Acta 288: 98-102.

TANIGUCHI, K. & IIDA, S. (1972b) The effect of phospholipids on the apparent activation energy of Na^+-K^+-ATPase. Biochimica et Biophysica Acta 274: 536-541.

TANAKA, R. & STRIKLAND, K.P. (1965) Role of phospholipid in the activation of the Na^+-K^+-ATPase of Beef brain. Archives of Biochemistry & Biophysics 111: 583-592.

TANAKA, R. & TERUYA, A. (1973) Lipid dependence of activity temperature relationship of (Na^+, K^+)-activated ATPase. Biochimica et Biophysica Acta 323: 584-591.

TANIGUCHI, K. & TONOMURA, Y. (1971) Inactivation of Na^{+}-K^{+}-dependent ATPase by phospholipase treatment and its reactivation by phospholipids. J. Biochemistry 69: 543-557.

TASHIMA, Y. & HASEGAWA, M. (1975) The mode of activation by potassium of potassium-dependent and ouabain sensitive phosphatase activity. Archives of Biochemistry & Biophysics. 171: 568-574.

TOBIN, T. & BRODY, T.M. (1972) Rates of dissociation of enzyme-ouabain complexes and $K_{0.5}$ values in (Na^{+}+K^{+}) Adenosine triphosphatase from different species. Biochemical Pharmacology 21: 1553-1560.

TOBIN, T., BANERJEE, S.P. & SEN, A.K. (1970) Allosteric interactions in (Na^{+}+K^{+})-ATPase. Nature 225: 745-746.

TOSTESON, D.C. (1962) Active cation transport, ATPase and phosphomonoesterase. Proceedings of the XXII Int. Congr. Physiol. Sciences 2: 615.

TRAUBLE, H. (1971) Phase transformations in lipids: Possible switching processes in biological membranes. Die Naturwissenschaften 58: 277-284.

UESEGI, S., DULAK, N.C., DIXON, J.F., HEXUM, T.D., DAHL, J.L., PERDUE, J.F. & HOKIN, L.E. (1971) Studies in the characterisation of the Sodium-potassium transport Adenosine triphosphatase. J. Biological Chemistry 246: 531-543.

UNEMOTO, T., SAKAKIBARA, Y. & HYASHI, M. (1975) On the specificity of the Folin and Lowry reagents for amino derivatives. Analytical Biochemistry 67: 198-205.

VAN WINKLE, W.B., LANE, L.K., & SCHWARTZ, A. (1976) The subunits fine structure of isolated purified Na^{+}-K^{+}-Adenosine triphosphatase. Experimental Cell Research 100: 291-296.

VEATCH, W.R. & STRYER, L. (1977) Effect of cholesterol on the rotational mobility of diphenylhexatriene in Liposomes: A nanosecond fluorescence anisotropy study. J. Molecular Biology 117: 1109-1113.

VEERKAMP, J.H. & BROEKHUYSE, R.M. (1976) Techniques in analysis of membrane lipids. In: Biochemical Analysis of membranes. Ed. Maddy, A.H. Chapman & Hall (London)

VOGEL, F., MEYER, H.W., GROSSE, R. & REPKE, K.R.H. (1977) Electron microscopic visualisation of the arrangement of the two protein components of (Na^{+}+K^{+})-ATPase. Biochimica et Biophysica Acta 470: 497-502.

WALLICK, E.T., & SCHWARTZ, A. (1974) Thermodynamics of the rate of binding of ouabain to the sodium, potassium, adenosine triphosphatase. J. Biological Chemistry 249: 5141-5147.

WATSON, D. (1960) A simple method for the determination of Serum Cholesterol. Clinica Chimica Acta Vol. 5: 637-643.

WEBER, K. & OSBORN, M. (1969) The reliability of molecular weight determinations by dodecyl sulphate polyacrylamide gel electrophoresis. J. Biological Chemistry 244: 4406-4412.

WHEELER, K.P. & WHITTAM, R. (1970) ATPase activity of the sodium pump needs phosphatidyl serine. Nature 225: 449-450.

WHITTHAM,R. (1962) The assymetrical stimulation of membrane adenosine triphosphatase in relation to active cation transport. Biochem. J. 84: 110-118.

WHITTHAM, R. & AGER, M.E. (1964) Vectorial aspects of adenosine triphosphatase in relation to active cation transport. Biochem. J. 93: 337-348.

WHITTHAM, R. & CHIPPERFIELD, A.R. (1975) The reaction mechanism of the sodium pump. Biochimica et Biophysica Acta 415: 149-171.

WHITTHAM, R. & DAVIES, R.E. (1953) Active transport of water, sodium potassium on α-oxoglistarate by kidney-cortex slices. Biochem. J. 55: 880-887.

WITTENBERG, J.B., KOREY, S.R. & SWENSON, F.H. (1956) The determination of higher fatty aldehydes in tissues. J. Biological Chemistry 219: 39-47.

WOLF, H.U. & PETER, H.W. (1972) Kinetics of $(Na^{+}-K^{+})$ATPase of human erythrocyte membranes. II.Inhibition by ouabain. Biochimica et Biophysica Acta 290: 310-320.

WREN, J.J. & SZCEPANOWSKA, D. (1963) Chromatography of lipids in the presence of an anti-oxidants,4 methyl, 2,6,di-tert-Butylphenol. J. Chromatography 14: 405-410.

WYNN -WILLIAMS, A.T. (1976) An explanation of apparent sudden change in the activation energy of membrane enzymes. Biochem. J. 157: 279-281.

YAGER, P. (1977) A novel mechanism for the $Na^{+}-K^{+}$-ATPase. J. Theoretical Biology 66: 1-11.

YELLIN, N. & LEVIN, I.W. (1977) Co-operative unit size in the gel-liquid crystalline phase transition of dipalmitoyl phosphatidylcholine-water multilayers: An estimate from Raman Spectroscopy. Biochimica et Biophysica Acta 468: 490-494.

YOSHIDA, H., IZUMI, E. & NAGAI, K. (1966) Carbamyl phosphate a preferential substitute of K^{+}, dependent phosphatase. Biochimica et Biophysica Acta 120: 183-186.

ZACHOWSKI, A., LeLIEVRE, L., AUBRY, J., CHARLEMAGNE, D. & PARAF, A. (1977) Role of proteins from inner face of plasmamembranes in susceptibility of $(Na^{+}+K^{+})$-stimulated Mg^{2+} adenosine triphosphatase to ouabain. Proceedings of the National Academy of Sciences, U.S.A. 74: 633-637.

APPENDIX 1

Variations in the sensitivity of proteins to protein assay procedures

INTRODUCTION

The estimation of protein is a routine standardising measurement made along the assay of protein based biological activities. Such measurements are rarely made on pure protein samples of known molecular weight and consequently, the standardisation of any given activity measurement is heavily dependent on the accuracy of the protein estimation. Most protein samples analysed are mixtures of diverse protein species which vary with respect to their molecular weight, amino acid composition, types of prostetic groups (if present), and structural organisation. This precludes any meaningful molar estimation of protein, and thus all protein assay procedures are geared to give an estimate of the total mass of protein in the assay sample. Such procedures usually involve the titration of the sample for a property fundamental to all proteins, followed by quantification by calibrating the titrated property against a known mass of purified protein.

The assay procedures commonly used are based on:

(a) Estimation of total nitrogen

(b) Estimation of amino acids

(c) Estimation of peptide linkages

(d) Estimation of basic groups

(e) Estimation of aromatic groups

In addition to these, turbidimetric and fluorimetric methods have also

been developed. However, such procedures depend on the physical state of the assay sample, and since there is no predictable stoichiometry associated with such assays, they will not be considered here. The properties commonly titrated in protein assays are mostly carried in the amino acid residues of the protein species in the sample. Given that protein species vary greatly in their relevant properties, all protein estimations must be statistical, and carry an error resulting from variations in the sensitivity of proteins to the assay procedures used. Thus the limiting errors in protein estimations are largely dependent on the nature of the assay procedure, and the choice of a suitable calibrating standard. Such errors will be small if the variation in the sensitivity of proteins to the assay procedure is small, in which case almost any purified protein would serve as an acceptable calibrating standard. However, if the variation is large, the choice of a calibrating standard will be difficult, especially in cases where the assay sample contains only a few protein species (e.g. the terminal stages of purification of a protein species). The design of an experimental procedure to circumvent these problems and to minimise such errors depends on knowing the extent of the variation in the sensitivity of proteins to the assay procedure used. This can be calculated from the amino-acid composition and molecular weight of the various proteins, however, this data, has only been recently available. Such data has been recently compiled (Krischenbaum, 1971, 1972, 1973), and this source is the input material for this study in which an evaluation of the sensitivity of proteins to the commonly used assay procedures, has been attempted.

METHODS

Source of data:

Krischenbaum (1971, 1972, 1973).

Calculation of sensitivity of a protein species to an assay procedure:

The sensitivity of a protein to a protein assay procedure was calculated in terms of m.moles of titrated group per gram of protein. This is given by equation

$$s = \sum \frac{n_i A_i}{M.W.} \times 10^3$$

A_i .. number of residues of the amino acid (or prosteric group) containing the titrated property

n_i .. number of groups of the titrated property per amino acid (or prosteric group)

M.W. . molecular weight of the protein

s .. sensitivity of the protein (m.moles per gram)

It was assumed that the reactive groups all contribute equally to the assay procedure and that the reported molecular weights included the associated prosthetic groups where present. The reactive groups titrated by the given assay procedures were taken to be:

(a) Total nitrogen assay (Kjeldahl procedure.... (Ballentine, 1957)
All amino acids and nitrogen containing prostetic groups.

(b) Total amino acid assay (Ninhydrin procedure (McGrath, 1972)):
All amino acids (except proline) and prostetic groups containing free or latent amino groups (the latent amino groups being those that may be liberated by hydrolysis).

(c) Total peptide bonds (Copper binding procedures ... i.e. Biuret procedure (Gornall, Bardawill & David, 1949) and Chloramine T procedure (Goldberg, 1973)):
All amino acids .... The stoichiometry of this procedure has been previously established (Strickland, Freeman & Gurule, 1961).

(d) Total basic groups (Procedures based on dye binding...
(Kiahara & Kuno, 1968; Nakao, Nakao & Nagai, 1973)).
Lysine, Arginine, Histidine, Glutamine, Asparagine and prosteric groups containing basic nitrogen .... the stoichiometry of this procedure has been previously established (Racusen, 1973).

(e) Total aromatic groups (Procedures based on the Folin-Ciocalteau phenol reagent (Lowry, Rosebrough, Farr & Randal, 1951), and the measurement of ultraviolet extinction. (Groves, Davis & Sells, 1968)):
Tyrosine and Tryptophan and prosteric groups containing these and or phenol groups (Note: Tyrosine and Tryptophan are not the only aromatic group containing amino acids in proteins. However, previous studies (Lowry et al, 1951; Groves et al, 1968) have shown these to be the only ones that contribute significantly when present in proportions normally found in proteins).

RESULTS

The calculations of the theoretical sensitivity to the various protein assay procedures were done for a sample of 350 proteins for which amino acid compositions have been reported (Krishenbaum, 1971, 1972, 1973). Figure AI.1 displays the results of the total nitrogen assay calculations as a frequency histogram. This shows that the distribution is not binomial since there was a 'tail' at the low sensitivity end. However, 86 per cent of the population were covered in the sensitivity range mean value (10.95 mMg^{-1} - see Table AI.1) $\pm$ 15 per cent of the mean. The apparent standard deviation (1.096 mMg^{-1}) was relatively large.

TABLE AI.1

Statistical Analysis of the Sensitivity of proteins to Protein Assay Procedures.

No. of proteins in sample - 350

Assay Procedure	Mean Sensitivity of sample. m.Moles g^{-1}	Std deviation m.Moles g^{-1}
Assay of total nitrogen	10.949	1.096
Assay of total amino acids (ninhydrin assay)	9.744	0.985
Assay of total peptide bonds	8.676	0.857
Assay of aromatic groups	0.419	0.193
Assay of basic groups	1.398	0.490

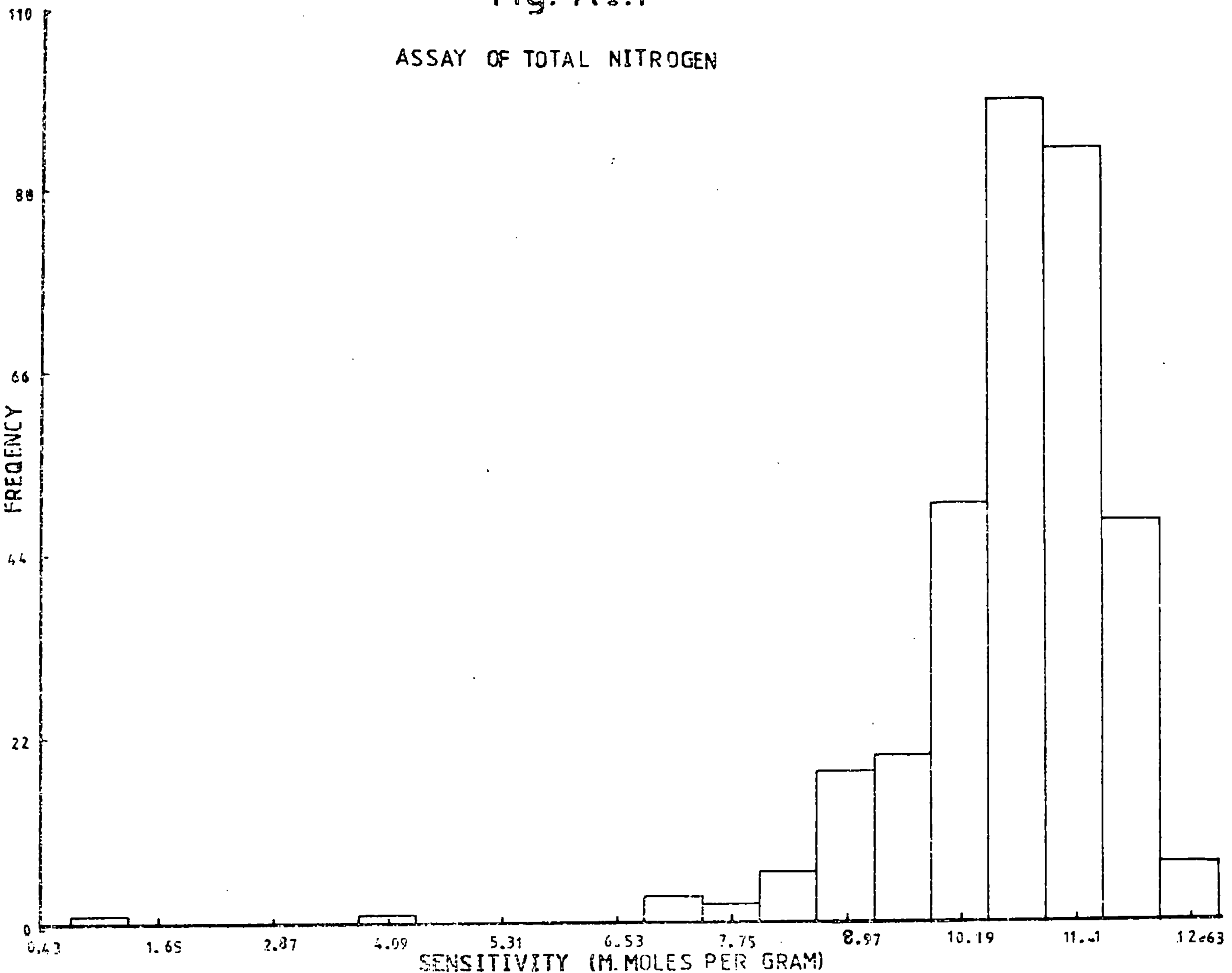


Fig. AI.1
ASSAY OF TOTAL NITROGEN
FREQENCY
110
88
66
44
22
0
0.43
1.65
2.87
4.09
5.31
6.53
7.75
8.97
10.19
11.41
12.63
SENSITIVITY (M.MOLES PER GRAM)

This undoubtedly reflected the presence of a 'tail' at the low sensitivity end. A closer inspection of the protein species forming the 'tail', revealed that they were antigenic glycoproteins with large complex prostetic groups which contained relatively little nitrogen.

The calculations of the sensitivity of the same sample of proteins to the amino acids and the total peptide bond assay procedures revealed a pattern similar to the total nitrogen assay. In both cases, the distribution (see figures AI.2, AI.3) was not quite binomial with a 'tail' towards the low sensitivity end, and in addition 88-90 percent of the sample was in the range mean value ($9.744 mMg^{-1}$ for amino acids and $8.676 mMg^{-1}$ for peptides (see Table AI.1 $\pm$ 15 percent of the mean. The apparent standard deviations calculated ($0.985 mMg^{-1}$ for amino acids and $0.887 mMg^{-1}$ for peptides) were larger than would be expected for a distribution in which 88-90% of the population was in the range mean value $\pm$ 15 percent. This undoubtedly reflected the presence of the 'tail' at the low sensitivity end which was attributable to the same proteins that produced the 'tail' in the distribution for the total nitrogen assay.

The results of the calculations for the basic groups and aromatic group assay procedures, showed a broader distribution of sensitivity than that calculated for the other procedures.
In the case of the aromatic group assay procedure, the frequency histogram (see figure AI.4) displayed a broad distribution with a tail towards the high sensitivity end. The range mean value ($0.419 mMg^{-1}$ - see Table AI.1)$\pm$ 15 percent of the mean covered only 48 percent of the total preparation. This along with the 'skew' in the distribution, was reflected in the relatively large apparent standard deviation ($0.193 mMg^{-}$

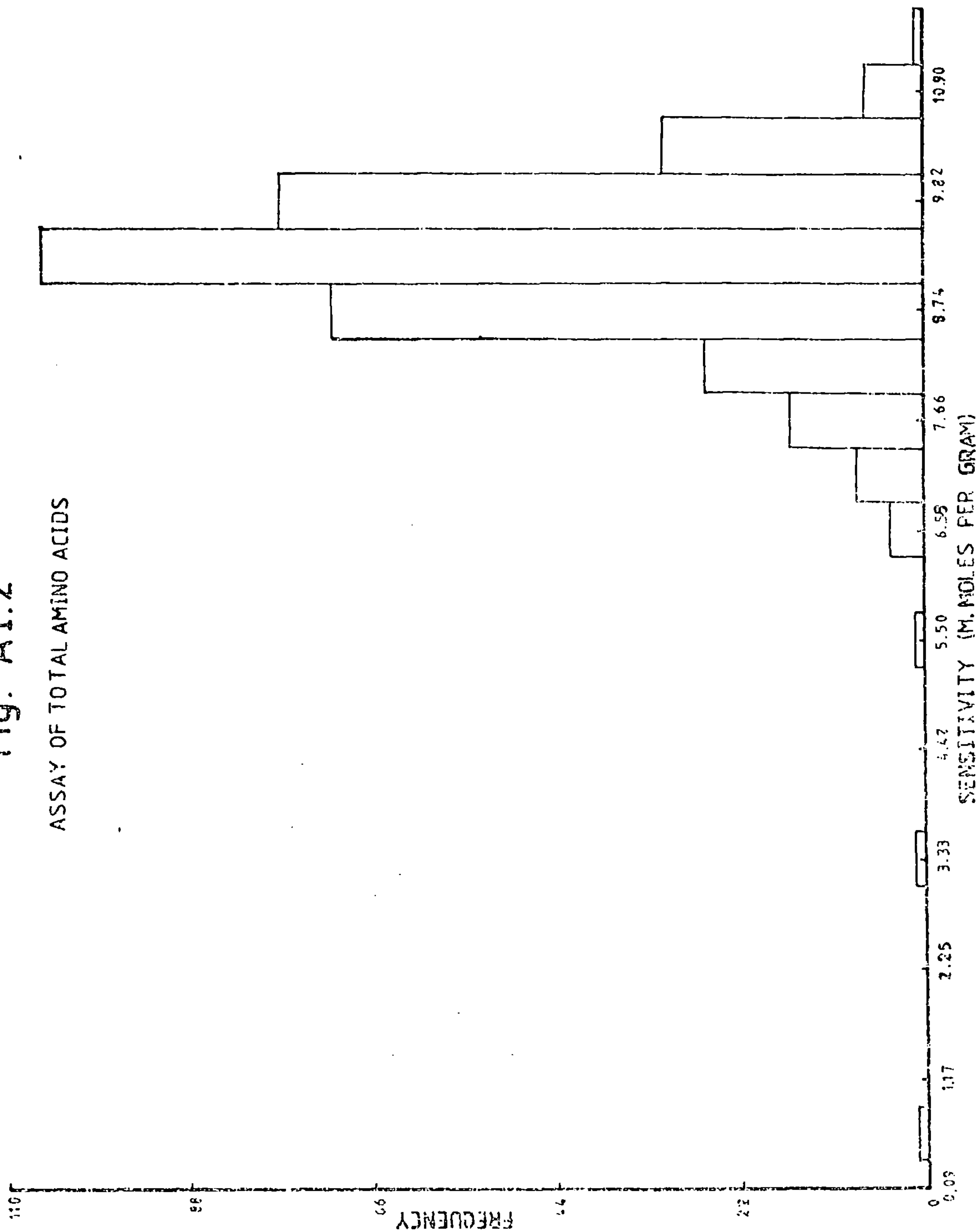


Fig. AI.2

ASSAY OF TOTAL AMINO ACIDS

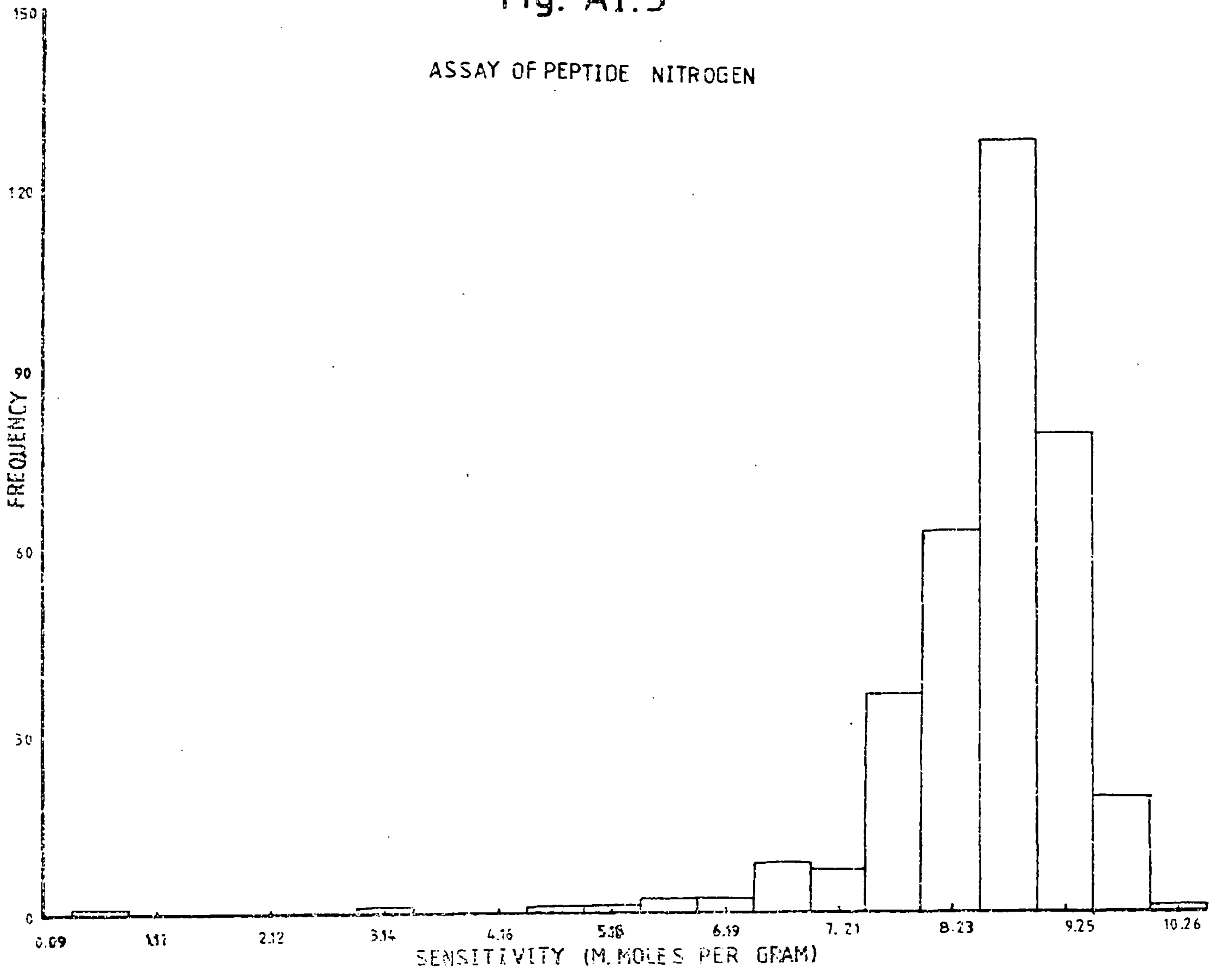


Fig. AI.3
ASSAY OF PEPTIDE NITROGEN
FREQUENCY
0
30
60
90
120
150
0.09
1.11
2.12
3.14
4.16
5.18
6.19
7.21
8.23
9.25
10.26
SENSITIVITY (M.MOLES PER GRAM)

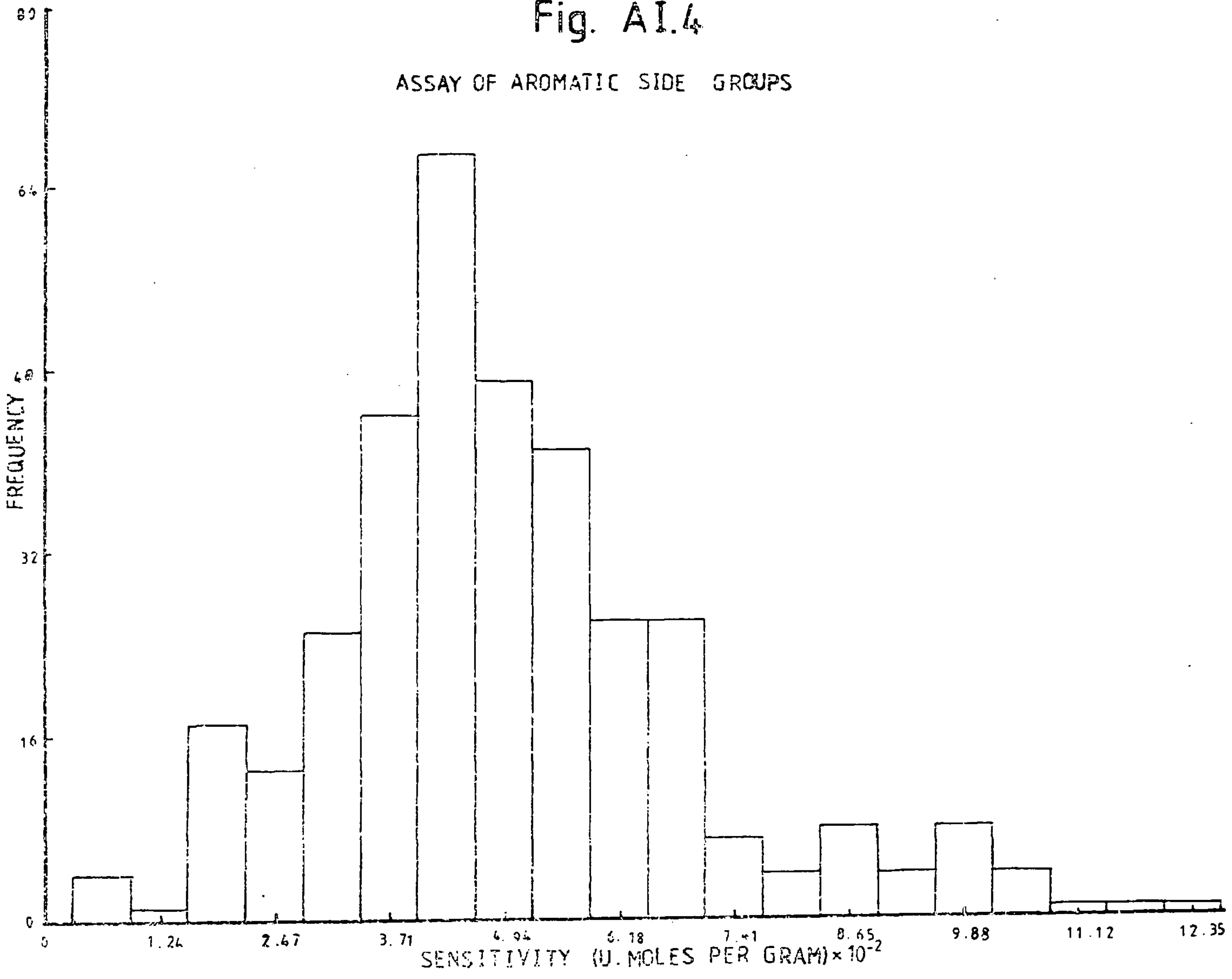


Fig. AI.4

ASSAY OF AROMATIC SIDE GROUPS

In the case of the basic group assay procedure, the frequency histogram (see figure AI.5) suggested that the data might be better interpreted as two populations, one centred near 1.2 $\pm$ 0.6mMg^{-1} and a much smaller population centred near 2.2 $\pm$ 0.3mMg^{-1}. The probable existence of two populations of proteins as regards their basicity has been suggested earlier (Mahler & Cordes, 1966), by the observation that the isoelectric points reported for protamines and histones were significantly higher than those reported for most other proteins. The distribution also showed significant 'tails' at the extremes of the sensitivity spectrum. The broadness of the overall distribution was reflected in the observation that only 42 percent of the overall population was covered by the sensitivity range mean value (1.398mMg^{-1}) $\pm$ 15 percent of the mean, and by the relatively large apparent standard deviation (0.49mMg^{-1} - see Table AI.1. In both cases (basic group assay and aromatic group assay), the antigenic glycoproteins which produced the 'low sensitivity tail' in the distribution described for the other three assay procedures, were not as distinct from the rest of the population as was the case with the three procedures previously mentioned.

DISCUSSION

The results of the above calculations suggested that the protein assay procedures based on the titration of total nitrogen, total amino acids, and peptide bonds, were equivalent as regards the error expected from the variations in the sensitivity of the proteins to the assay techniques. This was attributable to the fact that the properties assayed are generally the sums of contributions from all the amino acid

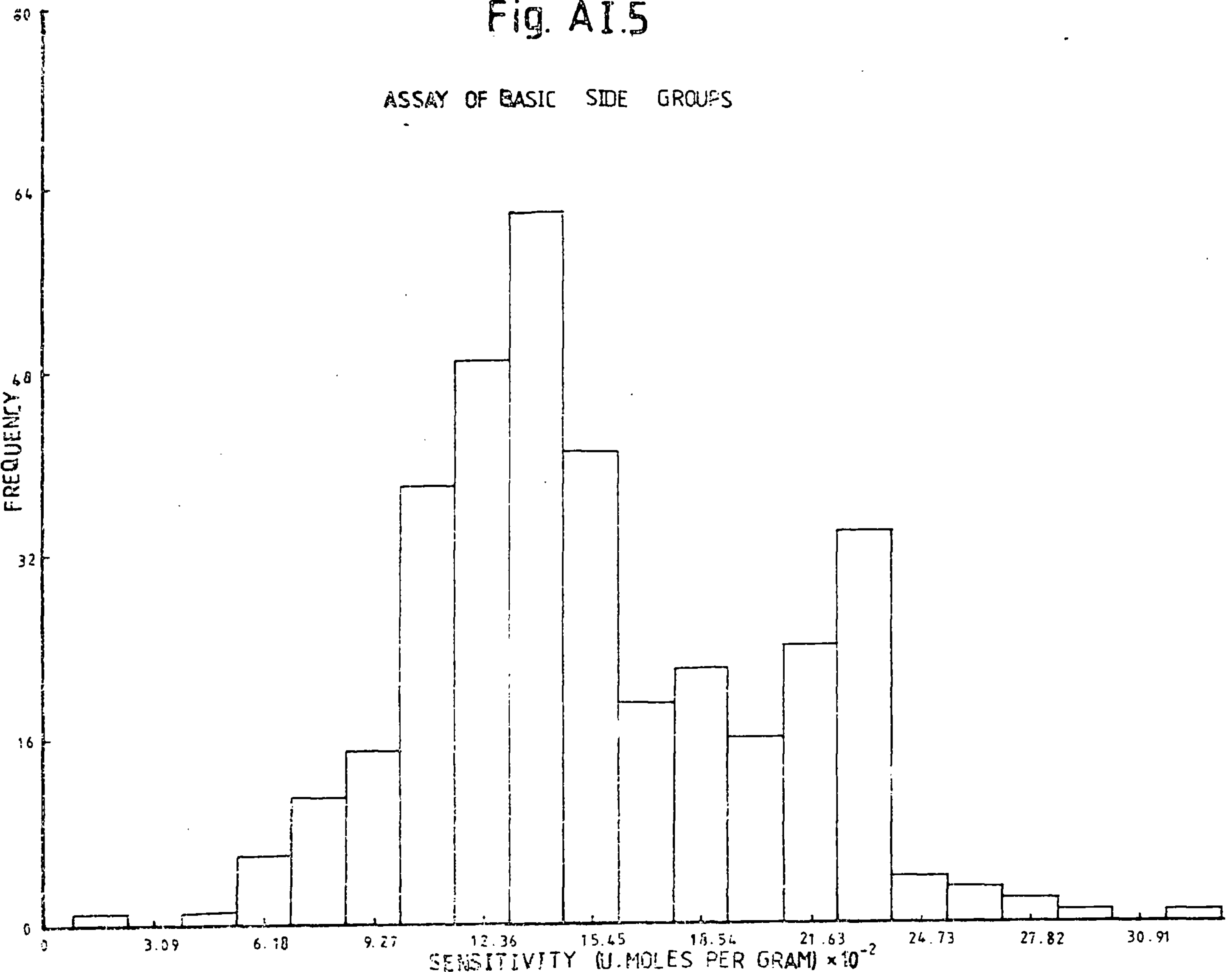


Fig. AI.5
ASSAY OF BASIC SIDE GROUPS
FREQUENCY
80
64
48
32
16
0
0
3.09
6.18
9.27
12.36
15.45
18.54
21.63
24.73
27.82
30.91
SENSITIVITY (U.MOLES PER GRAM) ×10-2

species normally found in proteins. The variations were thus mainly attributable to the variations in molecular weight and the composition of the associated prostetic groups. Since the distributions were generally narrow (the unusual antigenic proteins being ignored), the results also suggested that the limiting error expected for these techniques should be small (<5 percent) and that the choice of a suitable calibrating standard should not be difficult for these procedures.

In contrast to the above, the results suggest that the choice of a calibrating standard for the other two procedures (basic group assay and aromatic group assay), should be very difficult, especially if the experimental situation involves big changes in the protein composition of the assay sample (e.g. in the purification of a protein from a crude source). This problem should therefore be greatest in the case of the basic group assay where the data suggested that proteins may be broadly segregated into two populations as regards their basicity. Consequently the limiting error expected for a protein estimation by techniques based on these procedures should be considerably greater than that expected of the other three discussed. This can be directly attributable to the broadness in the overall distribution which in turn is attributable to the fact that the titrated properties are the sums of contributions from only a few of the amino acid species normally found in proteins.

The above considerations are not the only factors which affect the decision to implement a given protein assay procedure into a given experimental situation. Other important considerations include:

(a) Speed of the assay procedure.

(b) Quantity of material required for assay.

(c) Sensitivity of the procedure to interference by extraneous material.

Of these, the speed of the assay procedure is usually the least critical. However in cases where speed is important (e.g. in monitoring the effluent from a chromatography column), great accuracy is not usually required and for this the measurement of ultra violet extinction (Groves et al. 1968) is ideally suited. In general, the quantity of material needed for an assay is not very important, except in cases where the potential assay material is the product of long and expensive experimentation. Most of the assay techniques developed from the above procedures require between 10-100 micrograms of protein per assay, except the Biuret assay which on account of the small molar extinction coefficient of the chromaphore formed (Strickland et al, 1961) usually requires milligram quantities of protein per assay. Thus, in general, the Biuret type assay is avoided in cases where the assay sample is expensive. The sensitivity of the assay procedure to interference by extraneous material is perhaps the most important factor affecting the choice of an assay procedure. In most cases the potential assay sample contains extraneous material deliberately introduced by the worker so as to retain the biological activity of the sample. While, in principle, the assay in the presence of such material can be avoided by precipitation, washing and resolubilisation, this involves some loss of sample and is only feasible for use with large samples. Thus it is often necessary to assay for protein in the presence of extraneous material. Since the properties normally titrated in protein assays are not unique to proteins, it follows that a successful assay can only be carried out if the protein in the sample is the only significant contribution to the property being measured. This does not usually pose a problem since

the history of the sample is generally under the control of the worker or known. The problem usually arises where the extraneous material while itself not contributing to the measured property, interferes with the overall measurement by side reactions with the protein or assay reactants. The assay procedures using the Folin-Ciocalteau phenol reagent are especially sensitive to interference from this source. In a compilation of the reported interference by extraneous material (Unemoto, Sakakibara & Hayashi, 1975), almost all of the materials commonly used for the maintenance of the biological activity of proteins have been reported to interfere with this particular assay. The cause of these problems has been shown not to be a deviation from the Beer-Lambert law (Stauffer, 1975), and is believed to be related to the instability of the phenol reagent in alkaline solution and the interference of the said materials with the binding of the cupric ion necessary for the assay (Stauffer, 1975). The other assay procedures are generally less sensitive to interference by extraneous material.

Finally, the calculations also suggested that the accuracy of the determinations depends on the choice of the calibrating standard. The most widely used standard is Bovine Serum albumin (B.S.A.) since it is relatively inexpensive to obtain in a pure form. Table A1.2 compares the theoretical sensitivity of B.S.A. with the mean values calculated for the sample studied. This suggests B.S.A. to be a good standard for the assays based on total nitrogen, total amino acids and peptide bond determinations. In the other cases, the expected difficulty in choosing a good standard is reflected in the big differences between the B.S.A. value and that of the population mean (77 percent

TABLE AI.2

Comparison of mean assay sensitivity values with those calculated for Bovine Serum Albumin (B.S.A.)

Assay Procedure	▲Population Mean	*▲B.S.A. Value	B.S.A./Mean
Total nitrogen	10.949	11.674	1.066
Total amino acids	9.744	10.367	1.064
Total peptide bonds	8.676	8.783	1.012
Aromatic groups	0.4195	0.323	0.77
Basic groups	1.398	1.923	1.376

▲ Values in units of mMoles per gram

* Values calculated from amino-acid analysis as per King & Spencer (1970).

population mean for aromatic groups and 139 percent population mean for basic groups.)

The above considerations argued strongly in favour of the use of one of the three 'fundamental' assay procedures (i.e. total nitrogen, total amino acid and total peptide bond) for the determination of protein if problems associated with the variation errors were to be minimised. A recent publication (McGrath, 1972) described an assay procedure based on the assay of total amino acids after alkaline hydrolysis. As described the procedure was relatively quick (approx. 2 hours), sensitive, and tolerant to the presence of many extraneous substances. It, being ideally suited for the requirement of this project, was adapted for use in the experiments of this research study.

APPENDIX II

Computer assisted numerical aids to enzyme kinetic data

Enzyme kinetic measurements essentially involve the measurement of enzyme activity at known values of an independent variable. The worker usually attempts to fit the observations to the law(s) which relate the activity of the enzyme to the independent variable under consideration. Such laws usually describe the behaviour of the system in terms of some defining parameters (relative constants), and given that all experimental observations are subject to error, the basic problem is that of deriving the best value(s) for the defining parameters from the experimental estimates of the enzyme activity made under given experimental conditions. Such a problem can be readily handled by computer assisted least squares error minimising procedures.

The computer assisted procedures used here are all based on the assumption that the observed enzyme activity (Vi) differs from the 'real' enzyme activity (V_{calc} - the value calculated from the best fit values of the defining parameters) by a random error ($\delta v_{..} = |V_i - V_{calc}|$). Thus the best values for the defining parameters are those which minimise the sum of such errors. Thus, if enzyme activity (v) is a function of an independent variable (T), and the parameters Pa, Pb and Pc (i.e. v = f (T, Pa, Pb, Pc) then the least squares solution defines the minimum value for the sum of squared error (SSE) such that the partial derivative of the sum of squared error with respect to each parameter is equal to zero.

i.e.

for $SSE = \sum (V_i - V_{calc})^2$ and at a minimum value for SSE

$$\frac{\delta}{\delta P_a}(SSE) = 0 \; ; \frac{\delta}{\delta P_b}(SSE) = 0 \; ; \frac{\delta}{\delta P_c}(SSE) = 0.$$

Any procedure based on the above principle can be expected to return unbiased values for the required parameters provided that the errors at each of the data points are of similar magnitude. However, in many enzyme kinetic experiments the difference between the smallest and largest values can span two orders of magnitude. Consequently the procedures based on a minimisation of absolute error are likely to return values for the defining parameters which are biased towards a minimisation of the errors in the range where the magnitude of the experimental observations is fairly large. The resolution of this problem adopted here assumes that the size of the error in any determination is proportional to the magnitude of the determination. In most enzyme kinetic measurements this is generally true provided that the random error in the experimental observation is considerably greater than the limiting error of the methodology used to make that estimation. Since the latter limitation can be easily overcome through a judicious experimental design, the computer software developed for the handling of the data presented in this thesis was essentially based on the minimisation of the function given by the following equation:

$$SSE = \sum \left(1 - \frac{V_i}{V_{calc}}\right)^2$$

The procedures used here for the minimisation of the function given by the equation above depended on the number of parameters defined by the given problems. For problems involving two adjustable parameters (e.g. Michaelis Menten substrate activation kinetics, simple uncompetitive inhibition kinetics and Linear Arrhenius Temperature kinetics), a fibonacci search procedure was found to be efficient in its use of computer time and to be relative to the accuracy of the solution required. Such a procedure has been incorporated into part of a computer program for fitting the Hill equation (Atkins, 1973), and for the obtaining of Michaelis-Menten parameters (Bannister, Anastasi & Bannister, 1976) and the results reported by those authors and those observed here agree with respect to its reliability and its efficient use of computer time. In the instances where the problems contained more than two adjustable parameters (e.g. the kinetic models adopted in Chapters 3, 4, 5 and 7), the minimiser used was based on the principle described by Peckham (1970) and as implemented by the Numerical Algorithms Group (Routine E04FAF in the NAG Fortran library Mark 6 version). This was also found to be an efficient though slightly less reliable method of minimising the object function.

The above procedures were incorporated into purpose written computer programs using the high level language Fortran IV. The computer programs were designed to cope with some of the particular problems related to the design of enzyme kinetic experiments. The programs also used graphics package available at N.U.M.A.C. (Northumbrian Universities Multiple Access Computers), to display the processed data graphically. A combination of the numerical data processing and the graphical display facilities, thus provided an objective aid in the interpretation of the data presented in this thesis.

APPENDIX III

The vanadate ion impurity in Sigma grade A.T.P. and its probable effects on the data presented in this thesis.

The presence of an inhibitor of the Na^{+}-K^{+}-ATPase in some commercial sources of A.T.P. have been suggested in some recent reports (Beauge & Glynn, 1977; Josephson & Cantley, 1977). This inhibitor has since been identified as vanadate ion (Cantley, Josephson, Warner, Yanagisawa, Lechene & Guidotti, 1977; Quist & Hokin, 1978) which is found as an impurity in ATP that has been extracted from muscle. The presence of this inhibitor in Sigma grade A.T.P. has been considered responsible for some of the recently reported 'anomalous' kinetics of the Na^{+}-K^{+}-ATPase (Cantley & Josephson, 1976; Fagan & Racker, 1977). Since Sigma grade A.T.P. was used extensively in this study, the probability of perturbations in the data as a result of the effects of vanadate ion had to be considered. This was approached in the light of the previously reported characterisation of the effects of vanadate ion on the enzyme (Josephson & Cantley, 1977), by comparing the potassium activation kinetics when Sigma grade A.T.P. was used as substrate, with that using another commercial source of A.T.P. (Boehringer) which was not extracted from muscle.

The potassium activation kinetics of the Na^{+}-K^{+}-ATPase from deoxycholate extracted rat brain synaptic membranes were measured as previously described in Chapter 5, using Sigma grade A.T.P. and Boehringer A.T.P. as substrate in parallel assay tubes. In each case there was a sigmoidal increase in enzyme activity with increasing potassium concentration. The data points were fitted to a stoichiometric sigmoid curve as described in Chapter 5, and the defining parameters of these curves (mean of four preparations) are listed in Table AIII.1.

TABLE AIII.1

Comparison of Potassium activation parameters of DOC extracted rat brain Na^+-K^+-ATPase using different sources of ATP.

Parameter	Sigma grade ATP	Boehringer ATP
* V_{max}	100	103.5 ± 3.3
K_a (M)	$2.19 \times 10^{-4} \pm 7.1 \times 10^{-5}$	$4.2 \times 10^{-4} \pm^{\dagger} 2.32 \times 10^{-4}$
K_b (M)	$1.65 \times 10^{-3} \pm 1.35 \times 10^{-4}$	$1.55 \times 10^{-3} \pm 1.65 \times 10^{-4}$
$K_{0.5}$ (M)	$1.85 \times 10^{-3} \pm 2.1 \times 10^{-4}$	$1.89 \times 10^{-3} \pm 1.66 \times 10^{-4}$

* V_{max} values normalised against 100 for Sigma V_{max}

† The relative inaccuracy of this measurement is attributed to the higher levels of inorganic phosphate found in Boehringer ATP (see Text).

All values mean of 4 preparations ± 1 standard deviation.

This shows that the defining parameters calculated were not significantly different from each other ($P>0.05$) with respect to maximal activity, K_b and $K_{0.5}$. A higher value for K_a was calculated when Boehringer A.T.P. was used. However, as is evident from Table AIII.1, the accuracy of the estimation of K_a using Boehringer A.T.P. was a lot less than that estimated using Sigma grade A.T.P. This is because the latter source of A.T.P. contained lower levels of background inorganic phosphate, a fact which facilitated a greater accuracy in the estimations of the low levels of enzyme activity at low potassium concentrations. Thus the observed difference in the K_a values was not considered significant.

The above results suggested that there was insignificant inhibition of Na^+-K^+-ATPase activity, by the vanadate ion impurity in Sigma grade A.T.P., under the assay conditions used here. Although these results appear to be at variance with the reported presence of Na^+-K^+-ATPase inhibitors in Sigma grade A.T.P. described above, they can be explained if the characteristics of the inhibition process are considered. Josephson & Cantley (1977) reported that the inhibition of the Na^+-K^+-ATPase by the impurity carried in muscle A.T.P. is slow and requires 'free' magnesium ion and potassium ion. In the conditions used here (equimolar concentrations of Mg^{2+} and A.T.P.) it is calculated, using the value for the equilibrium constant for the formation of the Mg-ATP chelate (George, Phillips & Rutman, 1963), that the free magnesium ion concentration would be close to 10^{-4}M. Since optimal effects of the inhibitor has been reported to require at least milli-molar concentrations of free magnesium ion (Josephson & Cantley, 1977; Fagan & Racker, 1977; Quist & Hokin, 1978), the failure

to detect significant differences between the potassium activation kinetics of the Na^+-K^+-ATPase using Sigma grade A.T.P. and Boehringer A.T.P. in these studies, was considered consistent with the reported properties of vanadate ion inhibition of the Na^+-K^+-ATPase. Thus, the presence of vanadate ion in the Sigma grade A.T.P. used in this study was not considered to have had significant effects on the data presented in this thesis.

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