SOME STUDIES IN PURINE METABOLISM AND CONTROL

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PREFACE

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The work reported in this thesis was carried out in the Department of Molecular Sciences of the University of Warwick between October 1971 and June 1974.

All of the work was carried out by the author except for the bleeding of the rats and red blood cell counting which was carried out by Mr. P. McConnell and the gel isoelectric focusing which was done in collaboration with Mr. J. Wood both of whom he would like to thank.

SUMMARY

The flux of metabolites within the cell is discussed. Two approaches were used in the study of this subject:
(1) A preliminary investigation of the controlling factors of adenine metabolism in red blood cells was undertaken.
(2) A study was made of adenylate cyclase in red blood cells and of the cyclic-AMP binding proteins of the bovine adrenal cortex.

AMP deaminase was partially purified from human and sheep erythrocytes. This greatly facilitated the spectrophotometric assay of the enzyme's activity. Some properties of this enzyme were studied.

A method for examining the control of purine flux was developed by the use of radioisotopic tracers. The possibility of its use as a diagnostic assay for metabolic disorders is discussed. Adrenalin, which modifies the erythrocyte membrane, was tested for any effect it might have on the rate of transport of adenine into the red blood cell.

The adrenalin or fluoride ion stimulated activities of adenylate cyclase were measured in turkey, rat and human red blood cells. The assay used was the saturation analysis procedure of Brown et al. (1971) using a cyclic-AMP binding protein from the bovine adrenal cortex. The cyclic-AMP binding moiety is the cyclic-AMP dependent protein kinase regulatory subunit. The crude preparation used in the assay for cyclic-AMP was fractionated by several methods and cyclic-AMP binding studies were carried out on the different fractions.

Particular attention was paid to the interpretation of binding data. It was found that under different conditions certain preparations of the binding protein could produce either linear or curved binding plots ('bound' against 'bound/free' ligand). The causes of this are discussed. The result of the binding study has mechanistic implications

CHAPTER 1

INTRODUCTION

1.A. The control of metabolic flux: general considerations

The control of enzyme activities and the resultant metabolic fluxes is a topic that is receiving much attention now that the enzymes of the various metabolic pathways have been studied in some detail. There are many factors which contribute to the total control of a metabolic pathway and figure 1.1 is an attempt to summarise most of these. It may be helpful to look at some of the different factors that effect control under generalised headings.

1.A.a. Enzymes

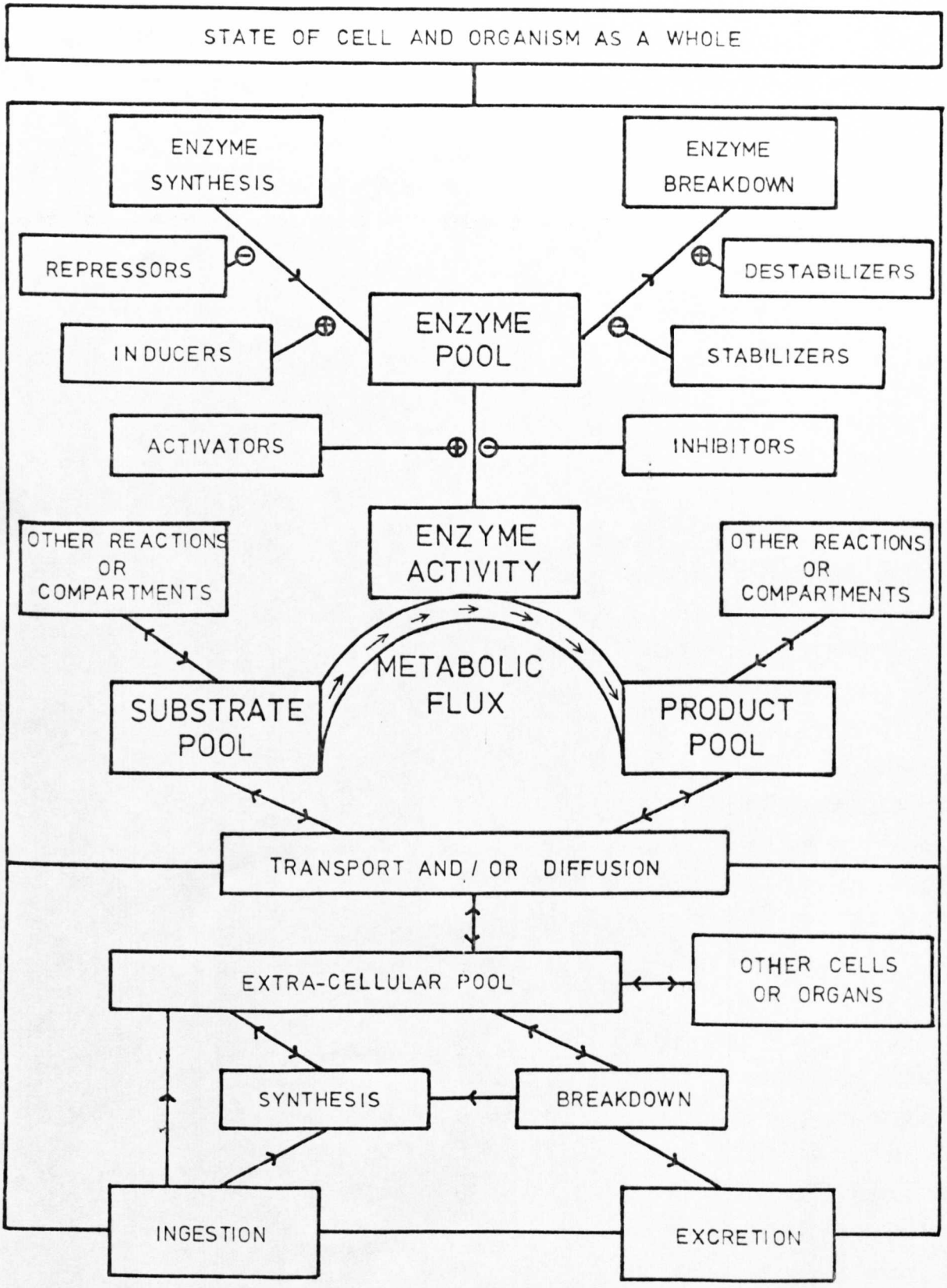
(i) The synthesis and degradation of enzymes themselves is undoubtedly a controlled process and may affect the metabolic flux. However, even the enzymes with the most rapid decay rates have half lives of a few hours (Schimke: 1969).

(ii) The kinetics of the enzyme catalysed reaction will depend on the substrate concentration and the concentration of any coenzymes, inhibitors or activators. Some enzymes have a sigmoidal response of activity against substrate concentration. This may be due to multisubstrate kinetics or some allosteric mechanism. Whatever the source of the sigmoidal kinetics, though, it has potential for control purposes.

(iii) The maximum activity of an enzyme (V_{max}) is an important consideration in understanding control. In any pathway control is exerted at the rate limiting step. In branched pathways there may be a number of such steps. If, therefore, the V_{max} is high an enzyme has little potential for control purposes unless in the cell it is strongly inhibited. Thus a knowledge of the enzyme's kinetic constants (V_{max}, K_m) and inhibitor constants (K_i) is important.

Figure 1.1

CONTROL OF ENZYME ACTIVITY and metabolic flux



1.A.b. Substrates

The concentration of the substrate will be a controlling factor in the metabolic flux only if it is non-saturating for the enzyme catalising the reaction. It is also unlikely to be a controlling factor if the enzyme catalised reaction is near equilibrium; that is, if the 'mass action ratio' of product concentrations and substrate concentrations is near the reaction equilibrium constant (Rolleston: 1972; see also section d). Determination of the mass action ratio is not necessarily straightforward. Compartmentalisation of substrate and/or product may seriously obscure the true situation (1.D).

In some situations the diffusion or transport of a substrate or product may affect the flux of a pathway if the enzymically catalysed reaction is near equilibrium. Such may be the case for lactate dehydrogenase in muscle. The diffusion of lactate into the blood and its consequent removal may be a limiting factor in the rate of the reaction (Newsholme and Start: 1973).

Cofactors can be considered under the general heading of substrate. It is unusual and unexpected to find control exerted by cofactor availability, although, the ratio between the concentrations of ATP and ADP, for example, is thought to be important in the control of phosphofructokinase activity. In general their widespread usage and the specific nature of control mechanisms are not compatible.

1.A.c. Inhibitors

Feedback inhibition is an important feature of control mechanisms. A related control feature is feed-forward activation. In each of these control mechanisms a metabolically related compound exerts a control on the pathway at a remote point. In the case of feedback inhibition this is usually the first committed step of a metabolic pathway. However, in some cases an inhibitor appears to bear no simple relationship to the substrate or the pathway in which it is involved. The

significance of such control is not immediately apparent.

1.A.d. Energy of the reaction

(i) In all reactions energy is conserved. Biochemical energy is conserved in the form of chemical bonds. In a reaction at equilibrium the change in free energy is zero, and there is no change in product or substrate concentrations.

$$\Delta G = RT\ln K + RT\ln\left(\frac{[\text{Products}]}{[\text{Substrates}]}\right)$$

The mass action ratio of product and substrate concentrations (strictly activities) is equal to the equilibrium constant. All the energy is conserved in chemical bonds but the net flux is zero. Biochemical flux requires reactions to be displaced from equilbrium. Reactions in which the mass action ratio is near the equilibrium constant will be highly efficient.

(ii) In some reactions energy is released in the form of work or dissipated as heat. This 'loss' of energy (biochemical energy) results in a reaction that is displaced from equilibrium towards the products. A reaction in which this displacement is very large is usually termed an irreversible reaction. It is these non-equilibrium reactions that both require and produce a constant exchange of material and are therefore important sites for the control of flux.

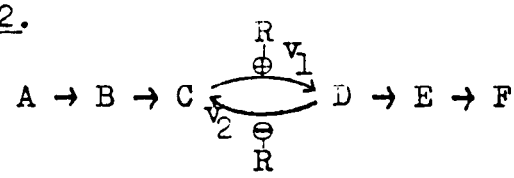
1.A.e. Situation of the controlling enzymes

On the basis of the previous discussion it can be seen to be necessary that a controlling enzyme should be rate limiting under the conditions in which its control is exercised. It is also necessary for the enzyme to be subject to inhibition or activation and it is usual for it to catalyse a non-equilibrium reaction.

A consideration of the position of enzymes in a pathway reveals probable sites for control albeit by teleological reasoning. The initial steps in a pathway are an obvious site for feedback inhibition. Branch points and the following steps are important points as are the final interconversions.

Substrate cycles (see fig. 1.2) can be a means of amplifying a response to an effector. The two reactions should be non-equilibrium reactions and subject to opposite controls by a regulator.

Figure 1.2.



A---F is a schematic representation of a metabolic pathway in which the interconversions between C and D are catalysed by two enzymes 1 and 2 with rates v_1 and v_2 respectively. If we stipulate that v_1 is greater than v_2 then the rate of substrate cycling is v_2 and the turnover rate is $v_1 - v_2$. If a regulator R activates 1 and inhibits 2 the the rate of cycling is decreased and the rate of turnover is increased by a larger amount than if it were subject to one controlling enzyme. (Newsholme and Start: 1973)

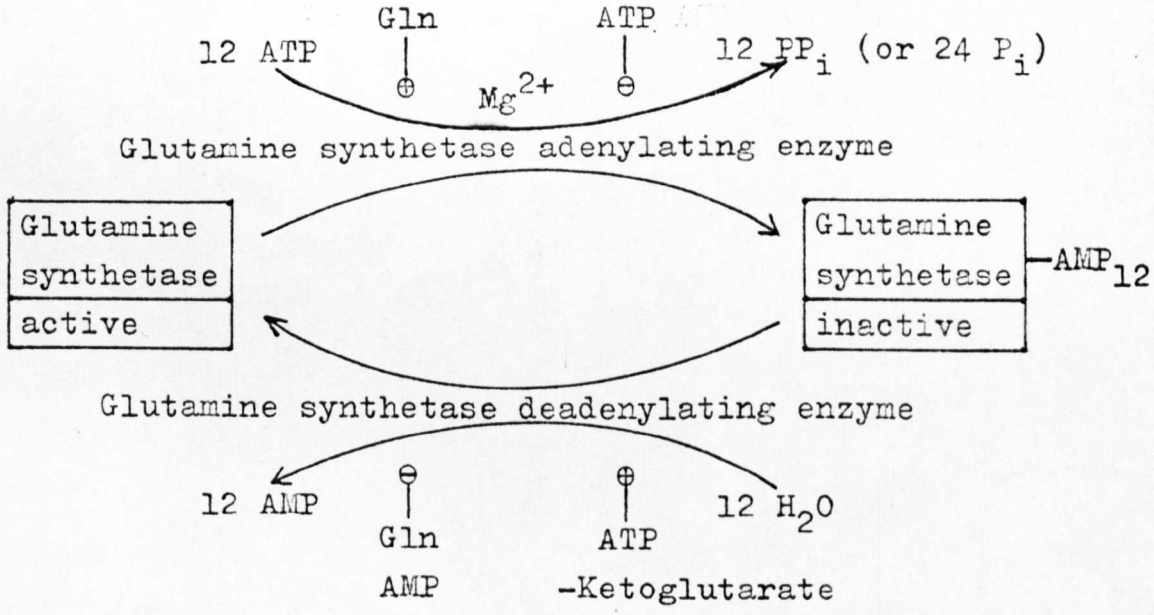
Another means of amplification may be termed 'enzyme cycles'. Here an enzyme converts another inactive enzyme into an active form or vice versa. An example of this is the adenylation of Escherichia coli glutamine synthetase (see fig. 1.3) or the phosphorylation steps involved in the activation of phosphorylase b (see fig. 1.4). This latter form of amplification appears to be a general feature of many hormonal actions mediated through the activation of adenylate cyclase and protein kinases (see section 1.G.).

1.B. Red blood cells

Erythrocytes were chosen as the tissue in which to study some aspects of the control of purine metabolism because the cells are readily available and uniform in nature. A separation on the basis of age can be made if necessary and a considerable amount of data has already been accumulated concerning the enzymology of the cell and in particular the purine pathways. Also the metabolism of purines in the red

Figure 1.3

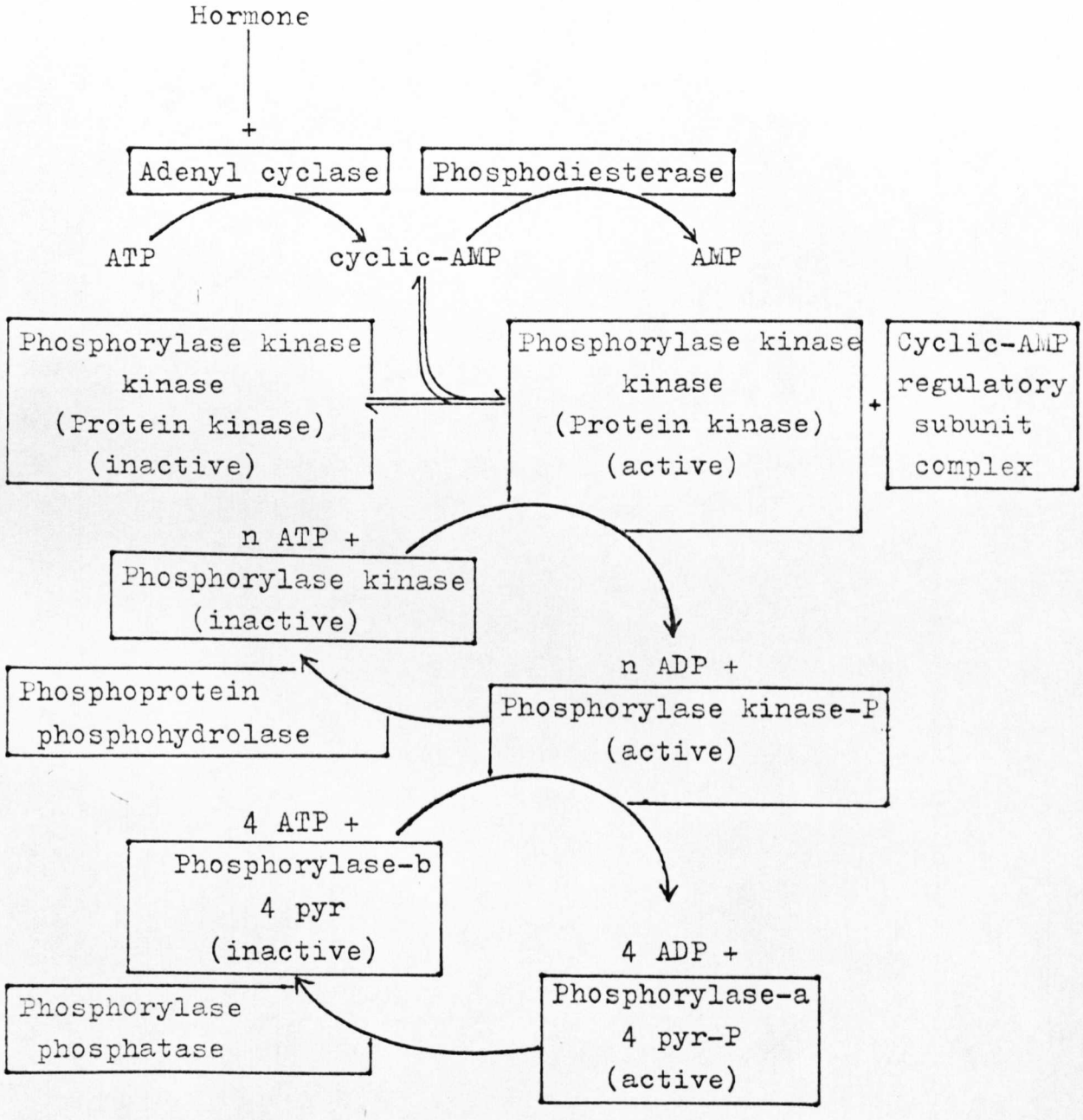
Activation of E. coli
Glutamine Synthetase



The regulation of glutamine synthetase from E. coli by enzyme catalysed chemical modification.
⊕ indicates a positive effector (stimulation)
⊖ indicates a negative effector (inhibition)
This figure is taken from Holzer (1969)

Figure 1.4

Activation of Phosphorylase-b



The regulation of glycogen phosphorylase from rabbit skeletal muscle by enzyme catalysed chemical modification. This figure was taken from Holzer (1969). It shows the 'enzyme cascade' by which the signal from a hormone (e.g. adrenalin) is amplified. Cyclic-AMP also causes the phosphorylation of glycogen synthetase which in the case of that enzyme inhibits it.

cell is simplified by the omission of a number of enzymes. The enzymic conversions in the pathway are shown in figure 1.5. The steps which do not occur in the human erythrocyte are indicated by dotted lines. Mammalian red cells have no nuclei and therefore no DNA. RNA is rapidly degraded during and after the reticulocyte stage of red cell development. The absence of the de novo purine synthetic pathway and of the enzyme IMP:L-aspartate ligase (GDP) means that erythrocytes rely on the supply of exogenous purines and have a specific requirement for adenine (or adenosine).

1.C. The enzymes of the purine pathway

Table 1.1 summarises much of the available information regarding the enzymes of this pathway. A study of most of these enzymes has been carried out though not in all cases with human erythrocytes as the source of the enzyme. Most attention has been directed to the enzymes involved in the metabolism of adenine and its derivatives.

1.C.a. Adenine phosphoribosyl-transferase (APRT)

APRT is a soluble enzyme that has been purified 2000 fold from human erythrocytes (Srivastava and Beutler: 1971). They studied the basic enzyme kinetics and the effects of temperature, metal ions, organic anions and sulphydryl reagents on the enzyme's activity. They proposed that the reaction mechanism was ordered (see figure 1.6). This is in agreement with the mechanism proposed by Henderson et al. (1967). Henderson also found that in 15% of a random sample of people the APRT was unusually heat stable. This suggests the possibility of there being isoenzymes of APRT. A partial deficiency of the enzyme has also been recorded (Henderson et al.: 1969) but there were no clinical manifestations of this. The actual role of the enzyme appears to be one of salvaging adenine, though it may do so in the process of keeping the adenine concentration below toxic levels.

Figure 1.5

The Purine Pathways

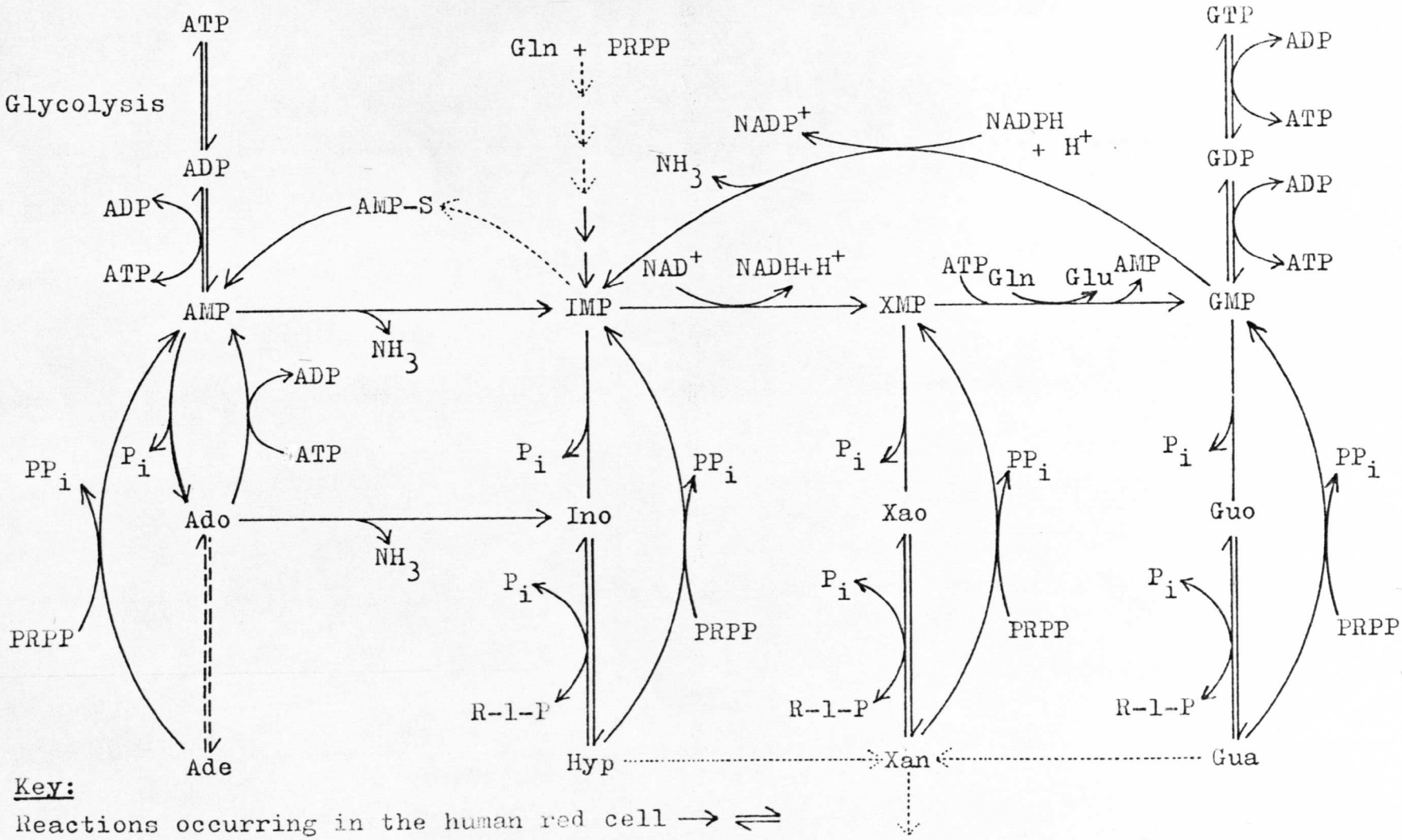


Table 1.1

Information concerning some Purine Enzymes of the Human Erythrocyte

Enzyme	EC No.	Substrates (K_m)	Products (K_m)	Metal ion requirement	Activator	Inhibitors (K_i)	V_m nmol/mg/h
APRT	2.4.2.7.	ade (62μM), PRPP (1.4μM)	AMP PP_i	Mg^{2+}		AMP (25μM), PP_i (0.9mM), ADP (50μM)	31
AMP kinase	2.7.4.3.	AMP (0.33mM), ATP (0.95mM)	ADP (0.12mM) ADP.Mg (0.22mM)	Mg^{2+}		AMP, ADP, GDP, IMP, ado, GMP	13,560
AMP deaminase	3.5.4.6.	AMP (hyperbolic with 2mM ATP)	IMP	K^+	K^+, ATP	GTP, 2,3-DPG	14
5'-nucleotidase	3.1.3.5.	AMP, IMP, XMP, GMP	ado, ino, xao, guo, P_i	Mg^{2+}		ATP, ADP	
Ado kinase	2.7.1.20.	ado (1.9μM), ATP (400μM)	AMP ADP	Mg^{2+}		ATP >1.25mM	<2
Ado deaminase	3.5.4.4.	ado (40μM)	ino			ado >0.4mM	250
PNPlase	2.4.2.1.	ado, ino (58μM), xao, guo, P_i	ade (0.41mM), hyp (19μM), xan, gua, R-1-P			Other purine bases and nucleosides	ino- 690 ade- <1
HGPRT	2.4.2.8.	hyp (11μM), xan, gua, PRPP (0.22mM)	IMP, XMP, GMP, PP_i	Mg^{2+}			hyp- 103 xan- 0.3 gua- 103
GMP kinase		GMP, ATP	GDP, ADP	Mg^{2+}			
Glycolysis		glucose	lactate				normal c.a. 7*

* Normal red cell lactate production: net ATP produced is twice this.

1.C.b. ATP:AMP phosphotransferase (AMP kinase)

AMP kinase has been partially purified from human erythrocytes and has been shown to exist as two commonly inherited isoenzymes. These isoenzymes differ slightly in heat stability, iodoacetamide inhibition and kinetic parameters. The proposed mechanism for their reaction is a random order rapid equilibrium (Brownson and Spencer: 1972 a & b). This enzyme has been found in both soluble and membrane bound cell fractions. This may be related to the observation that ADP can pass into the red blood cell but cannot pass out of the cell. AMP and ATP cannot traverse the cell membrane (Kashket and Denstedt: 1958). Rose (1968) has shown how the equilibrium constant of the reaction varies with magnesium ion concentration and pH.

1.C.c. AMP aminohydrolase (AMP deaminase)

Human erythrocytic AMP deaminase has been studied in crude haemolysate preparations. Rao et al. (1968) have shown the enzyme to be partially associated with the cell membrane. Askari (1963) showed that the enzyme required ammonia or a monovalent metal ion for activity. The activity was highest with potassium which is the physiologically activating ion. ATP was shown to enhance the metal ion activation (Askari and Franklin: 1965). The enzyme is strongly inhibited by 2,3-diphosphoglycerate (2,3-DPG) at physiologically significant concentrations. The response of activity to AMP concentration is sigmoidal except in the presence of sufficiently high concentrations of ATP (>2mM). The effect of 2,3-DPG is to enhance the sigmoidicity of this response (Askari and Rao: 1968).

1.C.d. 5'-Ribonucleotide phosphohydrolase (5'-Nucleotidase)

5'-Nucleotidase has not been studied from the human erythrocyte. There are numerous studies on the enzyme from various sources: human liver (Song and Bodansky: 1967); human aorta wall and placenta (Ahmed and Reis: 1958); human tissue distribution (Reis: 1951); calf intestine (Center and Behal: 1966); sheep brain (Ipata: 1967); Erhlich ascites tumour cells (Murray and Friedricks: 1969); rat heart (Sullivan and

Alpers: 1971) and others.

1.C.e. ATP: adenosine 5'-phosphotransferase (Adenosine kinase)

Adenosine kinase from human erythrocytes was studied as part of an investigation into the metabolism of adenosine by blood (Meyskens and Williams: 1971). The enzyme associated with erythrocyte ghosts has been studied by Mustafa et al. (1972) particularly with reference to the effect of detergents on the enzyme's activity. It is inhibited by high concentrations of ATP (>1.25mM) though this may depend on the magnesium concentration.

1.C.f. Adenosine aminohydrolase (Adenosine deaminase)

Meyskens and Williams also studied adenosine deaminase. Although this enzyme is associated with the membrane (Mustafa et al.: 1972) it is not strongly bound to it (Mitchell et al.: 1965). It is inhibited by high concentrations of adenosine (10 x K_m).

1.C.g. Purine-nucleoside: orthophosphate ribosyltransferase (PNPlase)

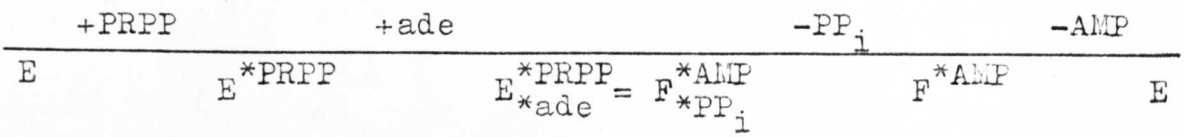
The PNPlase of human erythrocytes has been crystallised and extensively studied. It has a molecular weight of 81,000 and a single active site for the competing substrates (Kim et al.: 1968a). The reaction mechanism is considered to be an ordered bi-bi mechanism. There is no apparent ribosylation of the enzyme (Kim et al. 1968b). There has been controversy over the question of whether adenine is a substrate of the enzyme. Zimmerman et al. (1971) have demonstrated that the crystalline enzyme is capable of catalysing the conversion of adenine to adenosine at 0.0017 the rate of hypoxanthine conversion to inosine. The equilibrium constant for the reaction inosine to hypoxanthine is approximately 0.03.

Turner et al. (1971) have demonstrated seven electrophoretically different isoenzymes. The red cells were separated on the basis of age by density gradient centrifugation. The distribution of isoenzymes changed according to the age of the cell: from young cells with slower moving bands to old cells with faster (anodal) bands.

Figure 1.6

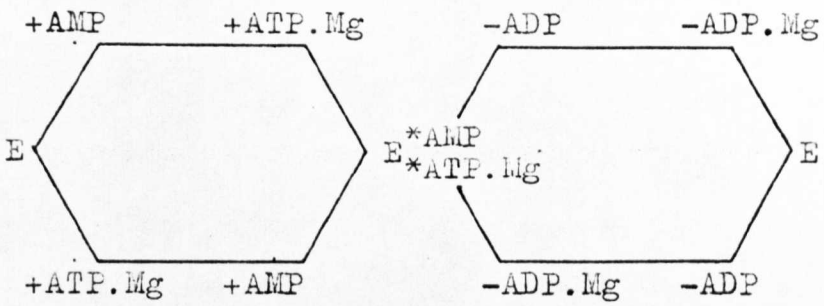
Reaction Mechanisms of Some Purine Enzymes

APRT ———— ordered reaction



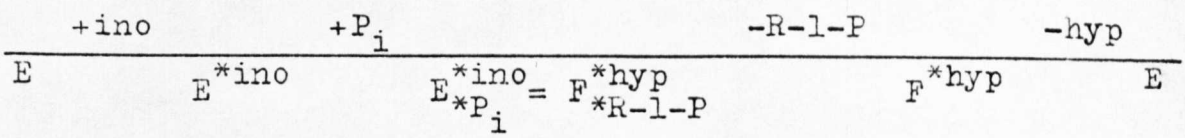
Henderson et al. (1969); Srivastava and Beutler (1971)(p. 5)

AMP kinase —— random order reaction



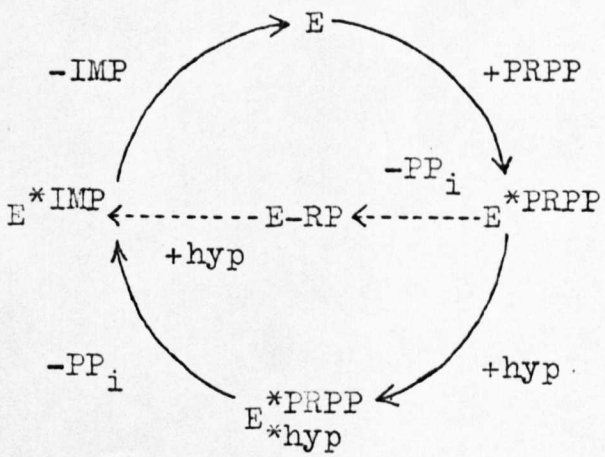
Brownson and Spencer (1972b)(p. 6)

PNPlase ———— bi-bi reaction



Kim et al. (1968)a (p. 7)

HGPRT ———— ordered reaction (possibly with a phosphoribosyl-enzyme intermediate)



Kelley et al. (1967b); Henderson et al. (1968); Krenitsky and Papiaoannou (1969) (p. 8)

1.C.h. Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)

Since the discovery of the Lesch-Nyhan syndrome (Nyhan: 1968) and its association with a deficiency of HGPRT (Kelley: 1968) this enzyme has received much attention. It is a single enzyme (M. Wt. 60,000) catalysing the reactions between 5-phospho-α,D-ribose-1-pyrophosphate (PRPP) and the three purine bases hypoxanthine, xanthine and guanine to give the respective nucleotides IMP, XMP and GMP. HGPRT has an ordered reaction mechanism involving a ternary complex (Kelley et al.: 1967a and Henderson et al.: 1968). The reaction may involve the formation of a phosphoribosyl-enzyme intermediate (Krenitsky and Papiaoannou: 1969). A partial deficiency of the enzyme is one of the many causes of gout (Kelley et al.: 1967b and Wyngaarden and Kelley: 1972).

1.C.i. Less well studied purine enzymes

ATP: GMP phosphotransferase (GMP kinase) has been isolated from human erythrocytes and been found to exist as four isoenzymes (Agarwal and Parks: 1971 & 1972).

Bishop (1960) recorded an absence of xanthine: O_2 oxidoreductase (xanthine oxidase) and guanine amino-hydrolase (guanase) from human erythrocytes. He found that C^{14}-hypoxanthine was converted to IMP and to GTP thus indicating the presence of active IMP: NAD^+ oxido-reductase (IMP dehydrogenase) and XMP: L-glutamine ligase (AMP) (GMP synthetase). The conversion of guanine to hypoxanthine (via GMP and IMP) was recorded by Bishop (1961) but was studied in more detail by Hershko et al. (1963). They demonstrated that the dialysed haemolysate required the addition of magnesium, ribose-5-P, ATP and reduced glutathione for the conversion to take place.

1.D. Compartmentalisation

The pool size of a metabolite is related to its concentration and distribution throughout the cell. If the distribution is uneven then it is said to be compartmentalised. This may represent actual physical areas of the cell such as organelles or a distribution between bound and free fractions.

Compartmentalisation of either form can lead to difficulties in determining the effective intra-cellular concentration of a substrate. For example human red blood cells have no organelles except for a few mitochondria that may be found in reticulocytes. These disappear very quickly in the maturation process. Therefore the only compartmentalisation possible is that of physical or chemical adsorption to proteins, to the cell membrane or of complex formation with metal ions. This is exemplified by the association of ATP and 2,3-DPG with haemoglobin which constitutes 97% of the total red cell protein (Gomperts: 1969). Table 1.2 gives the estimated concentrations of ATP, 2,3-DPG, haemoglobin and magnesium in human erythrocytes. The data are taken from Bunn et al. (1971) and are relative to cell water. They used the total concentration of

Table 1.2

Concentration (mM) (in cell water)

Substance	Oxygenated cells	Deoxygenated cells
Total ATP	2.00	2.00
'Free' ATP	0.37	0.08
$ATP.Mg^{2+}$	1.63	1.27
ATP.Hb	0	0.71
Total 2,3-DPG	7.20	7.20
'Free' 2,3-DPG	5.90	0.53
$2,3\text{-DPG}.Mg^{2+}$	1.30	0.39
2,3-DPG.Hb	0	6.29
Total Mg^{2+}	3.50	3.50
'Free' Mg^{2+}	0.57	1.90

these compounds and the known association constants to calculate the concentrations of the complexes with magnesium and haemoglobin (Hb) in oxygenated and deoxygenated cells.

This compartmentalisation has also been investigated by Till et al. (1973) who measured the exchange of radioactive phosphorus between phosphate esters in intact cells. The cells were maintained under an atmosphere of 95% O_2:5% CO_2 to maintain the cell pH.

The specific radioactivity of the medium was kept at a constant level. The combined specific activities of P_i, ADP and ATP rose to a maximum of 20% of the specific activity of the suspending medium. The specific activity of 2,3-DPG rose more slowly but eventually exceeded that of P_i, ADP and ATP together. Analysis of these results and other experiments showed that 60% of the P_i, ADP and ATP was 'inaccessible' to the exchange process. This result does not entirely accord with Bunn's calculation of the compartmentalisation but both results show that this is an important consideration in the case of phosphate esters in red cells.

In addition to the possible compartmentalisation of substrates that of enzymes should also be considered. Some enzymes are found to be associated with the cell membrane, as described earlier for adenosine kinase and deaminase and AMP deaminase. Some glycolytic enzymes are also associated with the bovine erythrocyte membrane (Green et al.: 1965).

1.E. Control features of the purine pathways related to the function of erythrocytes

The study of metabolic control is an attempt to bridge the gap between enzymology and cellular function. Known cellular functions can be used with care to direct one's investigation into controlling processes. The erythrocyte has several functions. It compartmentalises the haemoglobin necessary for the transport of oxygen and transports the oxygen and carbon dioxide for the purpose of respiration. (The haemoglobin also acts as the primary buffering agent for the maintenance of blood pH.) The red cell is also used for the inter-organ tranport of purines. Lajtha and Vane (1958) first showed the necessity of this transport and Henderson and Lepage (1959) showed that it was mediated by red cells.

In addition to cellular function the function of the various metabolites should be considered. The red cell requires ATP for the maintenance of cell shape (Nakao et al.: 1961). This is necessary for the free flow of blood especially in capillaries where the

diameter of the vessel may be as little as 4μ (Fung and Zweifach: 1971). Adenosine has been considered to be an important vasoactive substance released by myocardial cells during hypoxia for the regulation of coronary circulation (Berne: 1967 cited by van Belle: 1969a). ADP induces platelet aggregation whereas adenosine inhibits this action (Rozenberg and Holmsen: 1968). It is therefore reasonable to expect a strict control on the levels of adenosine and adenine nucleotides in the erythrocyte.

The control features concerned with the uptake, maintenance and deamination of the adenine containing compounds are summarised below.

(i) AMP deaminase is activated by ATP but inhibited by 2,3-DPG. The activated enzyme has a K_m for the substrate AMP of the order of 0.5mM (calculated from data of Askari and Rao: 1968). The normal concentration of AMP in erythrocytes, given an even distribution, is 0.01 - 0.02mM (Bartlett: 1959; Benesch and Benesch: 1967). Normally, therefore, the enzyme would be functioning at well below its maximum rate. The inhibitory levels of 2,3-DPG (compare information on table 1.1 and data of table 1.2) indicate that the enzyme is under strict control.

(ii) There is a substrate cycle about the adenosine kinase and 5'-nucleotidase reactions (see figs. 1.2 and 1.5). Although the rate of cycling and the net flux of this cycle are not known, it does appear that it has some properties which favour the conversion of adenosine to AMP as both a substrate and a product of the adenosine kinase reaction (ATP and ADP) inhibit 5'-nucleotidase. However, the alternative paths for adenosine are deamination, which is irreversible, or diffusion from the cell.

(iii) Adenine phosphoribosyl-transferase acts either as a salvage enzyme or as a detoxifying enzyme. Its activity in certain tissues appears to be limited by the availability of PRPP (Kelley et al.: 1968). Although the enzyme is subject to product inhibition, the enzyme is unlikely to be inhibited by the low

concentration of AMP in the cell (see table 1.1).

The 5'-nucleotidase reaction is also involved in a triple substrate cycle with the reactions of two other enzymes: PNPlase and HGPRT. It is possible that this cycle has some significance in the role of red cells in the transportation of purines as the major purines released from the cell are hypoxanthine, xanthine and xanthosine. This happens regardless of the original purine absorbed into the cell (Hershko et al.: 1967).

1.F. Metabolic control and the state of the cell

The effect of the state of the cell on the control of metabolism has been considered. Hershko et al. (1967) found that oxygenation of rabbit erythrocytes favoured the release of purines following the absorption of a labelled tracer. According to Bunn's calculations (table 1.2) the 'free'2,3-DPG concentration should be at its highest under these conditions and AMP deaminase will be inhibited. The 'free' ATP level is also high so 5'-nucleotidase should also be inhibited.

There are clearly more factors involved here than the level of these two metabolites. For example; Bunn's calculation also predicts a variation in the concentration of 'free' and ATP-bound magnesium ions with the state of oxygenation of the cell. This, together with the pH shift, alter the equilibrium constant of AMP kinase (Rose: 1968).

Other conditions of the cell which may effect the purine metabolism are its age and the relative abundance or lack of glucose or other metabolites.

In studying these enzymes and fluxes it was hoped that an understanding would be gained of the factors governing the release and uptake of adenine and its derivatives in the human erythrocyte.

A technique was developed to determine the inter-conversions of a radioisotopic tracer introduced into the pathway (chapter 5). The method was based on the incubation of the cells or haemolysate with a labelled purine. The conditions of this incubation could be

varied to simulate different conditions of the cell. The incubation was stopped by denaturing the protein with perchloric acid. After removal of the protein the distribution of the label between different purine compounds was determined. This was accomplished by separation of the components by thin layer chromatography and measuring their radioactivity by liquid scintillation counting.

This approach was backed up by the assay of relevant enzymes to gain information about their physiological roles and to investigate their control.

A new approach to the assay of APRT was tested (2. C.) and the assay and simple partial purification of AMP deaminase is dealt with in chapter 3. A brief comparison is made between the human and sheep erythrocyte enzymes.

1.G. Cyclic-AMP

1.G.a The second messenger role for cyclic-AMP

The term 'second messenger' has been coined for the action of cyclic-AMP (Sutherland et al.: 1965). It is produced in the cell by the enzyme adenyl cyclase. For the turkey erythrocyte Øye and Sutherland (1966) showed that the cyclase activity was to be found on the inside of the cell membrane and this appears to be the general site for the position of the enzyme. The assumption that this is always the case has been challenged by Rasmussen et al. (1972).

The activity of the enzyme is triggered by a hormone binding to a protein receptor on the outside of the cell. The hormone is therefore considered to be the first messenger. The activation of the adenyl cyclase across the membrane is equivalent to the transfer of information.

1.G.b. Control of cyclic-AMP concentration

The adenylate cyclase reaction is reversible (K=0.255, Takai et al.: 1971; K=0.065, Hayaishi et al.: 1971) but the high concentration of ATP (about 10^{-3}M) and the low concentrations of cyclic-AMP (about 10^{-7}M) and PP_i (about 10^{-5}M) in the cell mean that the reaction is

displaced far from equilibrium and therefore is essentially irreversible.

The concentration of cyclic-AMP in the cell is regulated by the activity of this enzyme and by the activity of cyclic-AMP phosphodiesterase (cyclic-AMP PDE). This enzyme cleaves the 3'-phosphoester bond in cyclic-AMP to give 5'-AMP. Its control appears to be independent of the hormonal control of adenyl cyclase. The cyclic-AMP PDE activity of rat cerebral cortex is controlled by a modulator protein and calcium (Teshima and Kakiuchi: 1974). The same finding has been made for the beef heart enzyme (Teo and Wang: 1973). The K_m for this enzyme was found to be 0.8μM and 25μM for the two electrophoretically distinguished isoenzymes (e.g. Tsou et al.: 1974). The K_ms for the two forms of the enzyme from human blood platelets are 50 and 500μM (Pichard et al.: 1973). These values are greater than the estimated concentration of cyclic-AMP in unstimulated tissue of 10^{-7}-10^{-6}M (Robison et al.: 1971 and Leeuput-Coutrez et al.: 1973). Therefore the rate of hydrolysis of cyclic-AMP is first order and this makes a significant increase in the cyclic-AMP concentration hard to achieve (Newsholme and Start: 1973).

1.G.c. Stimulation of cyclic-AMP dependent protein kinase

Cyclic-AMP activates a protein kinase by binding to a regulatory subunit (or cyclic-AMP receptor) which leads to the dissociation of the regulatory subunit - cyclic-AMP complex from the remaining catalytic subunit. The cyclic-AMP dependent protein kinase holoenzyme is much less active than the dissociated catalytic subunit (e.g. Yamamura et al.: 1971 ; Gill and Garren: 1971 and Kuo et al.: 1970).

The concentration of cyclic-AMP in unstimulated tissue (above) is also larger than the concentration required to stimulate cyclic-AMP dependent protein kinases in vitro. The concentration required to give half maximal stimulation is usually of the order of 10^{-8}-10^{-7}M (Reimann et al. (1971) found the value to be 1.5 and 3 x 10^{-8}M for the two kinases isolated from rabbit skeletal muscle). This does not necessarily mean that

the system is unresponsive to changes in cyclic-AMP concentration. There may be sufficient non-specific binding of cyclic-AMP by other proteins to result in a fairly low concentration of 'free' cyclic-AMP in the cell. Also the stimulation of the target pathway does not depend solely on the degree by which protein kinase activity is increased but on the relative activities of the protein kinase and phospho-protein phosphatase. It may be that only a small change in protein kinase activity is required for a significant response in the target pathway.

The partial purification of the bovine adrenal cortex protein kinase is dealt with in chapter 7. Binding studies on the enzyme are presented and discussed in chapter 8.

1.H. Adenylate cyclase in erythrocytes

Adenylate cyclase has been found in avian and frog erythrocytes (Davoren and Sutherland: 1963 and Rosen and Rosen: 1969). In mammalian red cells the adenylate cyclase activity appears to be confined primarily to the reticulocyte fraction (Sheppard and Burghardt: 1969 and Quiring et al.: 1973). The red cells do have active cyclic-AMP dependent protein kinases (Guthrow et al.: 1972 and Rubin et al.: 1973) and cyclic-AMP PDE (Sheppard and Burghardt: 1973). Therefore it appears that the developing red cell has a fully responsive cyclic-AMP second messenger system but that the adenyl cyclase is 'lost' during or soon after the maturation of the cell. This leaves the possibility open that the cyclic-AMP apparatus of the red cell is required for the very purpose of maturation or for the processes of differentiation taking place during the maturation of the cell.

Adrenalin has been observed to affect membrane properties of the red cell (Allen and Rasmussen: 1971). Noradrenalin and dibutyryl-cyclic-AMP have been shown to alter the ionic transport in red cells (Riddick et al.: 1971 and Kregenow: 1973). These results

indicate the possibility that general membrane changes are occuring which may be mediated to some extent by the cyclic-AMP second messenger system. In chapter 5 the effect of adrenalin is tested on the transport of purines across the red cell membrane.

Chapter 6 deals with the assay of cyclic-AMP and includes the results of some assays of the erythrocyte enzyme from turkey, rat and human blood.

1.J. Bovine adrenal cortex cyclic-AMP dependent protein kinase

A crude preparation of this enzyme was used for the assay of cyclic-AMP by the saturation analysis method of Brown et al. (1971). The preparation used was the 5,000 x g supernatant of the cell homogenate (section 6.B.a.). Some binding studies carried out on this preparation of the protein kinase gave non-linear binding curves (chapter 8). This indicates either the presence of multiple types of binding site differing in affinity for cyclic-AMP, or that there is a degree of positive cooperativity in the binding process. Further studies were carried out on partially purified protein kinase to examine the cause of this phenomenon.

This work shows that the simple equilibria often assumed to govern the function of the cyclic-AMP dependent protein kinases are not an adequate model for the activation of these enzymes.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.A. Introduction

This chapter contains general comments on the materials and methods used in the work for this thesis. For detailed comments on the methods and conditions used in any particular experiment the reader should refer to the relevant chapter.

2.B.a. Source of materials

Purine bases, ribonucleosides and ribonucleotides, 2,3-diphosphoglycerate, fructose-1,6-diphosphate, theophylline, adrenalin and calf thymus histone were all obtained from Sigma Chemical Co., London. 5-Phospho-α,D-ribose-1-pyrophosphate was initially obtained from PL Biochemicals, Milwaukee but subsequently from Sigma because theirs had a higher degree of purity. Adenosine-5'-triphosphate was obtained from Boehringer Mannheim GmbH. Adenosine-(8-H^3),cyclic-3',5'-phosphate and adenosine-5'-triphosphate-γ-P^{32} were obtained from the Radiochemical Centre, Amersham. All other chemicals used were of analytical or enzyme grade except where this degree of purity was not required.

2.B.b. Preparation of buffers and solutions

All buffers and solutions were made up using glass distilled water or twice glass distilled water. The pH of the solution was measured using a Pye pH meter with a combined glass and reference electrode or a glass electrode with a reference calomel electrode. The pH was measured at room temperature (about 16°C) and to within 0.05pH units of the stated value.

2.B.c. Source and preparation of blood cells

One day old samples of human blood were obtained from Warwick Hospital pathology laboratory. All such samples had potassium EDTA added as an anticoagulant.

Out of date bank blood was also used. Rat blood was obtained by cardiac puncture and turkey and sheep blood was obtained on slaughter. Blood from these sources was stabilised by sodium citrate and glucose. One volume of 3.13% sodium dihydrogen citrate was added to nine volumes of blood to prevent coagulation. 0.3M glucose was added to 1/20th the final volume in order to maintain the ATP levels in the cells.

Red cells were prepared by centrifuging the blood at 300 x g for 15 minutes at 0-4°C. (See section 2.B.g. for comments concerning centrifugation.) The supernatant, 'platelet rich plasma' (van Belle: 1969a), and buffy layer of 'white blood cells' covering the sedimented red cells were siphoned off. The precipitate, 'separated red blood cells', was resupended in about five volumes of Kreb's Ringer (table 2.1). The resuspended cells were centrifuged at 1,000 x g for 15 minutes. The supernatant and remaining buffy layer were discarded. This washing procedure was repeated twice to give 'Kreb's Ringer washed red blood cells'. Alternatively the cells were washed in 0.9% NaCl to give 'saline washed red blood cells'.

Table 2.1

Kreb's Ringer Solutions

Stock solutions	Parts by volume A	B
0.90% sodium chloride	80	100
1.15% potassium chloride	4	4
1.22% calcium chloride	3	3
2.11% potassium H_2 phosphate	1	1
3.80% magnesium sulphate.$7H_2O$	1	1
1.30% sodium H carbonate*	21	
0.16M sodium pyruvate	4	
0.10M sodium fumarate	7	
0.16M sodium glutamate	4	
0.30M glucose	5	
0.10M sodium phosphate buffer pH 7.4		21

* Gassed with CO_2 for 1h before mixing.
A Fortified Krebs Ringer; B Krebs Ringer
(Table taken from Dawson et al.: 1969)

2.B.d. Preparation of the haemolysate and stroma

Haemolysates were prepared by the addition of between two and nine volumes of distilled water to a red blood cell preparation. The lysate was then centrifuged at 3,000 x g for 15 minutes to remove stroma. The supernatant was termed the haemolysate. The precipitate was discarded unless required as stroma in which case it was washed with buffer or water before use.

2.B.e. Protein determination

Protein concentrations were determined by one of two methods. The determination of protein concentrations in general solutions was carried out by the method of Lowry et al. (1951) using the Folin-Ciocalteu phenol reagent. The concentration of stock reagents was changed, however, as it improved their storage. The commonly used solution of copper tartrate is super saturated at $0^{o}C$ and so crystallises out if kept in the refrigerator. A tenth dilution of this solution was found to be suitable for storage at 0^{o}. Therefore the concentrations of stock reagents was changed to:

A 0.1% sodium tartrate in 0.05% copper sulphate.$5H_2O$

B 2.5% sodium carbonate in 0.125N sodium hydroxide.

A mixture of 10ml A plus 40ml B gives an alkaline copper reagent C almost identical to that of Lowry et al.

The second method used in the determination of protein concentrations was to measure the absorbance of the protein solution at λ 280nm. This method was used where the protein concentration in a large number of samples had to be determined, for example the fractions eluted from a column. It is assumed that the absorbance of a protein solution with a concentration of 1mg ml^{-1} is 1.0.

2.B.f. Dialysis

All dialyses were carried out using Visking tubing which had been soaked in a solution of about 5mM EDTA and then washed with glass distilled water. Care was taken to avoid stretching the tubing. Dialysis was followed by a centrifugation step to clarify the solution.

2.B.g. Centrifugation

In the preparative stages of the work all centrifugation was carried out in a Sorvall RC2-B refrigerated centrifuge using a GSA or SS-34 head according to the sample volume. The rotor temperature was kept at about 0-4°C.

High speed clarifications were carried out either in the Sorvall or in a Beckman L-2 ultracentrifuge if a very high relative centrifugal force was required. The Beckman L-2 was also used for running sucrose density gradients with an SW 50L head.

The relative centrifugal force referred to in the individual chapters corresponds to the maximum value exerted at the tip of the tube.

Two unrefrigerated bench centrifuges were also used: a B.T.L. microangle centrifuge and a Coleman centrifuge. The former was used for routine work sedimenting small volumes of particulate material: charcoal, denatured protein and cells. The Coleman centrifuge was used for the rapid separation of blood cells from their suspending medium (section 6.B.).

2.B.h. Ammonium sulphate fractionation

Solid enzyme grade ammonium sulphate was used in fractionation procedures. All such fractionations were carried out in ice cold solutions. Additions of ammonium sulphate were made to give the required saturation according to a table in Dawson et al. (1969) for use at 0°C. Ammonium sulphate precipitates were collected by centrifugation at 10,000 x g for 30 minutes.

2.B.j. Column chromatography

The supports used for column chromatography were DEAE cellulose (Whatman DE 11 and DE 52), cellulose phosphate (Sigma) and Sephadex G-25 and G-100 (Pharmacia).

The DEAE cellulose was washed before use (and when recycled) in the recommended manner. The ion exchanger was equilibrated with concentrated buffer and the fines were removed before packing the column.

Cellulose phosphate was equilibrated with the buffer to be used. Fines were removed and the column when

poured was washed with the same buffer.

Sephadex gels were swollen in the elution buffer at 95°C to reduce the swelling time of the G 100 gel.

All columns were packed with the outlet open and the slurry was poured carefully so as to avoid forming bubbles and convection currents. The cellulose columns were protected by pieces of filter paper. The Sephadex G-100 column was protected by a layer of Sephadex G-25. All tubing connections were kept as short as possible to reduce the void volume. The chromatography was carried out in a coldroom maintained at 0-4°C.

2.B.k. Thin layer chromatography

Precoated plates from Machery and Nagel (Camlab, Cambridge - distributors) were used. They were washed before use as described in the text (section 5.B.b.).

2.B.l. Radioisotope precautions

Whenever possible protective gloves were used when handling radioisotopes and such work was limited to particular areas of bench space. Spillages were cleaned up immediately. Used or waste radiochemicals were disposed of according to the regulations laid down by the University Radiological Saftey Officer.

2.B.m. Liquid scintillation counting

Liquid scintillation counting was carried out in one of two machines: a Packard Series 4000 and a Packard Series 2450. The series 4000 counter was used for counting C^{14}, H^3 and double isotopes (C^{14} and H^3). The series 2450 counter was used for counting H^3 and P^{32}.

A simplified scheme for the molecular processes in scintillation counting is shown in table 2.2. The total efficiency E of the counting relates to the amount of radioactivity in the sample S to the count rate C

$$S = E \times C$$

and is the product of the efficiencies of the component steps in the scintillation process e_i.

$$E = \prod_i^n (e_i)$$

A quenching agent is any substance present in the scintillation vial which interferes with a step in the scintillation process. For instance, if compound M is

Table 2.2

Scintillation Processes

Process		Efficiency	
β decay	$H^3 \rightarrow He^3 + \beta + n$		
Solvent excitation	$T + \beta \rightarrow T^* + \beta'$	e_1	(1)
Excimer formation	$T + T^* \rightarrow D^*$		
PPO excitation	$D^* + PPO \rightarrow PPO^* + 2T$	e_2	(2)
PPO fluorescence	$PPO^* \rightarrow PPO + "h\nu_1"$	$e_3 \equiv \Phi_f$	
POPOP absorption	$"h\nu_1" + POPOP \rightarrow POPOP^*$	$e_4 \propto A$	
POPOP fluorescence	$POPOP^* \rightarrow POPOP + "h\nu_2"$	$e_5 \equiv \Phi_f'$	
Photocathode emission	$"h\nu_2" \rightarrow e^-$	e_6	

(1) The β particles collide with toluene molecules losing kinetic energy and converting the toluene to an excited state. (T = toluene, D = excimer and n = neutrino)

(2) Energy transfer requires 'contact' between PPO and either an excited state toluene molecule or more usually an excited state dimer (excimer). This must occur within the fluorescence lifetime of the excited state. The contact distance is about 6Å.

(Birks and Conte: 1968)

capable of interacting with PPO* causing transfer of the excited state energy to M which then loses its excited state energy by some non-radiative process. The overall effect will be a reduction in the fluorescence quantum yield of PPO which is a factor in the efficiency of the transfer of energy from PPO to POPOP.

The quenching agent most generally encountered is molecular oxygen. It is a paramagnetic quenching agent and has a high quenching efficiency.

To relate the count rate to the amount of radioactive sample it is necessary to know the efficiency of the counting and the degree of quenching.

The efficiency of counting tritium samples in the Packard series 2450 was found to be 0.65 for the channel preset for tritium counting. An external standard (Ra^{226}) radium pellet incorporated in the machine was used to calculate the degree of quenching. The relationship between the external standard ratio E_s and the degree of quenching Q was found to be volume dependent.

The relationship for 4ml scintillation fluid was found to be

$$Q = 1.1085(1 - E_s).$$

Now if C_o represents the observed count rate and C_t the true count rate then

$$C_t = \frac{C_o}{1.1085E_s - 0.1085.}$$

The equation holds true for E_s varying from 0.3 to 0.9.

P^{32} samples were counted using the preset channel for that isotope in the Packard series 2450. No corrections were made to allow for quenching.

Similarly C^{14} was counted in the series 4000 counter without any corrections being made to allow for quenching. The gain setting 10% gave optimal counts for a 50: 1000 window. The counting was at 88% efficiency.

When counting H^3 samples in the series 4000, however, quenching was significant and a quench curve was constructed using the channel ratio method. Figure 2.1 shows the quench curve and the machine settings are shown in table 2.3 together with the efficiency and and equation relating the channel ratio to the degree of quenching. The quench curve equation was obtained

Table 2.3

Settings of Packard 4000 for H^3 counting

Channel	Window	Gain	Background	Efficiency	Count
Red	50 - 1000	50%	135	0.41	
Green	50 - 150	100%	56		G
Blue	150 - 300	50%	44		B

The equation relating the degree of quenching Q to the channel ratio R is

$Q = (a + b.R^{-1} + c.R^{-2} + d.R^{-3})/(R - 1)$

where a = 1.0234, b = -0.74418, c = 0.095458 , d = -0.003471 and R = B/G.

by using a modification of "QUENCH" a computer programme for curve fitting quench data which is kept in the university computer library.

Double isotope counting for samples of C^{14} and H^3 was also corrected for quenching by the channel ratio method. The settings are shown in table 2.4.

Figure 2.1

Quench Curve for Counting H^3 in toluene: 2-EE:

PPO: POPOP Scintillation Fluid

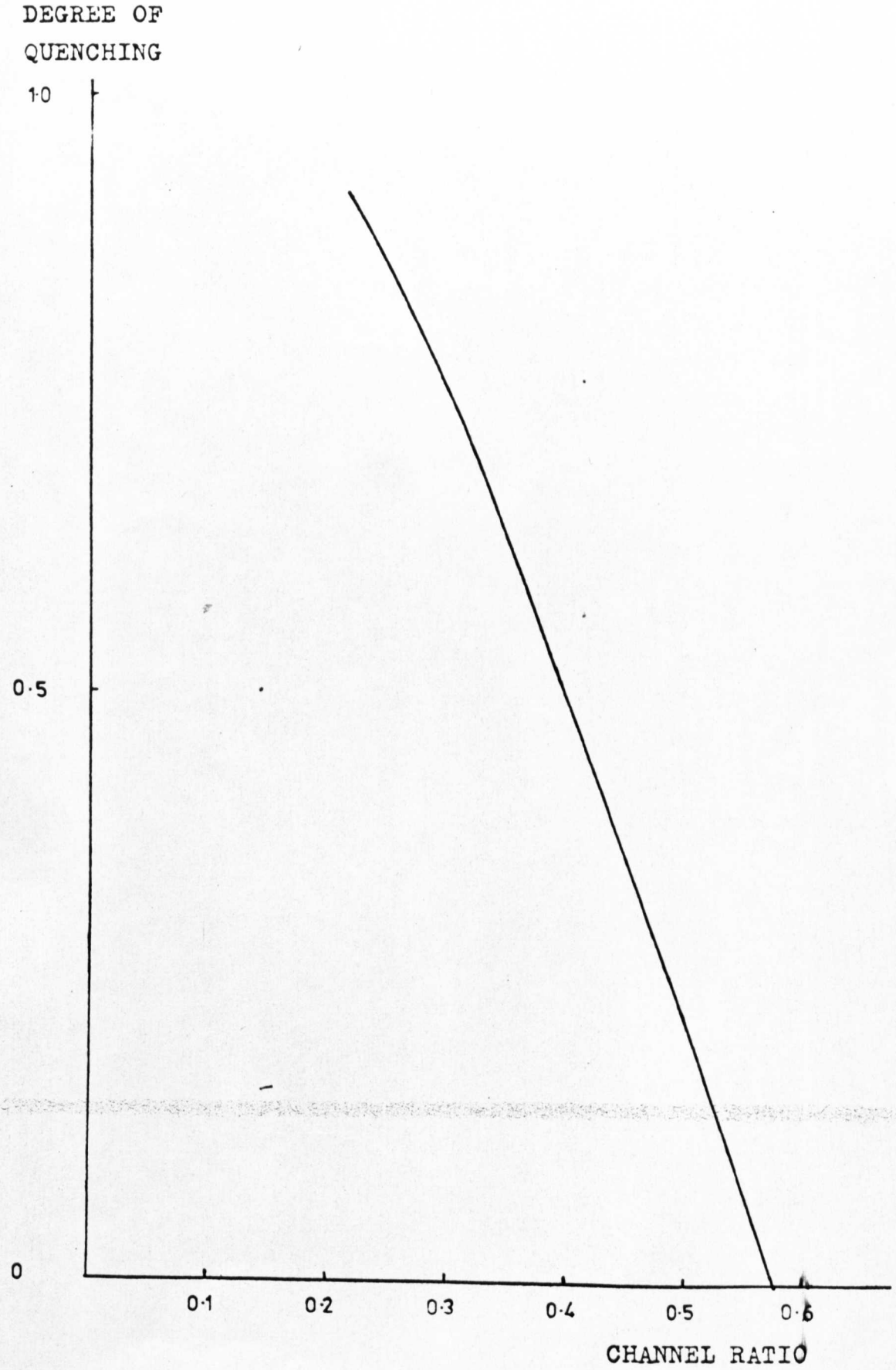


Table 2.4

Settings of Packard 4000 for C^{14} and H^3 counting

Channel	Window	Gain	Count
Red	50 - 335	50%	R
Green	220 - ∞	15%	G
Blue	220 - 370	15%	B

Equation relating channel ratio (R/G) = y to (B/G) = x is

$$y = (a.x + b.x^2 + c.x^3 + d.x^4)/(1 - x)$$

where a = -9.3141, b = 51.402, c = 90.526 and d = 53.731.

Another programme from the computer library "TRICAR" was used to calculate the C^{14} and H^3 counts from the channel readings.

In general it was found that there was little variation in the degree of quenching of tritium samples so that the quenching could be ignored without affecting the accuracy of the results. The external standard or channel ratio were still used to ensure that the variation was small.

The scintillation fluids used in the various parts of this work are denoted by their contents. So: toluene: PPO: POPOP consists of 4g PPO and 0.2g POPOP dissolved in 1l toluene and toluene: 2-EE: PPO: POPOP consists of 8g PPO and 0.4g POPOP dissolved in 1.2 litres of toluene and 0.8 litres of 2-ethoxyethanol.

2.B.n. Spectrophotometric measurements

A Zeiss PMQ-II u.v. - visible spectrophotometer was used for most fixed wavelength absorption measurements. An Eppendorf photometer was used for protein estimations by the method of Lowry et al. (1951)(see section 2.B.e.).

Fluorescence spectra were measured using a Farrand Mk I fluorometer. It is a single beam instrument. The fluorescence of a perspex block was used to check machine drift.

2.C. The stability of PRPP

It was proposed to assay the activity of adenine phosphoribosyl-transferase by hydrolysing the pyrophosphate which is produced in the enzyme catalysed reaction and determining the phosphate.

The assay for orthophosphate follows the procedure of Woolfolk et al. (1966) and is shown in table 2.5.

Table 2.5

Phosphate assay procedure

Room temperature
To test-tube add:
0.2ml sample containing 0 - 0.25μmol (25μg) ortho-phosphate
1.8ml 0.8% $FeSO_4$ in 0.015N H_2SO_4 (freshly made).
Shake well.
Add 0.15ml 6.6% $(NH_4)_2MoO_4.7H_2O$ in 7.5N H_2SO_4
Leave for 10 minutes and then measure the absorbance at 691nm.

It is a colourimetric assay which gives a linear response with up to 25μg of orthophosphate.

However, it was found that PRPP, the substrate for the enzymic reaction, was unstable to hydrolysis under the conditions of the phosphate assay. Indeed the PRPP was found to hydrolyse slowly at 0°C in pH 7.4 buffer (100mM Tris. HCl).

These findings precluded the use of a phosphate assay as a means of determining the activity of APRT. Subsequently all PRPP solutions were prepared shortly before use. A note was later obtained from Sigma Chemical Co. giving details of the stability of PRPP. Their data are in agreement with these results.

CHAPTER 3

AMP DEAMINASE

3.A. Introduction

AMP deaminase catalyses the hydrolytic deamination of AMP to give IMP and ammonia. The enzyme has been studied from a wide variety of sources though principally from rabbit muscle (Lee: 1957a and Smiley et al.: 1967).

Setlow and Lowenstein (1967) measured the activity of the enzyme by the quantity of ammonia produced in a fixed time. Burger and Lowenstein (1967) used a radio-isotope stop assay. They separated the product from the substrate by ion exchange chromatography. The enzyme is more usually assayed, however, by the method of Kalckar (1947). He demonstrated how the differences in U.V. absorption of the purine bases could be used in the measurement of their concentrations in a mixed solution. The difference in molar extinction is at its largest at λ265nm. The progress of the reaction is measured by the decrease in absorbance as AMP is converted into IMP.

A comparison is made here between the U.V. absorption and fluorescence properties of AMP and IMP. AMP deaminase was assayed by measuring the change in U.V. absorption at λ265nm (3.C.).

The assay of the enzyme in dialysed haemolysates was hindered on several counts. This was due

(i) to the presence of very large concentrations of protein

(ii) to the presence of undialysed inhibitor, even after prolonged dialysis

(iii) to the low rate of conversion of AMP into IMP.

Consequently a partial purification of the enzyme was investigated. The aim was to remove the main protein contaminant, haemoglobin, and to raise the activity of the enzyme solution.

A similar purification was carried out on the AMP deaminase from sheep erythrocytes. These red cells have a very low concentration of 2,3-DPG (Blunt et al.: 1971 and Rapoport and Guest: 1941).

3.B. Materials and methods

The sources of materials are as described in section 2.B.a.. All the methods used in the work comprising this chapter are dealt with in sections 2.B.b. - 2.B.j..

3.C. Determination of enzyme activity

3.C.a. Determination of AMP in the presence of IMP

A comparison was made between the use of the U.V. absorption of AMP and IMP and the use of their fluorescence as a basis for their determination.

The fluorescence of AMP is greatest in approximately 3N acid and has an excitation maximum at the wavelength 275nm and a fluorescence maximum at 390nm. IMP has almost negligible fluorescence under these conditions. However, the use of fluorescence measurements for the determination of AMP are limited in two respects. Firstly, the sensitivity of the fluorometer to the presence of residual protein and turbidity adversely affects the assay sensitivity and secondly, the range over which the assay response is linear is restricted by the absorbance of the AMP solution.

The relative fluorescence (I_{rel}) of a clear solution is given by the relationship (Udenfriend: 1961):

$$I_{rel} = I_o(1 - 10^{-\epsilon cd})\phi_f,$$

where c is the concentration of the fluorescent species, ϵ is the extiction coefficient, d is the optical depth and ϕ_f is the fluorescence quantum yield. In very dilute solutions the second order and higher terms in the exponential function can be ignored so

$$I_{rel} = I_o(2.3\epsilon cd)\phi_f.$$

So I_{rel} varies linearly with concentration until the absorbance of the solution becomes significant (c.a. 0.02).

The millimolar extinction for AMP at a wavelength of 275nm is approximately 4. The optical depth in a 1cm cell is 0.5cm. The concentration limit for the linear relationship is, therefore, 20μM. The relative fluorescence intensity of a 20μM solution of AMP was found to be only six times the background light intensity. This makes the difference in fluorescence between AMP and IMP an unsatisfactory measure of their concentration.

The U.V. absorbance of solutions of AMP and IMP was not affected to such an extent by limiting factors. The wavelength selected for the U.V. absorbance measurements was 265nm. This wavelength was chosen on the basis that it gave the largest percentage difference between the absorbance of AMP and IMP at the same concentration. This was the same wavelength as used by Kalckar (1947).

3.C.b. Assay of AMP deaminase activity

i) The assay of the enzyme activity in dialysed haemolysates was carried out by pipetting the solutions shown in table 3.1 into a small test-tube. The reaction mixture was incubated at 37°C for two minutes to establish thermal equilibrium before the reaction was started by the addition of haemolysate.

Table 3.1

Conditions used for the assay of AMP deaminase

Temperature 37°C

Shaking water bath (c.a. 2 c/s)

Solutions used:

0.5ml	200mM Tris.HCl pH 7.1 with 250mM KCl	
0.5ml	10mM ATP	
	10mM AMP	to a volume of 2.5ml
	haemolysate	
	water	

The reaction was terminated by deproteination with 8% perchloric acid. Denatured protein was removed by centrifugation. The progress of the reaction was measured spectrophotometrically (3.C.a.).

ii) This assay was used for determining the activity of fractions from columns where it avoids the use of large numbers of centrifugation steps.

The incubation mixture was essentially the same as above except that the volume was reduced to 1.5ml. (The other conditions are shown in table 3.1.) The reaction was terminated by the removal of a 50μl aliquot which was added to 2ml of 5mM EDTA (brought to pH 8.3 with Tris base). The absorbance of this solution was then measured at λ265nm. A correction for protein absorbance was made where necessary. The EDTA solution did not entirely stop the reaction as was shown by leaving the stopped reaction overnight and redetermining the absorbance. However, the reaction was slowed down sufficiently to consider it stopped over the period of time required for obtaining the absorbance measurements.

iii) The partially purified enzyme had a sufficiently high specific activity to enable the use of an assay involving the continuous reading of the absorbance at λ265nm. This necessitated, however, the use of low (non-saturating) concentrations of AMP and ATP (0.18mM and 0.12mM respectively). The change in absorbance against time was linear for 20 minutes and varied between 0.012 and 0.002 per minute. In view of the low concentrations of AMP and ATP required in this assay it was not often used.

iv) A correction was made by including a control tube in the assay incubation in which water replaced both AMP and ATP solutions. The control tube was deproteinated like the reaction tubes and the supernatant was diluted in the same proportions. The absorbance of the diluted solutions was measured at wavelengths 265nm and 290nm. If the control absorbance for each wavelength is A^P_{265} and A^P_{290} respectively and the absorbance for each reaction is A^R_{265} and A^R_{290} at the two wavelengths then the corrected absorbance is

$$A^C_{265} = A^R_{265} - A^R_{290}(A^P_{265} / A^P_{290}).$$

This is because the contribution of the nucleotide to the absorbance at λ290nm can be considered insignificant.

3.D. Partial purification of AMP deaminase from human erythrocytes

A summary of the individual steps described in detail here is given in table 3.2 at the end of section 3.D.d..

3.D.a. Preparation of the haemolysate

The haemolysates were prepared from washed red cells (2.B.c.) as described in section 2.B.d. The AMP deaminase specific activity of the haemolysates varied considerably, depending on the dialysis, from 0.014 to 0.194 µmol/h/mg. Some samples were not dialysed and had low activity presumably due to the presence of 2,3-DPG which is present in red cells at concentrations varying between 5 and 7mM. This assumption was not directly tested although dialysis resulted in increased activity of the enzyme. The yield of AMP deaminase could be increased about 10% by washing the stroma after the centrifugation following the lysis of the cells. However, this invariably led to a dilution of the enzyme and consequent decrease in activity of the enzyme and was not therefore generally used.

3.D.b. Ammonium sulphate fractionation

Addition of ammonium sulphate to 25% saturation to haemolysates with 35mg/ml protein gave very little precipitate. Increasing the concentration of ammonium sulphate to 55 - 57% saturation resulted in the precipitation of almost all the AMP deaminase activity. Haemoglobin, which accounts for 97% of the erythrocyte protein, precipitates at about 60% ammonium sulphate saturation.

The enzyme was therefore precipitated by adding ammonium sulphate to 57% saturation and collecting the pale pink precipitate by centrifugation. The pellet was resuspended in 20mM Tris.HCl pH 7.1.

The enzyme was thus purified about 30 fold with a yield of 60% but was no longer stable to dialysis.

3.D.c. DEAE cellulose column chromatography

The dialysed haemolysate (3.D.a.) was put on a 15 x 3cm column of DEAE cellulose equilibrated with 20mM Tris.HCl pH 7.2. The column was sequentially washed with 20mM Tris.HCl pH 7.2 and 50mM Tris.HCl pH 7.2 until no more protein was eluted. The haemoglobin was removed by these washes. The enzyme was eluted with 50mM Tris.HCl pH 7.2 containing 0.5M KCl. The active fractions were pooled. The column was used principally as a means of separating the enzyme from haemoglobin. The conditions described above ensured that this was done and that the enzyme was eluted in a small volume. (See table 3.2)

3.D.d. Cellulose phosphate column chromatography

A 77ml sample of DEAE cellulose purified enzyme was applied to the cellulose phosphate column which was equilibrated with 50mM Tris.HCl pH 7.2 containing 0.1% 2-mercaptoethanol. The column was washed with the equilibration buffer, 100mM Tris.HCl pH 7.2, containing 0.1% 2-mercaptoethanol and the same buffer containing 0.4M KCl. Each wash continued until no more protein was eluted. The enzyme was finally eluted with 1.0M KCl in 100mM Tris.HCl pH 7.2 containing 0.1% 2-mercaptoethanol.

3.E. Assay controls, enzyme activation and inhibition

3.E.a. Assay controls

The spectrophotometric assay cannot distinguish between AMP deaminase activity and deamination of adenosine produced by phosphatase activity in the presence of AMP.

Using an EDTA stopped assay (3.C.d) the activity of the enzyme eluted from a DEAE cellulose column was measured using 0.95mM AMP, 0.77mM adenosine and a mixture of both at the same final concentrations. The activities are shown in table 3.3.

Table 3.2

Summary of Purification Steps

Step	Volume ml	Protein conc. mg/ml	Activity µmol/ml/h	Specific activity µmol/mg/h	Yield %
Haemolysate	-	18	0.25	0.014	-
Dialysed haemolysate	-	62	7.4	0.194	-
Haemolysate	840	35	4.4	0.125	100
Ammonium sulphate 0 - 57%	116	7.5	19	2.5	58
Dialysed	138	6.3	2.7	0.42	10
Haemolysate	130	62	7.4	0.119	100
DEAE cellulose	73	3.8	10.8	2.84	84
Dialysed DEAE cellulose	77				
Cellulose phosphate	25.5	0.05	12.0	240	32

Table 3.3

Deamination of AMP and adenosine by 'DEAE cellulose enzyme'

Substrate concentration (mM)		Activity
AMP	adenosine	ΔA/h/ml
0.95	-	6.00
-	0.77	1.17
0.95	0.77	6.18

The K_m for adenosine deaminase is 40μM. Therefore the activity 1.17ΔA/h/ml in the presence of 0.77mM adenosine should represent the maximum rate of deamination. The deamination rate when both adenosine and AMP were present, 6.18ΔA/h/ml, should represent the maximum deamination rates for both deaminases. The rate of deamination by AMP deaminase alone, therefore, is the difference between these rates and is 5.01ΔA/h/ml. The contribution of the adenosine deaminase (combined with 5'-nucleotidase) to the deamination rate when only AMP was present was 17%. In another preparation this contribution was less than 14%.

3.E.b. Enzyme activation and inhibition

(i) The enzyme was assayed in the presence of 10mM KCl, 0.18mM AMP and 0.12mM ATP using the continuous assay (3.C.b.). The activity was 0.78 mol/mg/h. On the addition of 0.95, 2.38 and 4.75mM 2,3-DPG (final concentrations) the enzyme was inhibited by 41%, 84% and 100% respectively.

(ii) The enzyme was assayed as in (i) but with AMP concentrations of 0.18, 0.16 and 0.08mM and ATP concentrations of 0.12, 0.10 and 0.055mM respectively. The activities, 78, 64 and 12 mol/mg/h, were proportional to the product of the AMP and ATP concentrations.

(iii) The association of AMP deaminase with the cell membrane was measured using the EDTA stop assay (3.C.b.). The stroma had a low AMP deaminase activity which slowly increased with time (0.21 mol/ml/h when fresh and 0.42 mol/ml/h after 1 hour at 0°C). Addition of triton-X-100 or SDS increased the activity over threefold at concentrations of the detergents of 0.001% and 0.10mM respectively. In the presence of detergent the enzyme activity decreased with time. Higher concentrations of detergent resulted in loss of activity.

3.F. Sheep blood AMP deaminase: preparation and assay

Sheep blood was collected at slaughter. After 2h the coagulated blood was chopped and centrifuged at 3,000 x g. The supernatant, serum , was removed. The precipitate was washed twice with 4 volumes of 154mM NaCl. The washed coagulated cells were lysed by

homogenisation in a blender (Attomixer) with 2 volumes of 25mM Tris.HCl pH 7.4 containing 0.1% 2-mercapto-ethanol. Cell debris was removed by centrifugation at 10,000 x g for 20 minutes.

A portion of the supernatant (25ml) was dialysed for 2h against 2 litres of 25mM Tris.HCl pH 7.4 containing 0.1% 2-mercaptoethanol. Another 25ml portion had ammonium sulphate added to 57% saturation. The resultant precipitate was collected in the same buffer and dialysed as above. The ammonium sulphate precipitate contained a lot of haemoglobin.

The activity of the haemolysate 10,000 x g supernatant and ammonium sulphate precipitate were measured using the EDTA stop assay. The results are shown in table 3.4.

Table 3.4

AMP deaminase activity of sheep blood preparations

Enzyme sample	Age days	AMP mM	ATP mM	Activity ΔA/h/ml
Haemolysate before dialysis	<1	0.18	0	0.18
Haemolysate after dialysis	<1	"	"	0.15
0-57% ammonium sulphate ppt.	<1	"	"	0.33
"	2	"	"	0
Haemolysate	5	"	"	0.09

An ammonium sulphate precipitate (0-40% fraction) was obtained from the 25ml of the haemolysate 10,000 x g supernatant which was stored at 0°C. The precipitate was resuspended and dialysed as described above. The dialysed ammonium sulphate precipitate was assayed for AMP deaminase activity with the effectors shown in table 3.5.

Table 3.5

Activation of sheep blood AMP deaminase

Enzyme	AMP mM	Effector 0.67mM	Activity ΔA/h/ml
Ammonium sulphate ppt. (0-40%)		-	0
		ATP	0.253
		2,3-DPG	0
		Fructose-1,6-diP	0.053

3.G. Discussion

3.G.a. The relative change in fluorescence (excitation λ275nm and emission λ390nm) on the conversion of AMP to IMP is much larger (c.a. 94%) than the relative change in absorbance (c.a. 60%). However, the useful concentration range (0-30μM) for fluorescence measurements is much less than that for absorbance measurements (0-120μM).

Protein fluorescence, which is mainly due to tyrosine and tryptophan residues (excitation λ_{max} 275 and 287nm and emission λ_{max} 303 and 348nm respectively), represents a more serious problem in the measurement of purine fluorescence than the protein absorbance at λ265nm does in the measurement of purine absorbance.

3.G.b. The control assay (3.E.a.) showed that 5'-nucleotidase (or other phosphatases) and adenosine deaminase contributed to the apparent activity of AMP deaminase assayed spectrophotometrically. Generally 5'-nucleotidase requires magnesium for activity. The enzyme from calf intestine is irreversibly inactivated by EDTA (Center and Behal:1966). The magnesium can be reversibly removed by exhaustive dialysis, but the dialysis steps and other preparative stages here were either insufficient to remove the magnesium or the phosphatase activity was not magnesium dependent. ATP generally inhibits 5'-nucleotidase so assays with ATP should be free from this artifact if the phosphatase is 5'-nucleotidase.

3.G.c. A simple but satisfactory partial purification of human erythrocyte AMP deaminase was carried out using a DEAE-cellulose column. The resultant enzyme preparation

retained some of the characteristics observed by Askari et al. (1965 and 1968). It also contained a deamination route involving adenosine deaminase.

3.G.d. Rao et al. (1968) showed that water lysed red blood cells gave stroma fractions containing 58% of the whole cell AMP deaminase activity. Successive washes of this fraction reduced the activity to 16% of the total activity. Lee and Wang (1968) treated particulate fractions of rat brain and liver with various detergents to examine their effect on the AMP deaminase activity. They concluded that the detergents released activity from the particulate fractions and that anionic detergents SDS and deoxycholate inhibited the enzyme possibly by dual interactions with the substrate and activator sites. The inhibition was effective at approximately millimolar concentrations of the detergents.

Here SDS increased the activity of erythrocyte membrane AMP deaminase but inhibited at 1mM. There was also a time dependent loss of activity, presumably due to denaturation of the enzyme. Triton X-100, a non-ionic detergent, also gave increased activity at 0.01% but inhibited at higher concentrations. The time dependent loss of activity was also seen with triton.

3.G.e. The main difference observed between sheep erythrocyte AMP deaminase and human erythrocyte AMP deaminase was the lack of activity of the sheep enzyme in the absence of ATP. This has also been found for the enzyme from cat and dog erythrocytes (Askari and Franklin: 1965). Sheep erythrocytes have a much lower concentration of acid soluble phosphate esters (15.4mg/100ml) than human erythrocytes (55mg/100ml). The normal concentration of ATP and 2,3-DPG in sheep erythrocytes is 8.4 and 0.8mg/100ml respectively (Rapoport and Guest: 1941). Using a conversion factor to convert this to millimolar with respect to cell water (Bunn et al. 1971) the concentrations in sheep red cells are ATP 1.24mM and 2,3-DPG 0.2mM (c.f. table 1.2). This means that it might have been expected that sheep erythrocyte AMP deaminase has different regulatory properties to the human enzyme as appears to be the case.

CHAPTER 4

AN ATTEMPT TO DETERMINE THE FLUX IN PURINE PATHWAYS

4.A. Introduction

A technique for the separation of purine ribonucleotides, ribonucleosides and bases was developed. This technique was used to estimate the interconversions of radioactively labelled purines following an incubation of red blood cells with a labelled purine base. While this technique does not indicate the true flux in any pathway it does serve to indicate the relative importance of different paths.

Flux studies using radioisotopically labelled substrates have been used to show that purine bases are incorporated primarily into the corresponding nucleotides. Hypoxanthine was the only base which was significantly incorporated into the nucleotide fraction corresponding to a different base, namely guanine. This requires the prior formation of IMP and its subsequent conversion to GMP (Bishop: 1960). Lowy et al. (1958) demonstrated that adenosine uptake was dependent on the concentration of phosphate. Uptake was greater in phosphate buffer than in physiological saline. Only 20% of the adenosine was converted to nucleotide. The remainder was deaminated. These results were obtained by analysis of intra- and extra-cellular ribose, lactate and ammonia. Van Belle (1969b) demonstrated the rapid assimilation of adenosine by the blood cells of a variety of different species using an automatic colourimetric assay for adenosine (van Belle: 1969c). As adenosine is a vasoactive agent in regulating the coronary blood supply, its rapid uptake is an essential control on this action.

Hershko et al. (1967) used C^{14} labelled purines to demonstrate the pattern of uptake of purine bases in vitro and their subsequent release. Hypoxanthine and xanthine were the products released regardless of the purine incorporated into the cell. Some xanthosine was also released but this was shown to be due to the lack of glutamine in their incubations. Bertles and Beck

(1962) have shown that in the maturation process of rabbit reticulocytes the only purine released from the cells is hypoxanthine. The quantity released was proportional to the RNA breakdown in the cells. Hershko et al. (1963) examined the conversion of guanine to hypoxanthine. It was shown to take place via GMP and IMP. Most of the guanine was converted to GTP.

These studies on purine flux have demonstrated the possible functions of some of the pathways but the actual control and interrelationship of the pathways remains open to investigation. Subject to the limitations of examining flux by analysis of the products from a radioisotopic tracer (see discussion 4.D.) it was hoped that some headway might be made in understanding the controlling factors relating to the stabilisation of the adenine nucleotide pool and the control of the deamination routes.

4.B. Materials and methods

4.B.a. Incubation of labelled purine with red cell preparation

Washed red blood cells from human one day old blood samples were prepared as described in section 2.B.c. and haemolysates were prepared as described in section 2.B.d.

The incubation was carried out by adding 10 or 50µl of C^{14}-labelled purine base (2.4mM and 3.7µCi/µmol)* to 1ml of a 1 in 10 (or 1 in 4) suspension of red cells in Kreb's Ringer, or an equivalent haemolysate, in a small test-tube. The incubation was carried out at 37°C in a water bath with agitation.

The incubation was teminated by one of two methods. One was to add 0.5ml of 2.4M perchloric acid. The other was to separate the cells from the suspending medium by refrigerated centrifugation (5 minutes at 3,000 x g) and the supernatant removed. The cells were washed once with Kreb's Ringer and resuspended to the original volume of the incubation mixture, 0.5ml of pechloric acid was then added to the first supernatant and to the resuspended cells. All these operations were carried out at 0°C and as quickly as possible. The precipitated protein was

* Obtained from the Radiochemical Centre, Amersham.

removed by centrifugation and the supernatant decanted off and neutralised with $KHCO_3$. The neutralised supernatant was cooled to 0°C and the crystallised $KClO_4$ removed by centrifugation.

In general the final supernatant was reduced in volume on a rotary evaporator before separating the radioactive components.

4.B.b. The separation of purine bases, nucleosides and nucleotides by thin layer chromatography

i) Choice of method

There are a number of systems available for the separation of purine compounds (Randerath: 1964; Stahl: 1961; Randerath and Randerath: 1967; Lederer and Lederer: 1957; Caldwell: 1969; Meyskens and Williams: 1971; Hershko et al.: 1967 and others). The technique chosen was that of Crabtree and Henderson (1971) who used one dimensional tlc to separate the purine nucleotides and two dimensional tlc to separate the nucleosides and bases. The advantages of this system over other tlc methods are twofold. Firstly, the streaking of the spots is minimal. Secondly, it is not necessary to make an initial separation between the bases and nucleosides and the nucleotides. It is therefore relatively speedy and simple to use. The advantage over column chromatography (such as Caldwell: 1969) was that the separated compounds could be cut from the tlc plates and counted without the problems involved in counting dilute aqueous samples.

ii) Pre-treatment of the tlc plates

Precoated plates (see section 2.B.k.) were used. They had to be washed before use for the best results.

The PEI-cellulose sheets were developed for five hours with 4M sodium formate buffer pH 3.4. The plates were dried and developed overnight with 50% methanol. The sheet was dried before use.

Cellulose sheets were washed either with distilled water or the solvent used in the first dimension of the separation.

iii) Separation of purine ribonucleotides

The PEI-cellulose sheets were marked lightly with a very soft pencil with lines 2.0, 4.5, 9.0 and 19.0cm

from the base of the plate. A wick of Whatman 3MM chromatography paper (approximately 7 x 20cm) was stapled to the top edge of the sheet. Carrier nucleotides in a solution containing 30nmol of each nucleotide were applied to spots on the 2.0cm line so that the spots were at least 2.0cm apart. In this way up to nine samples could be chromatographed on the same plate.

The sample (prepared as described in section 4.B.a.) was then applied to the spot. The plate was washed overnight with 50% methanol and the wick containing the purine bases and nucleosides was discarded, and the plate dried.

The plate was developed by stepwise elution with sodium formate buffer pH 3.5 as follows:

0.5M to 2.5cm line above the origin,
2.0M " 7.0cm " " " " ,
4.0M " 17.0cm " " " " .

The plate was then dried and the separated nucleotides were visualised under λ254nm U.V. light. The spots were circled in pencil. The elution pattern is shown in figure 4.1.

iv) Separation of purine bases and nucleosides

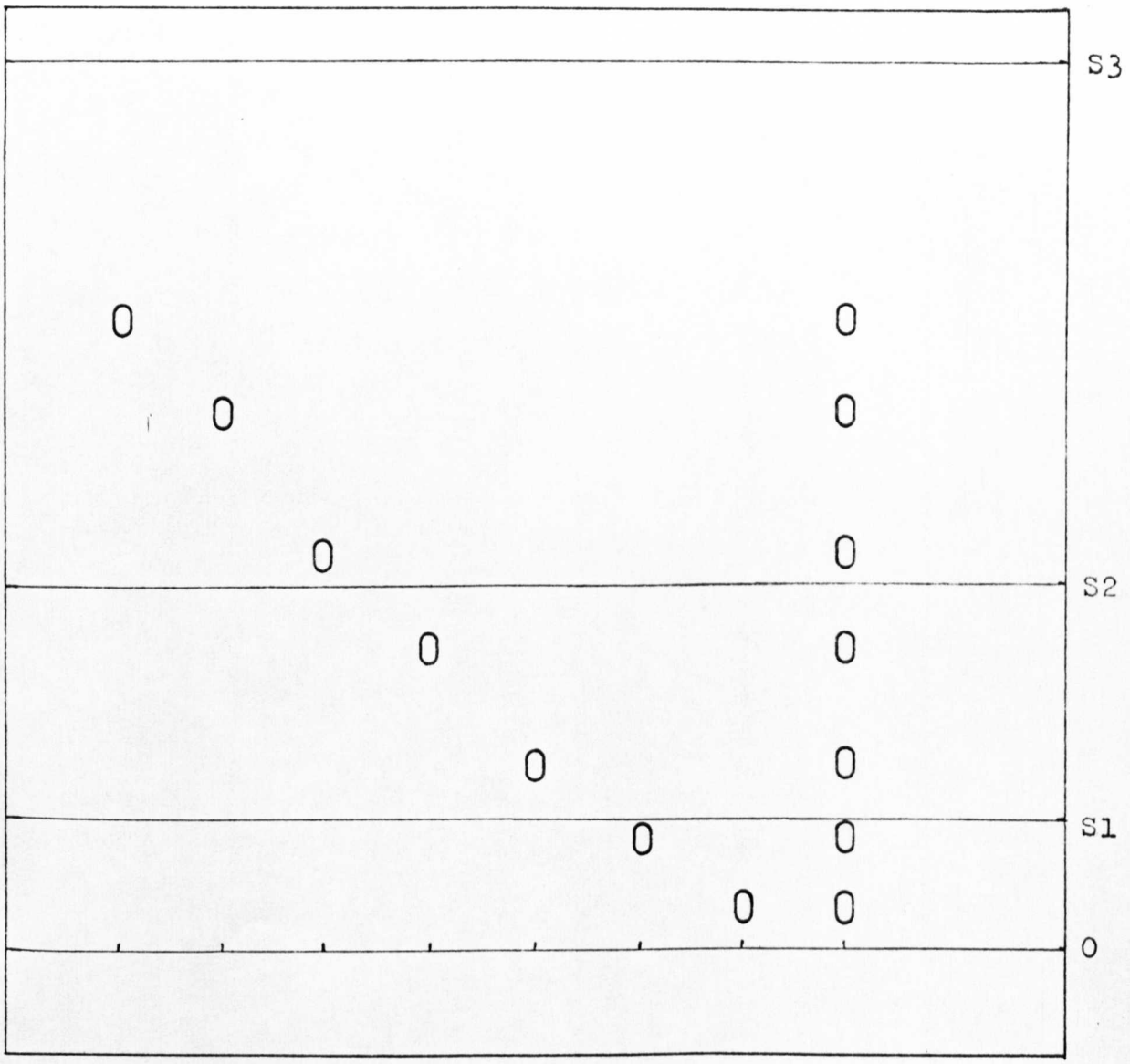
The cellulose sheets (section 2.B.k.) were cut into two 10 x 20cm plates. Carrier bases (30nmol each in 1-butanol solution) and carrier nucleosides (30nmol each in aqueous solution were applied to the origin which was a spot 2.5cm from either edge of the sheet. The sample was then applied to the spot. The solvent for the first dimension (20cm) was acetonitrile: 0.1M ammonium acetate pH 7.0: ammonia (SG 0.880) in the ratio 60:30:10 by volume. The plate was developed for 50 minutes in this solvent and dried.

The areas below the origin and 3cm from the top of the plate were removed. The plate was then developed in the second solvent which was composed of 1-butanol: methanol: water: ammonia in the ratio 60:20:20:1 by volume. The plate was developed to the top, dried and redeveloped in the same solvent. After drying the sheet the spots were visualised under λ254nm U.V. light. The elution pattern is shown in figure 4.2.

Figure 4.1

Elution Pattern for Purine Nucleotides

on PEI-cellulose tlc



0 = origin

S1 = limit of solvent 1 (0.5M sodium formate buffer pH 3.5)

S2 = " " " 2 (2.0M " " " " ")

S3 = " " " 3 (4.0M " " " " ")

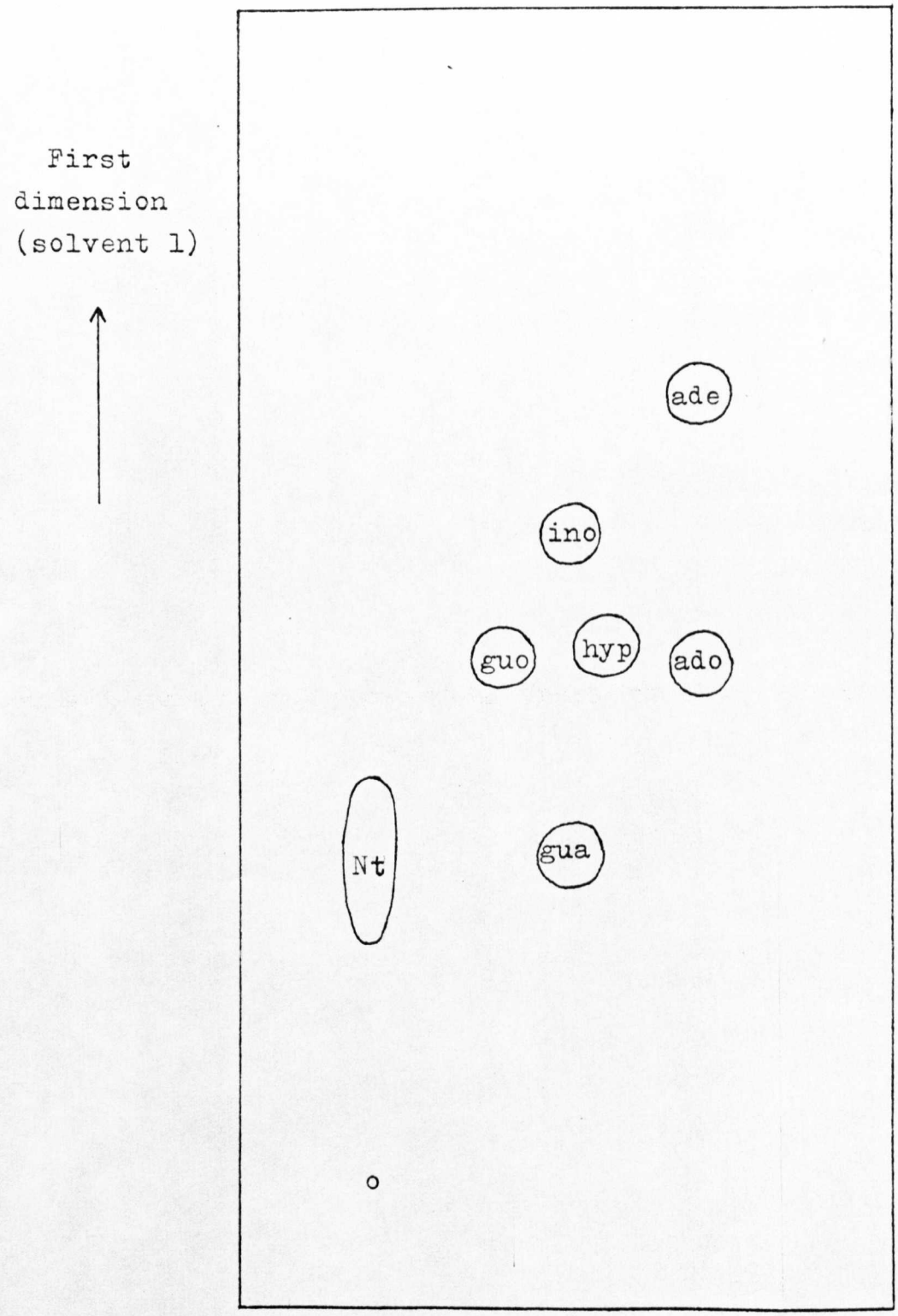
Figure 4.2

Elution Pattern for Purine Bases and Nucleosides on cellulose tlc

Solvents:

1) CH_3CN: 0.1M $CH_3CO_2^-.NH_4^+$, pH 7.0: NH_4OH (S.G. 0.88) 60:30:10

2) 1-butanol: methanol: water: ammonia (S.G. 0.88) 60:20:20:1



4.B.c. Liquid scintillation counting

The separated purine compounds were cut out from the tlc sheets in 1cm squares. (The backing of the tlc sheets was polyterephthalic acid and could easily be cut with either scissors or sharp knife.) The squares were placed into empty scintillation vials and the scintillation fluid was then added. The scintillation fluid was 4ml toluene: PPO: POPOP (see section 2.B.m.).

4.C. Results

The results of three incubations are shown in tables 4.1, 4.3 and 4.4. These tables contain details of the incubations and the results as described below.

i) (Table 4.1) Kreb's Ringer washed cells (2.B.c.), haemolysate (2.B.d.) and dialysed haemolysate were incubated with C^{14}-adenine and C^{14}-hypoxanthine under the conditions described above (4.B.a.). The incubation was terminated by the second method described there so as to measure the distribution of the label between the cells and the suspending medium. This is shown in the table together with the distribution of the label between the various compounds.

The uptake of adenine by rat erythrocytes (chapter 5: table 5.2) approached equilibrium in about 40 minutes. It is likely, therefore, that the percentage uptake shown here is an equilibrium value as the incubation time was 75 minutes.

A calculation of the change in concentration of the labelled adenine or hypoxanthine in the suspending medium and the apparent change in concentration of the labelled compound inside the cell shows that the incubations of 12μM label may have attained equilibrium across the cell membrane but the incubations with the 114μM label have not (table 4.2).

The uptake of purine was 14nmol/(ml of erythrocytes) in the case of the 12μM adenine and 12nmol/(ml of erythrocytes) in the case of the 12μM hypoxanthine. These figures correspond to the total uptake of purine and not to a rate of diffusion across the membrane. The comparable figure for washed rat red cells is 54nmol/ml.

Table 4.1

Incubation of Red Cell Preparations with Purine Bases

Suspension medium: Kreb's Ringer B* + 5% of 5.4% glucose solution
Cell preparations: A: 2ml 1 in 4 suspension of Kreb's Ringer washed cells. B: 2ml 1 in 5 haemolysate. C: 2ml 1 in 5 haemolysate (dialysed against phosphate buffer pH 7.4). Also used D: 2ml serum, and E: 2ml ringer.
(* see table 2.1)
Tracer: C^{14}-adenine and C^{14}-hypoxanthine (3.7μCi/μmol)
Temperature: 37°C. Gaseous phase: air. Incubation time: 75 minutes.

Sample	Fraction	Recovery of counts %	% uptake	% Coversion to nuclt.	Predominant compounds
A + 114μM adenine	Cells	12	12	4.5% ±1.5	AMP: ATP = 2:1
		16			ado:ade:ino = 10:9:1
	Supernatant	4	-	0	ade
	Cell wash	0	-	0	ade:hyp = 6:1
A + 12μM adenine	Cells	29	29	14% ±7	AMP:ATP = 2:1 ade:ino = 7:2
		94			
	Supernatant	66	-	0	ade
	Cell wash	0	-	0	ade:hyp = 6:1
B+ 114μM adenine	Haemolysate	55	-	1	AMP:ADP:ATP = 7:3:2
B+ 12μM adenine	"	99	-	5	AMP:ADP:ATP = 2:1:1
C+ 114μM adenine	Dialysed haemolysate	93	-	0.2	AMP; ade:hyp:ado:ino = 67:2:10:1
C+ 12μM adenine	"	104	-	1	AMP; ade:hyp:ado:ino = 55:2:3:1
A + 114μM hyp	Cells	14	14	5	IMP:GMP:GTP = 3:4:3
		100			hyp:ino:guo/gua = 2:1:1
	Supernatant	86	-	0	hyp
	Cell wash	0	-	0	-
A + 12μM hyp	Cells	25	25	4.5% ±3.5	IMP:GMP:GTP = 4:1:1
		99			hyp
	Supernatant	74	-	0	"
	Cell wash	0	-	0	-
B+ 114μM hyp	Haemolysate	116	-	0.5	IMP:GTP = 2:7 hyp:ino:guo/gua = 11:2:1
B + 12μM hyp	"	58	-	0.2	IMP; hyp
C+ 114μM hyp	Dialysed haemolysate	98	-	0.3	IMP:GTP = 1:3 hyp:ino:guo/gua = 5:3:1
C + 12μM hyp	"	74	-	0.5	IMP:GTP = 1:1 hyp:ino:guo/gua=6:5:1

Table 4.2

Uptake of Labelled Purine Bases by Human Erythrocytes					
C^{14} Tracer	Volume of Tracer	Time (min)	Concentration in suspending medium	Apparent concentration in cells	Conversion to nucleotide for equilibrium
ade	100µl	0	150µM	-	-
		75	132µM	58µM	Not equm.
ade	10µl	0	15.9µM	-	-
		75	11.3µM	14.0µM	1.35nmol (5.6%)
hyp	100µl	0	150µM	-	-
		75	129µM	68µM	Not equm.
hyp	10µl	0	15.9µM	-	-
		75	11.9µM	12.0µM	0.05nmol (0.2%)

This correlates with the difference in the content of acid soluble phosphorus (particularly ATP) between rat erythrocytes and human erythrocytes (Rapoport and Guest: 1941). They measured the concentrations of ATP in rat and human erythrocytes. The concentrations were 34.1 and 13.5mg/100ml respectively. (These are equivalent to 3.67 and 1.45mM.)

The amounts of adenine and hypoxanthine taken up were comparable, but more of the adenine (25-60%) than of the hypoxanthine (4-35%) was converted to nucleotide. This is surprising considering the relative activities of APRT and HGPRT in the human erythrocyte. A more expected result was the lower conversion to nucleotides in the haemolysate and dialysed haemolysate incubations.

ii) (Table 4.3) Kreb's Ringer washed cells (2.B.c.) in a 1 in 10 suspension were incubated with 0.114mM H^3-hypoxanthine (83.3µCi/µmol). The conditions of the incubation are described in the table and in section 4.B.a.. Four separate blood samples were used. The cells were not separated from the suspending medium before the addition of the perchloric acid. The table shows the percentage of the total counts for each incubation mixture that was found in the nucleoside sheet corresponding to hypoxanthine, inosine, nucleotides, adenine and adenosine. Separate chromatographic runs of the mixtures to determine the nucleotides showed that there

Table 4.3

Blood sample	Percentage of total counts					Poor separation between
	hyp	ino	IMP	ade	ado	
1	76.5	3.8	18.9	0.3	0.6	
	70.5	5.4	22.4	1.8	0.6	
	87.1	2.3	9.8	0.5	0.2	
2	47.5	33.8	16.4	2.2	0.2	hyp + ino
	83.0	4.3	8.2	4.5	0	
3	88.0	3.4	6.9	0.7	0.9	
	53.4	8.6	32.2	4.5	1.5	hyp & IMP?
4	57.7	29.5	10.5	1.0	1.2	hyp & ino
	90.9	2.9	5.0	0.4	0.8	
Overall average ± S.D.*	82.7 ±7.8	4.4 ±2.1	12.3 ±6.2	1.8 ±1.7	0.7 ±0.5	

* Averages do not include counts where there was a poor separation of the components concerned, those being the figures underlined. They are more than 3 x S.D. from the average given.

was negligible incorporation into any nucleotide apart from IMP.

A discrepancy appeared in the determination of the hypoxanthine and inosine counts in samples 2 and 4. This was because of the rather close proximity of the chromatographic spots. Figure 4.2 shows the position of these spots as found for aqueous or 1-butanol solutions of the compounds. The impurities in a cell extract caused the slurring of the spots so that complete resolution was not always possible.

The conversion to adenine and adenosine was barely significant. The counts involved were little over the background count and the percentage conversion was almost equal to the standard deviation.

iii) (Table 4.4) Four samples of blood were prepared and incubated in the same way as for ii) above except that, one sample was incubated with 50µl H^3-adenine (2.4mM, 83µCi/µmol) and with 50µl of C^{14}-adenine (2.4mM, 3.7µCi/µmol) in separate tubes. Samples 2 to 4 were incubated with C^{14}-adenine only. When the incubations

were stopped aliquots from the incubation medium with H^3-adenine were cochromatographed with each of the other incubation media.

The table shows the count rate for the C^{14}-purines with an above background count. Adjacent to each count rate is the ratio between that count rate and the count rate for H^3-purines formed by incubation with sample one and cochromatographed with the C^{14}-labelled samples.

Table 4.4

Incubation with C^{14}- and H^3-adenine to get C^{14}- and H^3-purine ratios

	Sample 1		Sample 2		Sample 3		Sample 4	
Protein concentration	10.5mg/ml		25.5mg/ml		30.1mg/ml		26.9mg/ml	
Compound	Count rate (C^{14})	C^{14}/H^3	Count rate (C^{14})	C^{14}/H^3	Count rate (C^{14})	C^{14}/H^3	Count rate (C^{14})	C^{14}/H^3
Base Sheet								
adenine	3690	0.350	3875	0.414	3550	0.382	3695	0.385
inosine	49	0.290	2	0.061	24	0.142	22	0.192
hypoxanthine	125	0.369	69	0.376	15	0.374	16	0.191
nucleotides	174	0.322	220	0.559	147	0.386	152	0.481
Nucleotide sheet								
AMP	52	0.138	69	0.195	80	0.197	82	0.269
ADP	53	0.205	67	0.630	57	0.485	71	0.491
ATP	99	0.313	101	0.900	87	0.433	120	0.667
IMP	14	0.189	11	0.275	15	0.218	27	0.159
Average ratios ±S.D.		0.279 ±0.081		0.420 ±0.250		0.328 ±0.115		0.356 ±0.175

The object was to see if the ratio between the C^{14}-purine count rate and the H^3-purine count rate was constant within a sufficiently narrow range for it to be used as a means of detecting different fluxes. This may have use in the study of control factors and as a diagnostic tool for metabolic disorders.

It would be expected that with identical blood samples the specific activities of the derivatives of a C^{14}-tracer and an H^3-tracer would vary in a similar manner. This follows if there are no kinetic isotope

effects or tritium exchange between the H^3-tracer and the solvent. The ratio between the C^{14} and H^3 count rates should, if that is the case, be constant.

Take a simple example of a reaction in a pathway in which a compound P is converted into another compound Q.

A tracer P* (mol) with a specific activity of p (Ci/mol) will combine with any endogenous P on addition to the reaction mixture to give a total amount of P, $P_T = P + P^*$. The specific activity of P_T will be $p' = p.P^*/P_T = p.P^*/(P + P^*)$. This assumes that the tracer and endogenous P are thoroughly mixed.

In the enzymic conversion of P_T to Q* the specific activity of Q* formed from P_T will be the same as the specific activity of P_T, p', if there are no isotope effects. Now if there is endogenous Q, the specific activity of the total amount of Q, Q_T will be q' given by $q' = p'.Q^*/(Q + Q^*)$.

The total radioactivity associated with Q will be $q'.(Q + Q^*) = p'.Q^*$. It is clear that this quantity is dependent on the isotopic dilution of the tracer P* (p') and on the amount of Q formed from P in a given time (Q*). A measurement of the total radioactivity is, therefore, a means of determining the flux of a pathway, and its state. The use of two isotopes enables the comparison of the same pathway in two different cell samples. The cochromatography of the two isotopes is to help eliminate errors due to manipulation.

The protein concentration of the reference sample (sample 1) was considerably lower than that of the other three samples. This was reflected in the average ratios shown in table 4.4. However, the standard deviations for the ratios are as large as the differences that are due to the protein concentration. The standard deviation for the reference ratios was found to be lower than that for the ratios of the other samples. This may be a reflection of the varience to be expected in 'normal' samples, or else it may be due to real differences in the fluxes of the samples. The errors involved, however,

were too large for the technique to be of practical use without considerable refinement.

4.D. Discussion

The results have been discussed to a certain extent in their respective sections. The conversion of the purine bases primarily to their corresponding nucleotides was evident in all the results. This is in accord with the work of Bishop (1960) and Henderson and Lepage (1959).

It is clear from the tables 4.1 and 4.2 that the 0.114mM purine bases had not attained equilibrium across the erythrocyte membrane. Therefore the transport of purines across the cell membrane should also be considered in a study of the flux of these pathways.

The results of these experiments do not give a direct measurement of the flux in a pathway, but in suitably controlled experiments the determination of flux would be quite feasible. A number of difficulties were encountered in this work and they are discussed below.

The chromatography of the nucleosides and bases was the poorest part of the separation proeedure. This was partly due to the slurring of the spots which probably resulted from impurities carried over from the incubation medium. It was also due to the use of half sheets and quarter sheets rather than whole sheets. This was a fiscal economy. In the same way the specific activity of C^{14} labelled compounds was kept at 3.7μCi/μmol. An increase in specific activity may have enabled a more accurate determination of the minor products in the reactions.

Tritium labelled tracers were used to try and improve this situation but a tritiated tracer with a specific activity of 83μCi/μmol only resulted in a three-fold increase in counts. This was due to the severe quenching that occurred when counting tritium adsorbed to a solid support.

In attempting this work the limitations imposed by the use of radioisotopic tracers was recognised. For example, they give no measure of the actual pool sizes and therefore of total flux. They do, however, enable

the estimation of the proportion of any pool metabolised in a particular direction along a pathway. Inherent in their use are the problems of re-utilisation of the isotope in cyclic paths and the exchange of the isotope with the solvent (in the case of tritium particularly). These are serious drawbacks. An alternative technique would have been the enzymic or chemical analysis of the compounds involved but even quantitative analysis does not elucidate the effects of compartmentalisation.

A further problem was the time involved in the use of this technique. On the one hand it recommended itself in that washing procedures could be carried out overnight (4.B.b.) and actual chromatography stages were of relatively short duration. On the other hand they required careful observation and the sample and carrier application was time consuming. To compensate for the low counts in the minor reaction products the volume of the stopped reaction mixture was reduced using a rotary evaporator.

The quantitative separation of the purine bases, nucleosides and nucleotides was found to be feasible but not practicable. The study of the control of purine metabolism hinged on the success or failure of this technique.

CHAPTER 5

THE TRANSPORT OF PURINES ACROSS BLOOD CELL MEMBRANES

5.A. Introduction

The transport of purine bases across the erythrocyte membrane has been shown to be a rapid simple diffusion process (Whittam: 1960). Lassen (1961 and 1967) and Overgaard-Hansen and Lassen (1959) have shown that while hypoxanthine diffuses across the red cell membrane, uric acid is actively transported across the membrane.

Red blood cells have been implicated in the transport of purines throughout the body, as shown by Henderson and Lepage (1959). They injected mice with C^{14}-adenine and followed its incorporation into red blood cells and other tissues. Felig *et al.* (1973) have shown that erythrocytes are important in the transport of aminoacids in humans. Alanine, representing about 40% of the aminoacids released by muscle, was transported primarily to the liver where it would be used for gluconeogenisis. A significant difference between this aminoacid transport and purine transport is that although all of the purine bases are absorbed by the red cell, Hypoxanthine and xanthine are the major purines released by the cell (Hershko *et al.*: 1967).

As a measure of transport the uptake of C^{14}-adenine by rat blood cells is measured. The kinetics of the process is in agreement with it being diffusion limited.

5.B. Materials and methods

Rat blood, obtained by cardiac puncture, was treated as described in section 2.B.c. to obtain 'separated red blood cells' and 'Kreb's Ringer washed red blood cells'. (8-C^{14})-Adenine was obtained from the Radiochemical Centre, Amersham.

The cells were incubated by pipetting the solutions described in table 5.1 into a test-tube. The incubation was terminated by removing aliquots at set times and layering them on 100µl of dibutyl phthalate in Coleman centrifuge tubes. The tubes were centrifuged at the time recorded for the end of the incubation. Using this technique it was possible to effect a separation between the red cells and their suspending medium within 15s. An aliquot of the suspending medium was counted for radioactivity. A total count was obtained for each incubation by counting an aliquot of the whole incubation mixture.

Table 5.1

Conditions used for the Transport Assay

Temperature:	37°C
Gaseous phase:	air
Solutions used:	
0.2ml	blood cells in 50% suspension (by volume)
0.05ml	1mM adrenalin (optional)
0.70 or 0.75ml	serum or Kreb's Ringer A (table 2.1)
	Preincubate to equilibrate temperature
0.05ml	C^{14}-adenine (8.5nmol, c.a. 80,000cpm.)

Dibutyl phthalate is a hydrophobic liquid with a density of 1.046g/ml which is between that of red cells and water or serum. It has been used by other workers for the separation of red cells (see Danon and Marikowsky: 1964 and Gunn and Tostesson 1971).

A mixture of dibutyl phthalate, 1ml, and 50µl of C^{14}-adenine in 1ml of 0.9% saline were thoroughly mixed using a vortex mixer. The two phases were separated by centrifugation and an aliquot from each phase was counted to see if a significant proportion of the adenine was found in the dibutyl phthalate layer. The dibutyl phthalate was found to contain less than 1.5% of the radioactivity.

5.C. Results

Blood cells were incubated with 9.4μM C^{14}-adenine in the presence and absence of 55μM adrenalin. The cells were prepared in the different ways described in the methods section (5.B.). Figure 5.1 shows the result of an incubation of C^{14}-adenine with washed red blood cells and adrenalin. Each incubation gave results which produced a straight line on a semilog. plot. The uptake, measured by the decrease in the counts in the suspending medium, was approaching equilibrium at about 40 minutes after the addition of the labelled adenine. The data from these incubations is summarised in table 5.2. All the figures represent the average of duplicate experiments.

Table 5.2

The Uptake of Adenine by Red Cells in the presence and absence of Adrenalin

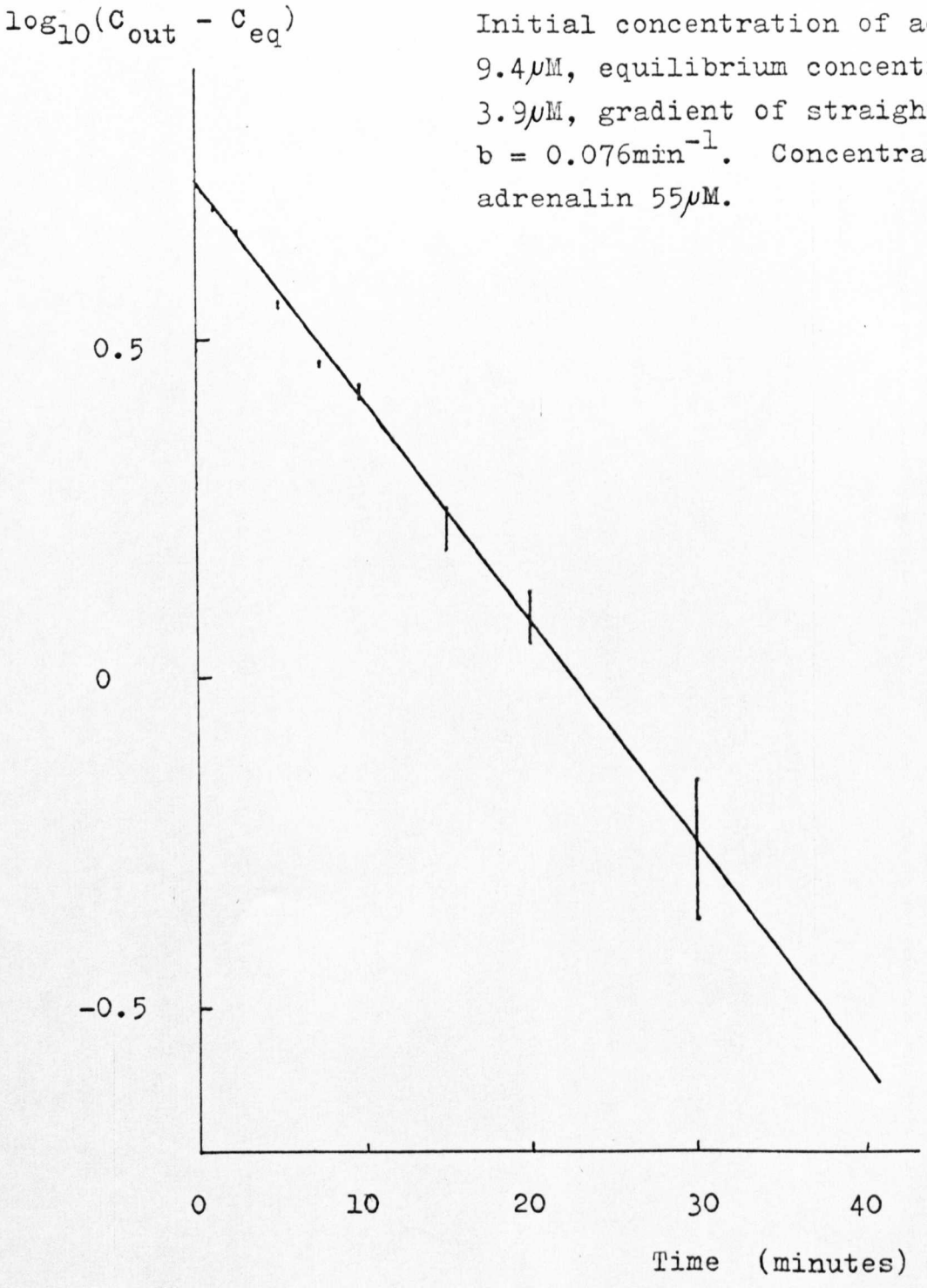
Cell condition	Gradient semilog. plot min^{-1}	C_{eq} μM	ΔC_{out} μM	$(\Delta C_{in})_{app}$ μM	$(\Delta C_{in})_{app} / C_{eq}$	Uptake %
Whole blood + Adr	0.104	7.3	2.4	21.6	3.0	25
Whole blood - Adr	0.079	6.8	3.0	27.0	4.0	31
Rbc in serum + Adr	0.048	4.9	4.3	38.7	7.9	47
Rbc in serum - Adr	0.038	4.6	4.4	39.6	8.6	49
Washed rbc + Adr	0.077	3.9	5.5	49.5	12.7	59
Washed rbc - Adr	0.086	4.4	5.3	47.7	10.8	55

The gradient of the semilog. plot (min^{-1}) corresponds to b in equation (1) below. C_{eq} is the equilibrium concentration of adenine in the suspending medium estimated from the equilibrium count rate. ΔC_{out} is the difference between the initial adenine concentration and C_{eq}. $(\Delta C_{in})_{app}$ is the apparent change in internal adenine concentration calculated from ΔC_{out} and the volume of the cells and the medium. The percentage uptake is the percentage of the total counts removed from the suspending medium.

Figure 5.1 (a)

The Uptake of Adenine by Rat Erythrocytes in the Presence of Adrenalin

Conditions as described in text. Initial concentration of adenine 9.4μM, equilibrium concentration 3.9μM, gradient of straight line, b = 0.076min^{-1}. Concentration of adrenalin 55μM.

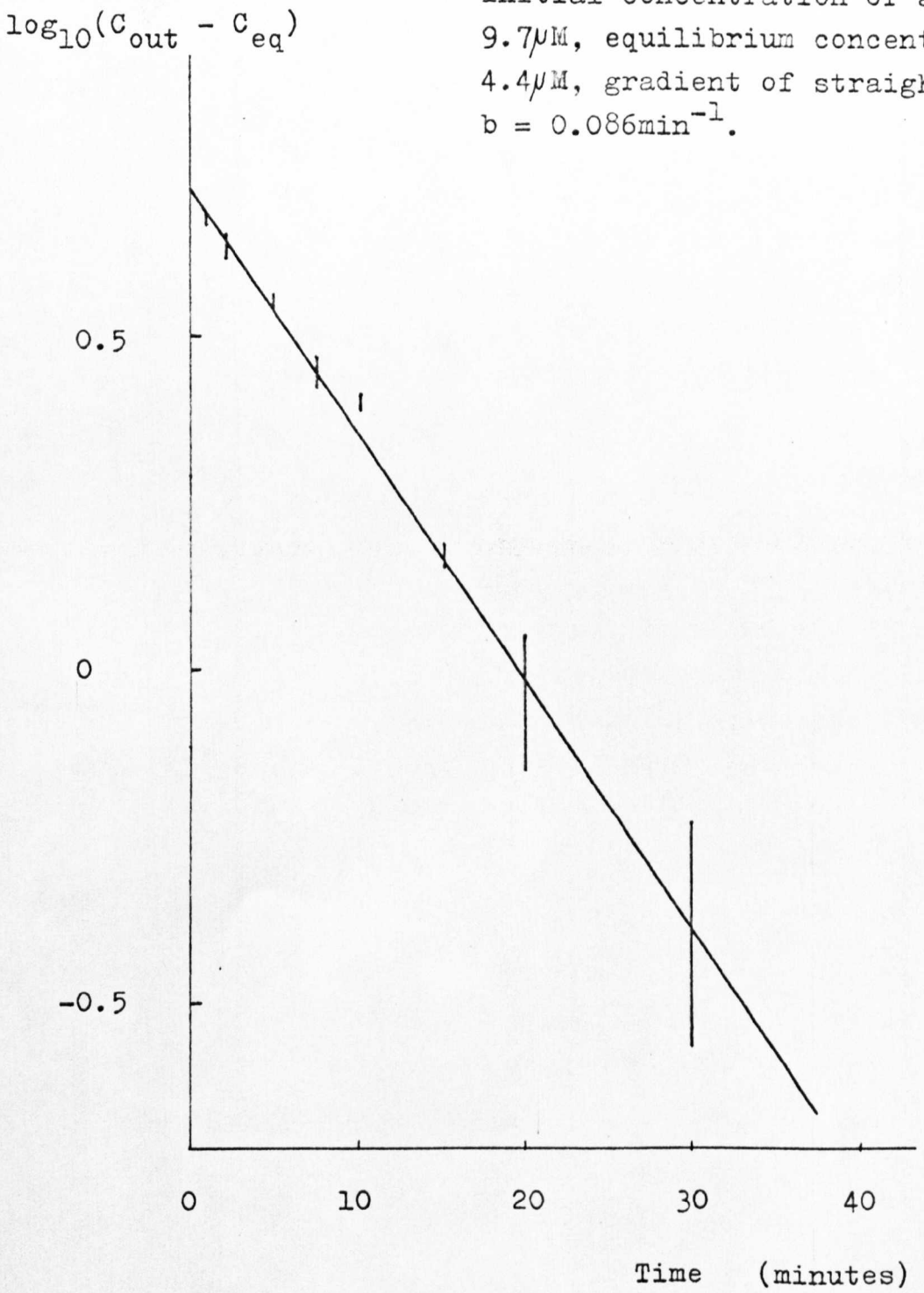


Error bars show the deviation of duplicate experiments.

Figure 5.1 (b)

The Uptake of Adenine by Rat Erythrocytes in the Absence of Adrenalin

Conditions as described in text. Initial concentration of adenine 9.7μM, equilibrium concentration 4.4μM, gradient of straight line, $b = 0.086 min^{-1}$.



Error bars show the deviation of duplicate experiments.

The equation to which the results were fitted in order to get a straight line was:

$$\log_{10}(C_{out} - C_{eq}) = \log_{10}(C_{tot} - C_{eq}) - bt \qquad (1)$$

where C_{tot} is the initial or total concentration of adenine, t is the time in minutes and the other terms are described in table 5.2.. The adenine did not reach an equilibrium distribution between the cells and the medium so the results were fitted to the equation manually to determine this constant. The gradient of the line, b, is related to the permeability constant (5.D.).

If the volume of the packed cells is taken to be an approximate measurement of their internal volume then, assuming that the internal compartment is well mixed, the apparent change in the internal concentration of adenine ($C_{in})_{app}$ is given by:

$$(C_{in})_{app} = - C_{out} \times \frac{V_{out}}{V_{in}} \qquad (2)$$

where V_{out} and V_{in} are the volumes of the suspending medium and the internal cell compartment respectively. The ratio $(C_{in})_{app}/C_{eq}$ gives the minimum factor by which the internal concentration of adenine exceeds the equilibrium concentration in the suspending medium.

In making these calculations the count rate was directly related to the concentration as the degree of quenching was nearly constant.

5.D. Discussion

The diffusion of any substance into red cells is governed by the relationship (Whittam: 1964):

$$\frac{dC_{in}}{dt} = \frac{PA}{V_{in}}(C_{out} - C_{in}) \qquad (3)$$

From equation (2) it can be seen that:

$$\frac{dC_{in}}{dt} = - \frac{V_{out}}{V_{in}}\left(\frac{dC_{out}}{dt}\right) \qquad (4)$$

It follows that

$$\frac{dC_{out}}{dt} = \frac{PA}{V_{out}} (C_{in} - C_{out}) \qquad (5)$$

Equation (1) may also be written in a differential form:

$$\frac{dC_{out}}{dt} = b (C_{eq} - C_{out}) \qquad (6)$$

Comparing equations (5) and (6), b is related to the permeability constant:

$$b = \frac{P A}{V_{out}} \qquad (7)$$

where A is the total surface area of the red blood cells and V_{out} is as before. As the cell volume was not monitored the permeability constant cannot be accurately determined.

The other difference between equations (5) and (6) is that in equation (6) C_{eq} is used in place of C_{in}. This is necessary because as shown in table 5.2 the calculated apparent internal concentration of adenine, though only an approximate figure, exceeds the external concentration of adenine by a factor of between 3 and 12 at equilibrium. The approximation is that the internal cell volume is the same as the packed cell volume and that this did not change during the experiment.

Simple diffusion must always take place down a concentration gradient. It cannot create an adverse gradient, therefore, the excess label in the red cells must be in a chemically modified state so that it cannot diffuse across the membrane. The enzyme, APRT, is responsible for the conversion of adenine to AMP and Bishop (1960) has shown that adenine once incorporated into the cell is rapidly converted into AMP. It appears, therefore, that the equilibrium level of adenine is related to the availability of substrates required to convert the internal adenine to other compounds that cannot diffuse out of the cell. This is in accord with the finding (table 5.2) that the percentage uptake at equilibrium was highest for washed red cells and lowest for fresh whole blood.

The evidence obtained here does not exclude the possibility that the adenine is incorporated into the cell by carrier facilitated diffusion. Facilitated diffusion is indistinguishable from simple diffusion at concentrations of the substrate much lower than the dissociation constant of the membrane bound carrier.

Adrenalin was tested for any effects it might have on adenine transport because it has been observed to alter red cell deformability (Allen and Rasmussen:1971). This was observed in the absence of adenyl cyclase activity. Also noradrenalin and dibutyryl-cyclic-AMP have been shown to cause an increased flux of potassium into duck erythrocytes (Riddick et al.: 1971 and Kregenow: 1973).

Adrenalin does not appear to affect the diffusion of adenine into rat erythrocytes.

CHAPTER 6

THE ASSAY OF CYCLIC-AMP AND ADENYLATE CYCLASE

6.A. Introduction

There are numerous methods available for the assay of cyclic-AMP. It can be determined by a radioisotope dilution assay in which the cyclic-AMP dependent protein kinase is used to bind the labelled cyclic-AMP in the presence of the unknown cyclic-AMP. The radioactivity of the 'bound' fraction is a measure of the cyclic-AMP in the unknown sample (Gilman: 1970; Walton and Garren: 1970 and Brown et al.: 1971). Another method is to use specific cyclic-AMP binding antibody. Cailla et al. (1974) have found that succinylating the cyclic-AMP in the 2'-hydroxyl position leads to a much improved sensitivity. The detection limit for this immunoassay is claimed to be 50 amol.

Cyclic-AMP has also been determined by the activation of protein kinase (Wastila et al.: 1971) for which (γ-P^{32}) labelled ATP was used as the substrate. The incorporation of P^{32} into protein was measured. Rall and Sutherland (1958) used the activation of glycogen phosphorylase as a measure of cyclic-AMP. The assay was later automated (Davoren and Sutherland: 1963).

Other methods involve the direct measurement of cyclic-AMP. This normally involves the use of labelled ATP as a substrate for adenyl cyclase (for example: Streeto and Reddy: 1967). Brooker (1972), however, uses high pressure liquid chromatography to separate cyclic-AMP from other components and determines it on the basis of U.V. absorption in a special flow cell.

The following requirements have been delineated for the assay of cyclic-AMP (Johnson: 1972)

(i) The treatment of the sample with an excess of cyclic-AMP phosphodiesterase should result in a nil response.

(ii) The addition of known amounts of cyclic-AMP

to any test sample should give the expected increments in the response parameter.

(iii) The assay should not be responsive to impurities in the sample, so its purification should not effect the result of the assay.

A cyclic-AMP binding protein assay which meets these requirements was used for this work. The procedure followed was the saturation assay of Brown et al. (1971). This assay recommended itself because of its simplicity, accuracy, economy and reliability.

It is simple because it is a one step assay and the techniques involved in setting it up are in essence elementary (6.B.); although this is not to say that the theoretical analysis is simple. Ekins et al. (1968) have documented an elaborate theory of saturation analysis (including radioimmuno-assay) in which the factors limiting sensitivity and precision were analysed. The recurrently expendable materials are cheap and therefore the method is economical. The wide use of the techniques involved indicates their reliability.

Some features of the assay were examined. The **adenylate cyclase activity of whole cell** incubations of red blood cells from three species were measured.

6.B. Materials and methods

The materials used in this chapter have been described previously (2.B.a.).

6.B.a. Preparation of the cyclic-AMP binding protein

The binding protein was prepared from the bovine adrenal cortex largely as described by Brown et al. (1971). All steps were performed at 0-4°C. Frozen adrenal glands were thawed. The cortices were removed, chopped and homogenised with 1.5 volumes of ice-cold Littlefield's medium (Littlefield et al.: 1957) in a blender (Attomixer) at full speed for 1 minute. The homogenate was centrifuged for 5 minutes at 2,000 x g. The supernatant was then centrifuged at 5,000 x g for 15 minutes. The resultant '5,000 x g supernatant' was stored frozen in 1 ml portions. The samples were thawed and diluted as required (6.D.a.).

The 5,000 x g supernatant was frequently contaminated heavily with yellow lipid. When this was the case the supernatant was skimmed before it was aliquoted for storing. When necessary a 10,000 x g centrifugation was used to bring the lipid to the surface so that it could be removed.

6.B.b. The assay of cyclic-AMP

An aliquot of diluted 5,000 x g supernatant (6.B.a., 6.D.a.) was incubated with the sample with an unknown quantity of cyclic-AMP and a standard amount of radioactively labelled cyclic-AMP. The cyclic-AMP binds to the protein. The incubation time was sufficient for the system to reach equilibrium. All unbound cyclic-AMP was removed by the addition of coated charcoal (6.E.b.) and centrifugation in a bench centrifuge at full speed for 3 minutes. An aliquot of the supernatant (bound fraction) was counted in a liquid scintillation counter (2.B.m.). The degree of isotopic dilution is a measure of the unknown cyclic-AMP (6.C.). The procedure for the assay is shown in table 6.1.

Table 6.1

The Assay of Cyclic-AMP

Temperature 0-4°C.

To a plastic centrifuge tube add: 100µl dilution buffer[1]
50µl unknown sample or standard containing 0-15pmol cyclic-AMP
50µl ($8\text{-}H^3$) cyclic-AMP (60nM, 8nCi)[2]
100µl diluted binding protein 5,000 x g supernatant
Mix and leave for 90 minutes or more.
Add 100µl freshly mixed charcoal suspension[4].
Mix thoroughly (Whirlimixer) and centrifuge.
Remove 100µl aliquot from the supernatant for determination of the radioactivity in the bound fraction and add to 4ml of scintillation fluid in a counting vial.[5]

1) 50mM Tris.HCl pH 7.4 with 8mM theophylline and 6mM 2-mercaptoethanol.
2) The tracer is made up in dilution buffer.
3) The binding protein is diluted in dilution buffer (6.D.a)
4) Suspension of 1g Norit GSX charcoal in a 2% solution of B.S.A. in dilution buffer.
5) Toluene: 2-EE: PPO: POPOP (2.B.m.).

6.B.c. Assay controls

The total counts were determined by adding all the solutions except the charcoal suspension to a tube and making up the volume of charcoal with dilution buffer. This results in a slight underestimate of the total counts because of the volume of the charcoal suspended in the other tubes. This was determined by adding 10 g of charcoal to 100 ml of 2% B.S.A. in dilution buffer. The volume of the suspension was 106 ml, so the charcoal contributes 5.6% of the volume of the charcoal suspension. The contribution of the charcoal to the volume of the experimental tubes was therefore 1.4%. This was the factor by which the total counts control underestimated the actual total count relative to the other tubes. As this affected all results in the same way and did not affect the determination of the cyclic-AMP it was ignored.

A control in which no binding protein was added to the control tube and in which the volume was made up with dilution buffer gave the proportion of radioactivity not absorbed by the charcoal. Where there has been an exchange of tritium between the cyclic-AMP and the solvent this would be expected to increase this control count. The cyclic-AMP must also be distributed between the charcoal and the solvent. The amount of cyclic-AMP remaining in the solvent was found to vary with the composition of the solvent. The count rate for the control was independent of added cyclic-AMP up to 100nM concentration and it was proportional to the concentration of the tracer cyclic-AMP up to 100nM also. Ethanol at concentrations as low as 4 to 5% was found to increase the count rate by 50%. Sodium chloride did not affect the count rate for 10nM tracer **(up to 1 M NaCl tested).**

The necessity for controls because of variations in the constituents of the assay sample which might affect the binding protein and the charcoal and therefore alter not only the zero reading but also the shape of the standard curve has been emphasised by Albano et al. (1974). The assays for cyclic-AMP in this chapter had zero cyclic-AMP controls but none for variations in the shape of the standard curve.

6.C. Theory

6.C.a. The most convenient linear representation of results for a standard response curve is that of Hales and Randle (1963). In the assay for insulin using the insulin anti-body they derived the following relationship on the basis of the isotope dilution of the anti-body precipitate by the sample or standard insulin:

$$\frac{C_o}{C_i} = \frac{i}{i_o} + 1 \qquad (1)$$

i is the concentration of sample or standard; i_o the concentration of tracer. C_o is the count rate of the precipitate (bound fraction) when no sample or standard is present and C_i is the count rate when the sample concentration is i.

The equation only holds if the anti-body is fully saturated in the presence of tracer alone. This is seldom the case and does not happen in this assay. The relationship was extended by Wunderwald et al. (1974) to take account of the degree of saturation of the binding protein in the assay of cyclic-AMP. The equation has the form (following the notation above):

$$\frac{C_o}{C_i} = \frac{(i_o)_{Bd}}{(i + i_o)_{Bd}}\left(\frac{i}{i_o} + 1\right) \qquad (2)$$

where $(i_o)_{Bd}$ and $(i + i_o)_{Bd}$ correspond to the concentrations of the bound species in the presence of tracer alone and tracer and sample respectively. When the tracer nearly saturates the binding protein the curve does not deviate significantly from a straight line.

6.C.b. The accuracy of the assay has been described by Ekins et al. (1968) in terms of the precision and the sensitivity as defined below:

(i) Precision $$\Delta p = \frac{\Delta R}{\partial R/\partial p} \qquad (3)$$

(ii) Sensitivity $$\Delta p' = \frac{\Delta R}{\partial R/\partial p}\Big|_{p=0} \qquad (4)$$

where ΔR is the error in the response parameter; Δp is the error in measurement of the substance P being assayed and $\partial R/\partial p$ is the gradient of the response curve.

Theoretically the assay can only be optimised for one concentration of cyclic-AMP, that is, one point on the response curve is more precisely defined than all the other points. Neighbouring points will be less precise but never-the-less still accurate. The further a point is from the most precise region of the curve the less precise it will be. The range of satisfactory precision depends on the error in the response parameter and the gradient of the response curve (equation 3).

6.C.c. The experimental errors are likely to be the limiting factor in the optimisation of the assay. The relative error in the result of the assay, ΔR, has two basic components, namely the relative experimental error and the systematic error which is primarily the scintillation counting error. These may be represented as ϵ and σ_n/N respectively where N is the count for the sample and $\sigma_n = N^{\frac{1}{2}}$.

The experimental error arises mainly from the pipetting of the various solutions in the assay, which involves six steps, and the preparation of the standard solutions. As piston pipettes were used the approximate error in each of these steps was 0.01 (Ellis: 1973) and the combined error is approximately 0.03.

The error in the response parameter ΔR is given by:

$$\Delta R = \left(\epsilon^2 + \frac{1}{N}\right)^{\frac{1}{2}} \qquad (5)$$

If the counting error is less than ϵ then:

$$\epsilon < \Delta R < 2^{\frac{1}{2}}\epsilon. \qquad (6)$$

This will be so if $N > 1110$. This is an experimental condition that can be easily met. If the linearised form of equation is used involving C_o/C_x where both C_o and C_x are subject to these errors then the total error is increased so that

$$2^{\frac{1}{2}}\epsilon < \Delta R < 2\epsilon. \qquad (7)$$

6.D. Results

6.D.a. The dilution of the binding protein

Using the standard procedure (table 6.1) with the dilution buffer replacing the sample, the tritiated cyclic-AMP bound by six serial dilutions of the 5,000 x g

supernatant (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) was measured. The results are shown in fig. 6.1. The dilution required in the assay is that which gives approximately 30-40% binding of the tracer. The appropriate dilution was selected by referring to the dilution curve.

6.D.b. Cyclic-AMP standard curve

The assay was carried out as described in section 6.B.b.. A stock solution of 10μg ml^{-1} cyclic-AMP (30.4μM) was diluted 1 in 50.7 to give a 600nM solution. This was then diluted to give the concentrations used in the preparation of the standard curve, namely, 2, 5, 10, 15, 25, 30, 50, 75, and 100nM. The assay volume was 0.3 ml so the amounts of cyclic-AMP used were 0.7 to 30 pmol.

The standard curve is shown in figure 6.2 in which the results are plotted in four of the forms commonly used for saturation analysis data.

6.E. Method for the assay of adenylate cyclase

The enzyme was assayed in turkey, rat and human erythrocytes. The cells and stroma were prepared as described in sections 2.B.c. and 2.B.d.. The cells were washed and resuspended in glucose supplemented saline (154mM NaCl plus 1/20th volume of 5.4% D-glucose).

The cells or stroma were incubated in a small plastic test-tube with the solutions shown in table 6.2. The test-tube had to be capable of withstanding boiling and centrifuging at full speed in a bench centrifuge.

Table 6.2

Incubation for the Assay of Adenylate Cyclase

Temperature: 37°C.

Contents of the incubation medium:

0.35 ml	50 mM Tris.HCl pH 7.4 containing	2mM ATP 3mM $MgSO_4$ 8mM theophylline
0.10 ml	650mM NaCl containing 1/20th vol. 5.4% glucose	
0.05 ml	1 mM adrenalin or 1 M NaF	
0.10 ml	blood cells in a 50% suspension	

Figure 6.1

A Dilution Curve for the 5,000 x g Supernatant

The assay was as described in the text (6.B.b.) except that serial dilutions of the binding protein were used and the sample was replaced with dilution buffer.

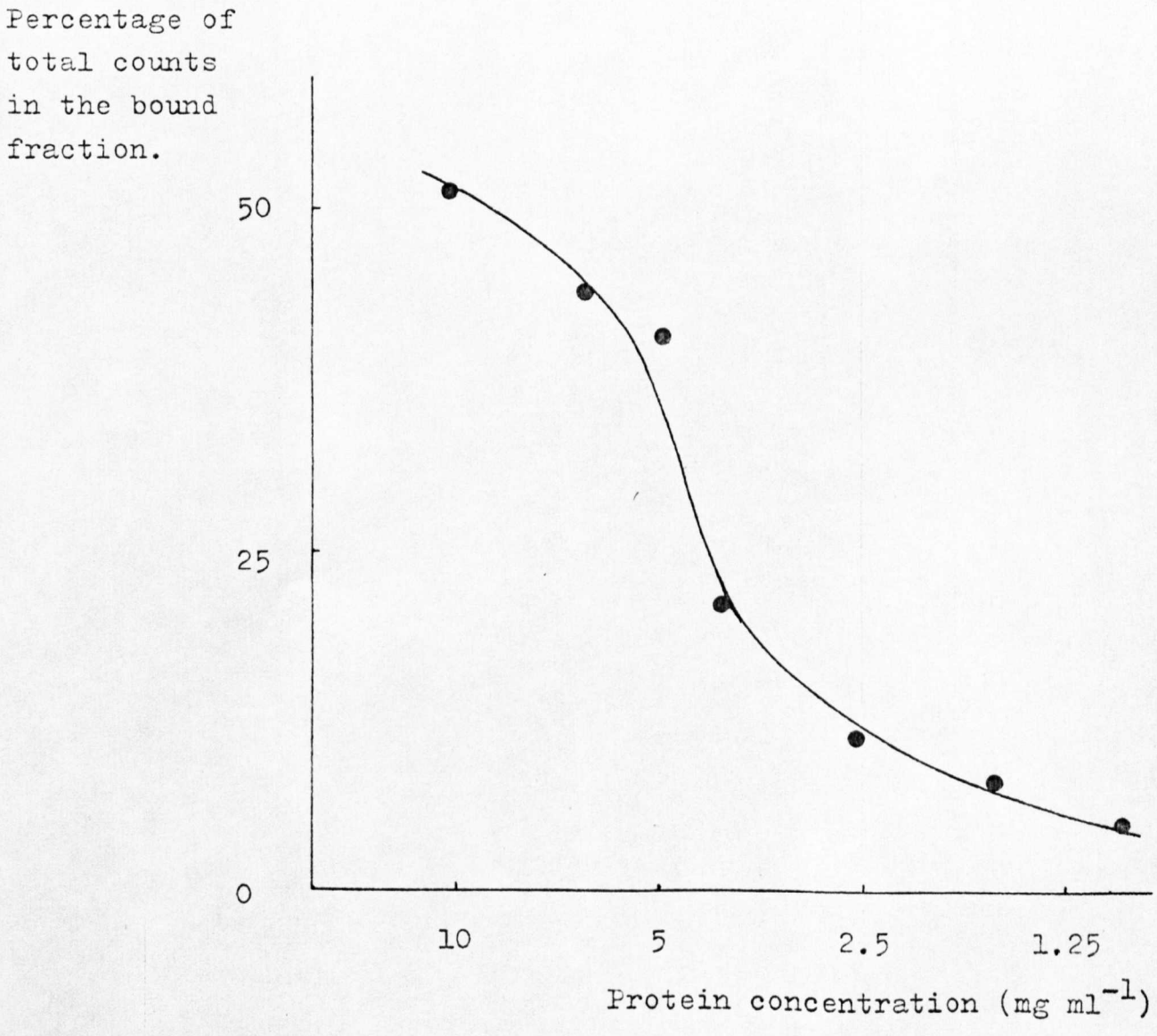
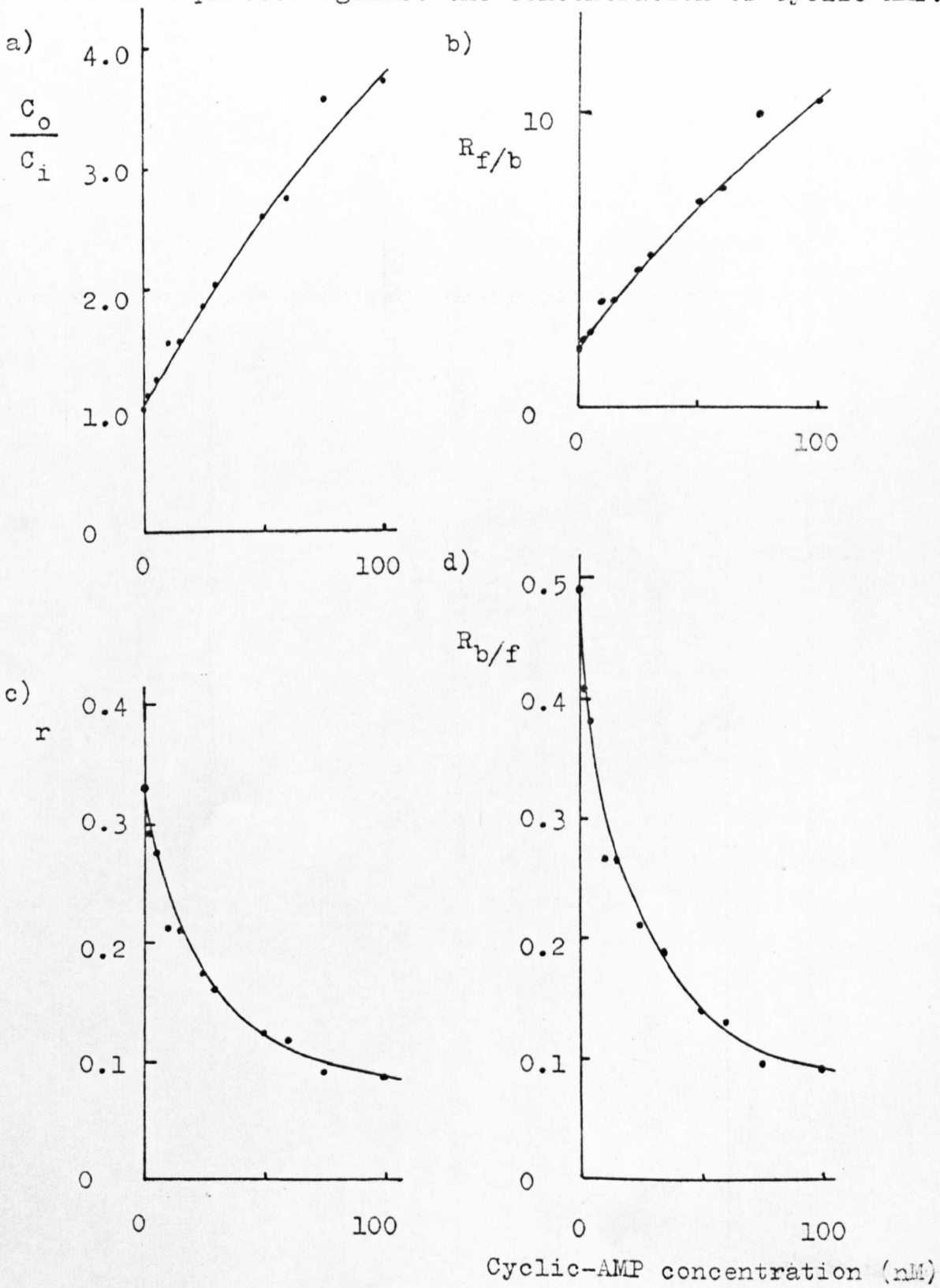


Figure 6.2

Standard Response Curves for the Assay of Cyclic-AMP

a) C_o / C_i as defined in 6.C.a.; b) $R_{f/b}$, the ratio of free and bound fractions; c) r, the proportion of tracer bound and d) $R_{b/f}$, the ratio of bound and free fractions are each plotted against the concentration of cyclic-AMP.



The reaction was started by the addition of the 'enzyme' or adrenalin. The reaction was terminated either by putting the tube in a bath of boiling water for 3 minutes or by rapidly cooling in an ice-salt bath and centrifuging at $0^{o}C$ to separate the cells from the suspending medium. The supernatant was drawn off and the cells resuspended in the same volume of cold saline as the supernatant. The supernatant and resuspended cells were put in a bath of boiling water for 3 minutes. These procedures were carried out as quickly as possible. The denatured protein was removed by centrifugation and the supernatants were then assayed for cyclic-AMP. This procedure was taken from the work of Davoren and Sutherland (1963).

6.F. Results

6.F.a. Adenylate cyclase in turkey erythrocytes

In an initial test for the activity of adenylate cyclase in turkey erythrocytes the stroma were prepared (as described in sections 2.B.c. and 2.B.d.). The time course of the reaction was ten minutes and the enzyme was activated by 0.083 M NaF. The activity of the enzyme was 380±150 pmol cyclic-AMP formed/h/mg protein. The protein concentration was estimated by the method of Lowry et al. (1951), see section 2.B.e. The large error was due to the heterogeneity of the suspension used for the assay.

The production and release of cyclic-AMP by turkey erythrocytes was measured over a period of 30 minutes. The results are shown in figure 6.3. The packed volume of cells used was 0.05ml and the incubations were set up as described in section 6.E.. The activating agent was 0.083 mM adrenalin.

6.F.b. Adenylate cyclase activity in rat red blood cells

Haemopoiesis was stimulated in rats by bleeding. Blood samples with approximately 20% reticulocytes were assayed for adenylate cyclase activity alongside normal blood samples in order to compare the activities of the respective enzymes. The results of this together with the assay details are shown in table 6.3.

Figure 6.3

Time Course for the Formation and Release of Cyclic-AMP by Turkey Erythrocytes

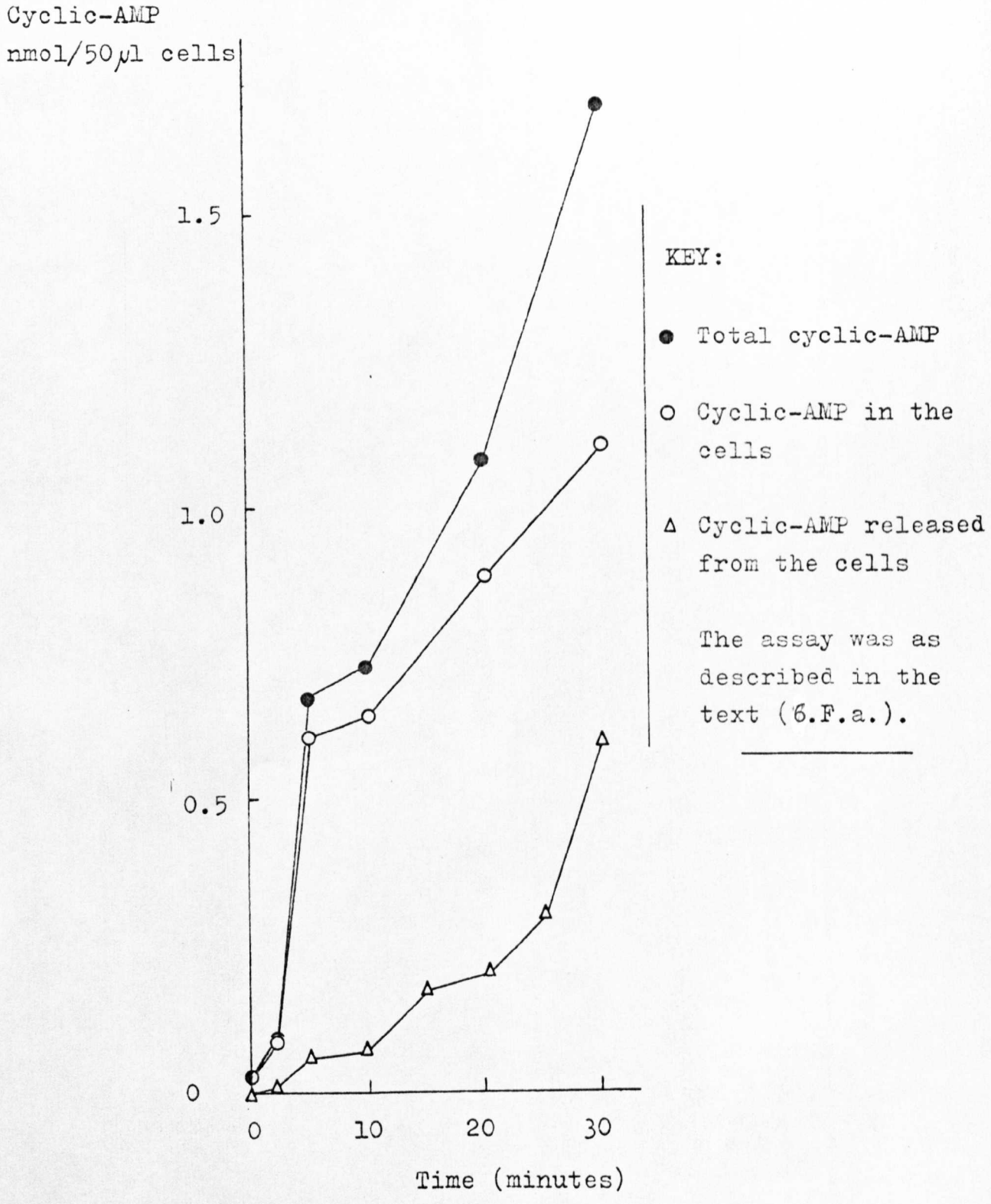


Table 6.3

Assay for Adenylate Cyclase Activity in Rat Red Cells

The assay procedure was as described in table 6.2. Blood samples 1 and 2 were assayed as whole blood. Samples 3 and 4 were washed red cells.

Sample	Reticulocyte %	Cyclic-AMP pmol/50µl cells		Activity nmol/ml/h
		0 min.	10 min.	
1	1	147	219	8.6
2	20	260	360	12.0
3	1	100	204	11.4
4	18	142	396	27.7

It is clear that the major activity of adenylate cyclase is to be found in the high reticulocyte blood.

6.F.c. Adenylate cyclase activity in human red blood cells

One day old samples from two patients at the Coventry and Warwick Hospital who were suffering from macrocytic anaemia and a third patient suffering from ulcerative colitis were assayed for adenylate cyclase activity using an 8% suspension of the cells.

i) Assay of 10% reticulocyte (ulcerative colitis) and normal human blood.

The assay was as described in table 6.2 with an incubation time of 10 minutes and 0.083mM adrenalin added to activate the adenylate cyclase. There was no cyclic-AMP produced in the incubation as determined by the normal cyclic-AMP assay (6.B.b.).

ii) Assay of 18 and 20% reticulocyte (macrocytic anaemia) and normal blood.

Both the washed blood cells and stroma (section 2.B.c and 2.B.d.) were assayed in the presence and absence of activators. Both adrenalin (0.083mM) and NaF (0.083M) were used to activate the enzyme.

It was noticed that in the presence of activators cyclic-AMP appeared to be formed. However, the no-enzyme controls in which there was activator also showed the same deviation from the normal count rate. The net result therefore was that no cyclic-AMP was deteeted.

All the assays on human red blood cells were carried out in duplicate. The average value for C_o / C_i for sample i) was 0.99±0.03 and for sample ii) 0.94±0.16. The larger error in the case of sample ii) was due to the variations shown by the control results. The detection limit of this assay was about 1 pmol.

6.G. Discussion

The assay for cyclic-AMP described by Brown et al. (1971) was used to measure the adenylate cyclase activity in the redblood cells of three species. The assay was not used under optimal conditions for sensitivity. This would only have been desirable in the assay of the enzyme from human red cells.

The tritiated cyclic-AMP was obtained as a solution in 50% ethanol and was diluted for use. This meant that there was a small amount of ethanol in the assay. This did not appear to unduly affect the results (c.f. chapter 8.D.b.). It also meant that any tritium exchanged between the cyclic-AMP and the solvent would be carried over into the assay. This contributed to the count rate observed for the control in which there was no binding protein added. This control determines the radioactivity not absorbed by the charcoal. The count rate was found to be proportional to the amount of H^3-cyclic-AMP added and was not normally affected by the addition of unlabelled cyclic-AMP or salts, though it increased with the concentration of ethanol. Care had to be exercised in the use of charcoal as it was easy to disturb the sedimented charcoal when sampling for the counting procedure.

A labelled cyclic-AMP solution was chromatographed after two months to check whether it was still chemically pure. The chromatographic system was that described in section 4.B.b. using the first solvent only. The cyclic-AMP had an R_f of 0.57 and AMP an R_f of 0.76. Nine percent of the counts were found associated with the AMP spot, the remainder were located in the cyclic-AMP spot.

The assay results for the turkey erythrocytes show a similar activity of adenylate cyclase to those assayed by Øye and Sutherland (1966). The time course and release of cyclic-AMP are similar to those observed for pigeon erythrocytes by Davoren and Sutherland (1963) except that the concentration of the released cyclic-AMP is much lower in this case. This may be due to the difference in concentration of blood cells in the two assays. The erythrocytes contributed 50% of the volume of Davoren's incubation medium whereas for these results the percentage was 8.3%.

Gauger et al. (1973) assaying rat red blood cells concluded that the activity of the adenylate cyclase was localised in the reticulocytes. The results here show that the activity is much greater in the reticulocytes, and perhaps young erythrocytes also, than in a normal blood sample.

Sheppard and Burghardt (1969) who were the first to show the activity of adenylate cyclase in rat erythrocyte ghosts also assayed the enzyme in human erythrocyte ghosts. They found that there was a very slight activity in the red cell preparation but considered that it may have been due to impurities. Adenylate cyclase has been found in human white blood cells (Stolc: 1973; Constantopoulos and Najjar: 1973) and also in platelets (Murakami et al.: 1971).

The possibility of the enzyme being found in samples of red blood cells with a high reticulocyte count was tested.

The blood with the high reticulocyte count from a person suffering from ulcerative colitis may be compared with the rat blood in which a high reticulocyte count had been caused by bleeding. Ulcerative colitis is characterised by mucosal inflamation of the rectum and colon and is usually accompanied by a discharge of blood and mucus with the stool. This results in stress erythropoiesis like that of the rat induced by bleeding.

The reticulocytes formed in the case of macrocytic anaemia, however, are larger and have a different nuclear structure from the normal (normoblastic) reticulocyte.

They arise from an alternative differentiation pathway, megaloblastic haemopoiesis, rather than the normal pathway, normoblastic haemopoiesis.

No adenylate cyclase activity was detected in either cell type under the conditions of the assay.

The lack of adenylate cyclase activity in mature erythrocytes in human and rat blood would appear to indicate that cyclic-AMP has no function in such cells. Its presence in rat reticulocytes, however, means that this conclusion cannot be taken to include the developing red cell. The formation of red cells is triggered by the hormone erythropoietin, and it may well be that the function of this hormone requires the action of adenylate cyclase.

CHAPTER 7

PARTIAL PURIFICATION OF THE CYCLIC-AMP BINDING PROTEIN

7.A. Introduction

The occurrence of non-linear binding curves (section 8.D.) for the binding of cyclic-AMP to the 5,000 x g supernatant indicated that the binding was more complicated than a 1:1 complex formation between the cyclic-AMP and the protein kinase.

The non-linear nature of the binding curves may be caused by the presence of more than one type of binding protein characterised by different dissociation constants for the binding process. Other plausible explanations are that the dissociation of the regulatory subunit from the catalytic subunit changes the affinity of the receptor site for cyclic-AMP or that there is some degree of cooperativity in the binding process.

The 5,000 x g supernatant was fractionated and the cyclic-AMP binding protein partially purified in order to investigate the origin of the non-linear binding curves. The binding studies are dealt with separately in chapter 8.

7.B. Materials and methods

General comments relating to the techniques used in sections 7.C.a.- 7.C.d are to be found in chapter 2 sections B.h.- B.j..

7.B.a Protein kinase assay

The protein kinase assay used in section 7.C.c. was a development of the assay of Kuo et al. (1971). The solutions used are shown in table 7.1. They were all made up in 67mM sodium acetate buffer pH 6.0. The enzyme was assayed by pipetting 50µl of solutions 1), 2) and 3) or 4) into a small plastic tube. The contents were brought to the reaction temperature (30°C) and 50µl of enzyme solution were added. The contents were mixed and left at 30°C in a shaking water bath. The

Table 7.1

Solutions used in the assay of protein kinase

1) 0.8mg/ml histone and 40mM magnesium acetate in buffer
2) 5μM (γ-P^{32})ATP: 1Ci/mmol in buffer
3) buffer (67mM sodium acetate buffer pH 6.0)
4) 2μM cyclic-AMP in buffer
5) stop mixture of 5% TCA and 0.25% sodium tungstate pH2.0

reaction was stopped by removing a 50μl aliquot of the incubation mixture and placing it on a filter pad (1.5cm^2, Whatman 3MM filter paper) which was placed immediately into a beaker containing solution 5). This follows the filter paper disc technique of Bollum (1959 and 1966). The time at which the reaction was stopped was taken as the moment when the filter paper disc was placed in the stop mixture. Sodium tungstate was included in the stop mixture as polylysine (a model compound for histone) is not completely precipitated by TCA alone but has been shown to be insoluble in tungstic acid (Gardener et al.: 1962). The amount of stop mixture used per filter pad was 10ml.

The advantage of this technique was that it enabled a batch of samples to be treated together rather than the individual washes required for the protein precipitate in the Kuo method. The filter pads were washed twice in each of the following solvents: stop mixture, 95% ethanol, and ether. The pads were stirred in each solvent (5ml per pad) to ensure that they remained separate. The wash solution was then decanted off and after the final wash the pads were dried in a stream of air. The pads were then placed in scintillation vials with 4ml of toluene: PPO: POPOP and counted in a Packard 2450 (2.B.m.) on preset settings for determining P^{32} (efficiency estimated to be 37%). A total count was obtained by counting a filter pad that had had 50μl of incubation mixture placed on it and had then been allowed to dry in air.

This technique depended upon the precipitated

protein becoming enmeshed in the paper but the smaller molecules were free to diffuse out of the paper during the wash procedures. Most of the radioactivity was removed in the first two washes.

Although the technique allowed a number of samples to be treated at once, care had to be taken to ensure that the filter papers were not left in the stop mixture too long otherwise they began to fray. It was found that 50 reaction tubes could be handled in less than 20 minutes. The fraying did not become serious until after 30 minutes had passed.

7.B.b. Sucrose density gradient centrifugation

Linear sucrose density gradients were prepared using a perspex block gradient mixer with equal bore chambers. The gradient volume was 5ml so each chamber was filled with 2.5ml of 20% and 5% sucrose made up in the standard dilution buffer (table 6.1). The centrifuge tubes were filled by gravity feed from the gradient mixer. The outlet tap was partially closed to limit the flow rate into the tube. A 0.4ml sample of protein was then added to the density gradient.

The gradients were centrifuged in a Beckman L-2 ultracentrifuge using an SW 50L rotor. The head was centrifuged at its maximum rating (reduced through usage to 38,000 rpm) for 18 hours, following the procedure of Martin and Ames (1961).

7.B.c. Polyacrylamide gel isoelectric focusing

The isoelectric focusing was carried out using an LKB 2117 multiphor. The gel was prepared by J. Wood*. Two samples were applied to the anodal end of the gel on a piece of filter paper 1 x 1.5 cm^2. The instrument was set initially to a voltage of 1,000V and 30mA. The final settings were 1,100V and 4mA. The two samples were removed and then one was stained with Coomassie blue for 20 minutes. Excess stain was removed by immersing the gel in a water, ethanol and acetic acid mixture (8:3:1 by volume) for 15 minutes at 60°C. The other gel was soaked for 2 hours in a solution of

* A research student in Molecular Sciences Dept.

H^3-cyclic-AMP (60nM in dilution buffer: table 6.1). The gel was then washed twice in dilution buffer by immersing it in the buffer for about two hours. It was then sectioned into 24 bands and these were mashed and counted using the toluene: 2-EE: PPO: POPOP scintillation fluid.

7.C. Results

7.C.a. Ammonium sulphate fractionation

Ammonium sulphate fractionations were carried out on several preparations of the 5,000 x g supernatant (6.B.a.). Most of the cyclic-AMP binding activity was precipitated in the range of 20-60% ammonium sulphate saturation (table 7.2).

Table 7.2

Ammonium sulphate fractionation of the 5,000 x g supernatant

Fraction A(%)	Relative activity*	Fraction B(%)	Relative activity*	Fraction C(%)	Relative activity*
0 - 20	5	0 - 15	12	0 - 15	12
		15 - 30	10	15 - 35	9
20 - 40	79	30 - 45	38	35 - 50	50
40 - 60	16	45 - 65	40	50 - 65	29

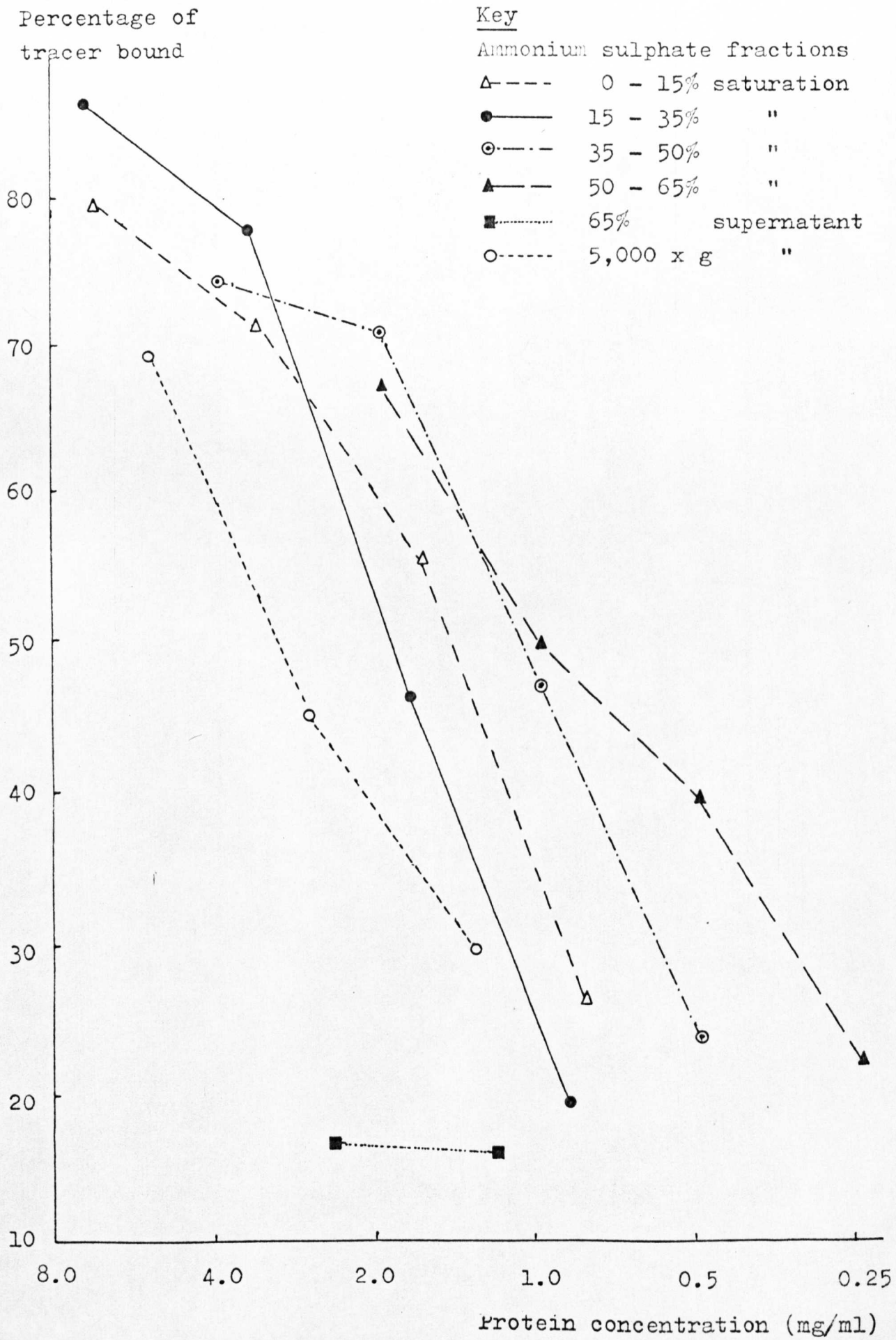
* The relative activity was determined by plotting B against B/F (see section 8.C. and 8.D.b.) for each fraction. The figures represent the percentage of total binding sites for each fraction.

Binding studies indicated that the protein precipitated at about 20% saturation and at 40 - 50% saturation had a K_d of 3 - 4 nM. The protein precipitated in between these two had a higher dissociation constant. (These results are shown in section 8.D.b. and are discussed there.)

The dilution curves (6.D.a.) are shown in figure 7.1 for fractionation C above (table 7.2). The dilution curves show that the ammonium sulphate fractionation enabled an increase in the cyclic-AMP binding specific activity for fractions above 15% saturation and below

Figure 7.1

Cyclic-AMP binding dilution curves for ammonium sulphate fractions of the 5,000 x g supernatant



65% saturation.

7.C.b. Cellulose phosphate chromatography

A cellulose phosphate column was used to fractionate the binding proteins. The column was used initially at pH7.4 but after a gel-isoelectric focusing experiment (7.C.f.) the pH was reduced to 5.1. The fractions eluted from both columns were determined for cyclic-AMP binding activity at pH7.4.

The elution profiles are shown in figures 7.2 and 7.3. The conditions used in the chromatography are described in the corresponding legends.

In both figures it is seen that the cyclic-AMP binding activity was partially resolved into two peaks. In the first column labelled cyclic-AMP was added to the 5,000 x g supernatant before adding it to the column. The quantitiy of cyclic-AMP added was insufficient to saturate the binding protein. The radioactivity of the fractions was measured before and after the addition of coated charcoal. The difference between these counts represents the free cyclic-AMP shown in figure 7.2.

The sample for the second column was an aliquot of the 5,000 x g supernatant. The binding activity of the fractions was measured by taking aliquots of 0.5ml from each fraction. They were brought to pH 7.4 by adding 0.05ml of 0.08M Tris base, and 0.1ml of 60nM H^3-cyclic-AMP was added. After equilibrating at 0^oC, 0.1ml of the charcoal suspension was added, the contents of the tube were thoroughly mixed and centrifuged. The radioactivity of the supernatant was measured in the usual manner.

The binding proteins were separated as in the previous column into two peaks of binding activity. A third minor binding peak was also noticed.

7.C.c. DEAE-cellulose column chromatography

The use of DEAE-cellulose in the purification of bovine adrenal cortex cyclic-AMP dependent protein kinase has been reported by Gill and Garren (1970).

Figure 7.2

Cellulose phosphate column pH 7.4

The elution profile for 2.0ml of the 5,000 x g supernatant mixed with 2.0ml of 60nM H^3-cyclic-AMP in dilution buffer (table 6.1) and added to a 3 x 12cm column of cellulose phosphate equilibrated with 0.025M phosphate buffer pH 7.4 containing 6mM 2-mercaptoethanol and eluted with a linear gradient of sodium chloride (0 - 0.3M).

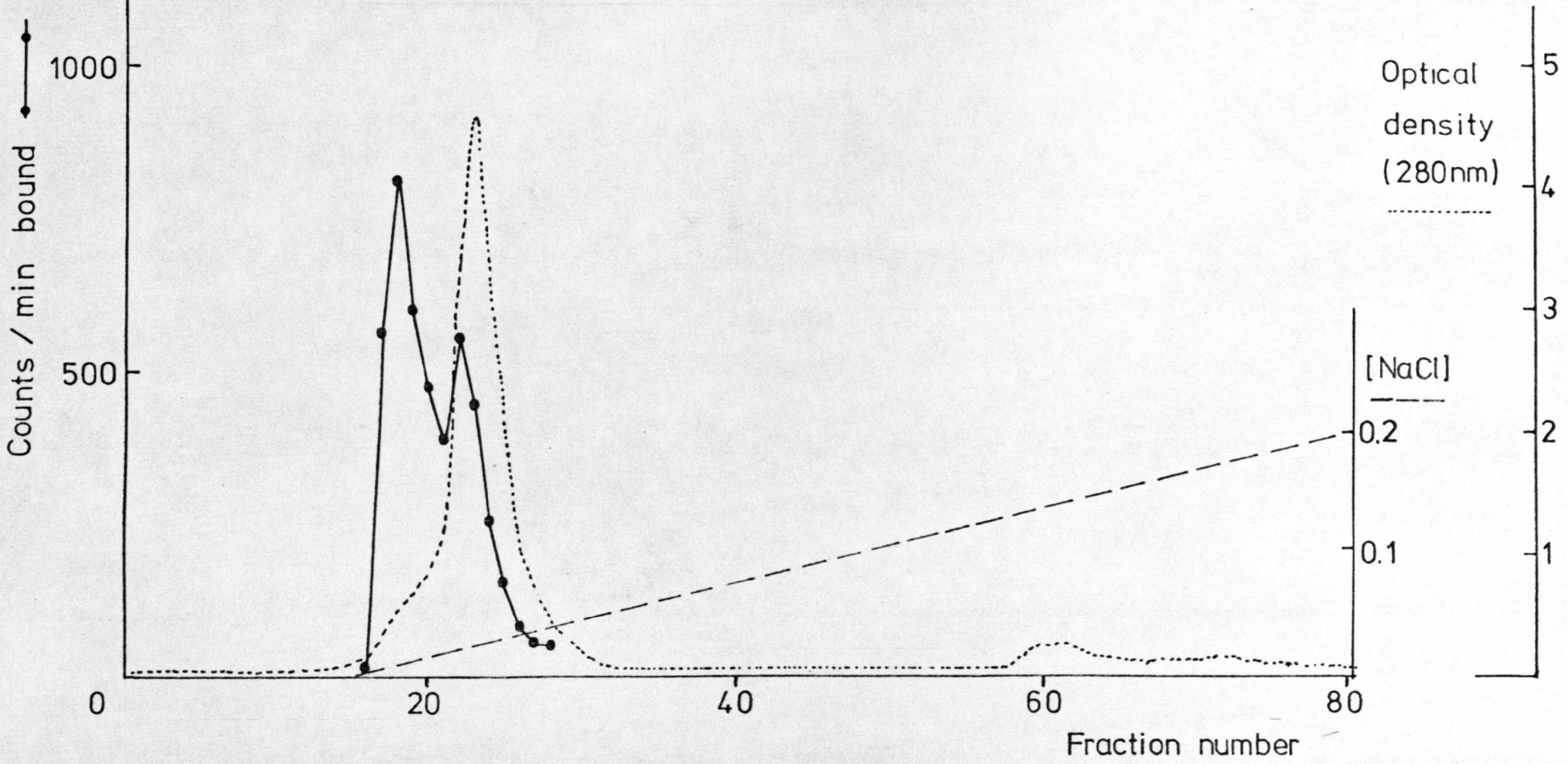
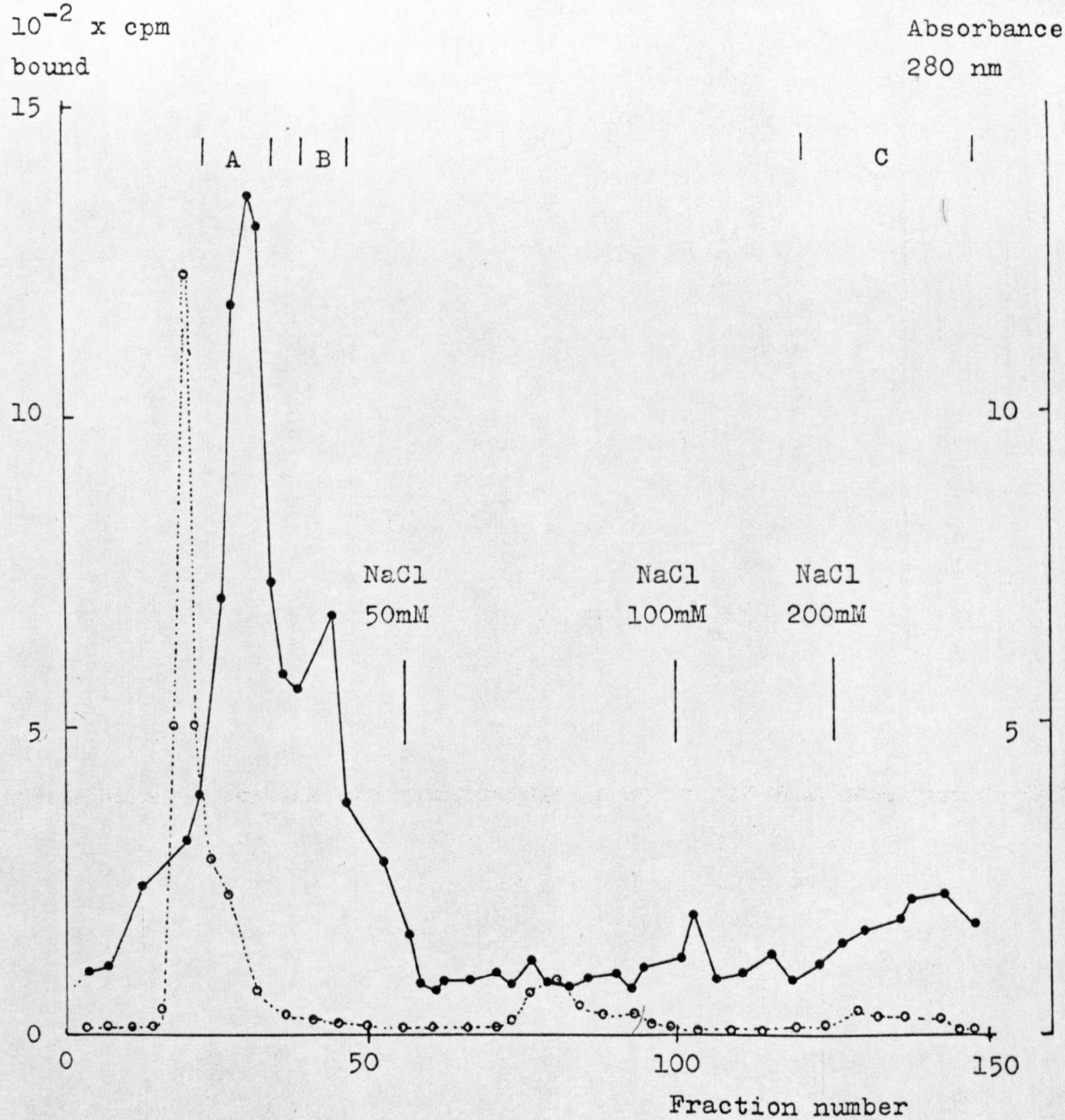


Figure 7.3

Cellulose phosphate column pH 5.1

The elution profile for a 10ml sample of the 5,000 x g supernatant added to a column of cellulose phosphate (3 x 12cm) equilibrated with 0.025M sodium acetate buffer pH 5.1 containing 6mM 2-mercaptoethanol and eluted with the same buffer. Addition of sodium chloride in concentration steps (50mM, 100mM and 200mM) was made to elute any further binding activity.



Figures 7.4 - 7.6 show the elution profiles of the 5,000 x g supernatant and two ammonium sulphate fractions from the 5,000 x g supernatant. Each sample was dialysed over night against 10 l of the equilibration buffer for the column. The dialysed sample was then centrifuged at 10,000 x g for 30 minutes before putting on the column.

For the first two columns (figs. 7.4 and 7.5) only the cyclic-AMP binding activity was measured. For the third column both cyclic-AMP binding activity and protein kinase activity were measured. The conditions used in each case for the elution and assay for activity are described in the respective legends.

I (figure 7.4) DEAE-cellulose chromatography of the 5,000 x g supernatant.

The cyclic-AMP binding activity was divided into two major peaks (A and B) presumably corresponding to Gill and Garren's cyclic-AMP dependent protein kinase and the cyclic-AMP receptor protein (Gill and Garren: 1970). However, there appeared to be cyclic-AMP binding activity in other peaks (C and D) not detected by Gill and Garren. Their partial purification of the protein kinase and cyclic-AMP binding activities by ammonium sulphate fractionation (0 - 54%) and calcium phosphate gel chromatography may have removed these bands.

Pooled fractions from this column were used in the sucrose density gradient work (7.C.d.) and in the study on the effects of cyclic-GMP on the binding of cyclic-AMP (8.F.a.)

II (figure 7.5) DEAE-cellulose chromatography of a 30 - 65% ammonium sulphate fraction.

The fractions eluted from the column were assayed for cyclic-AMP binding activity at two concentrations of cyclic-AMP, namely 10nM and 100nM.

There were two major peaks of binding activity. The first (A) was detected using 100nM cyclic-AMP and was therefore thought to be a more weakly binding protein than the second peak (B) which was more apparent when 10nM cyclic-AMP was used. The fractions comprising the

Figure 7.4

DEAE-cellulose column I

Elution profile for 20ml of the 5,000 x g supernatant dialysed against 20mM Tris.HCl pH 7.3 and added to a 2 x 23cm column of DEAE-cellulose equilibrated with 20mM Tris.HCl pH 7.3 containing 6mM 2-mercaptoethanol and eluted with a linear gradient of NaCl 0 - 0.25M followed by 0.4M NaCl all in the equilibration buffer. Binding activity was measured by removing 0.1ml aliquots from the fractions and assaying as in section 6.B.b..

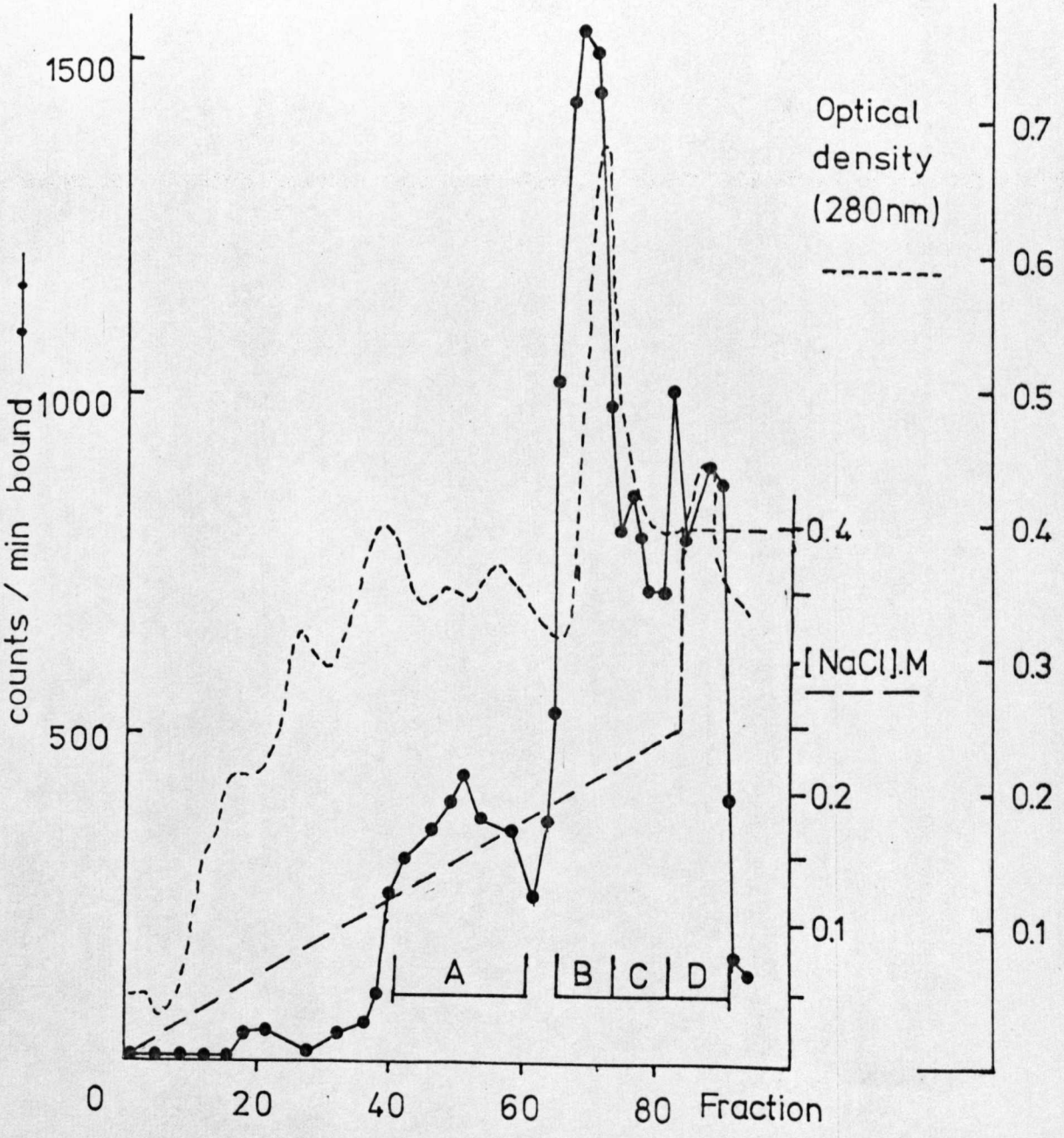
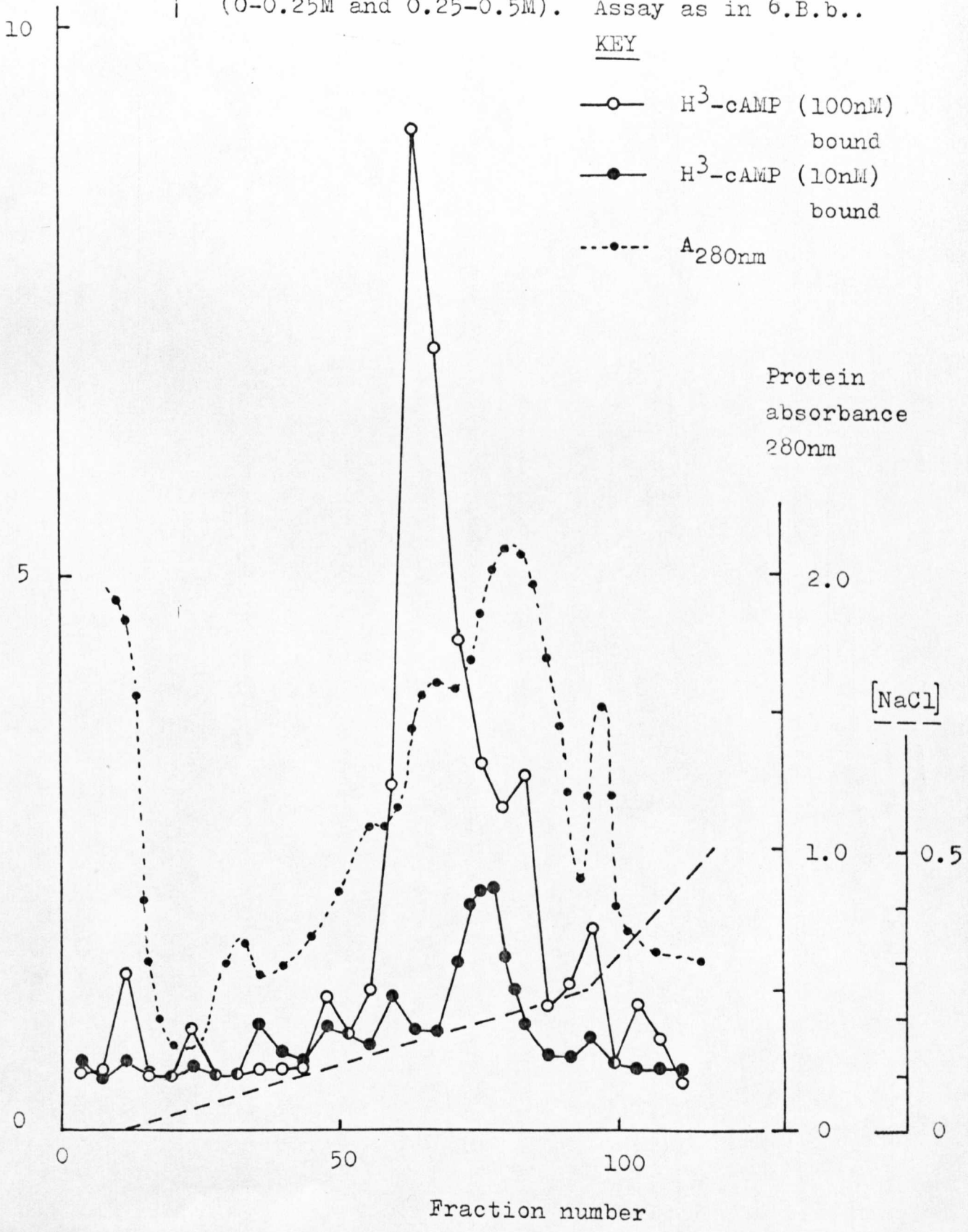


Figure 7.5

DEAE-cellulose column II

Elution profile of 50ml dialysed 30-65% ammonium sulphate fraction from a 2 x 23cm DEAE-cellulose column equilibrated with 20mM Tris. HCl pH 7.3 containing 6mM 2-mercaptoethanol and eluted with two linear sodium chloride gradients (0-0.25M and 0.25-0.5M). Assay as in 6.B.b..



two peaks were pooled and frozen. Samples were thawed for determination of the dissociation constants of the two proteins (8.D.c.) and subsequently were used in a study on the effect of other column fractions on the binding of cyclic-AMP (8.E.c.).

III (figure 7.6) DEAE-cellulose chromatography of a 0 - 50% ammonium sulphate fraction.

The fractions from the column were assayed for cyclic-AMP binding activity using a 100nM solution of H^3-cyclic-AMP. (Assays were also carried out using 10nM and 1μM H^3-cyclic-AMP but unlike the previous column the elution pattern was not changed.) The fractions were also assayed for protein kinase activity in the absence and in the presence of 0.67μM cyclic-AMP. The results obtained are similar to those of Gill and Garren (1970) except for the high peak of cyclic-AMP dependent protein kinase activity which just precedes the major cyclic-AMP binding peak. The active fractions from this column were used on a Sephadex column so as to distinguish between the peaks on the basis of molecular weight (7.C.d.). Dilution curves were constructed for the cyclic-AMP binding activity and protein kinase activity of the active fractions (figure 7.7).

A summary of the results obtained from these columns is given in table 7.3.

7.C.d. Sephadex G-100 gel filtration

Sephadex G-100 was chosen for use as the bovine adrenal cortex protein kinases have molecular weights of about 145,000 and 60,500 (Gill and Garren: 1971). This meant that the protein kinase was near the exclusion limit for the gel and the length of time the proteins were on the column was minimised.

A column 4.5 x 55cm was prepared using gel swollen in 50mM Tris.HCl pH 7.4 containing 0.1M NaCl and 6mM 2-mercaptoethanol. The gel was protected by a layer of Sephadex G-25.

The DEAE-cellulose column III fractions containing protein kinase and cyclic-AMP binding activity were pooled and reduced in volume by precipitating the protein

Figure 7.6

DEAE-cellulose column III

Elution profile of 400ml dialysed 0-50% ammonium sulphate fraction from a 4.5 x 60cm DEAE-cellulose column equilibrated with 20mM Tris HCl pH 7.4 containing 2-mercaptoethanol and eluted with a linear gradient of sodium chloride (0-0.5M).

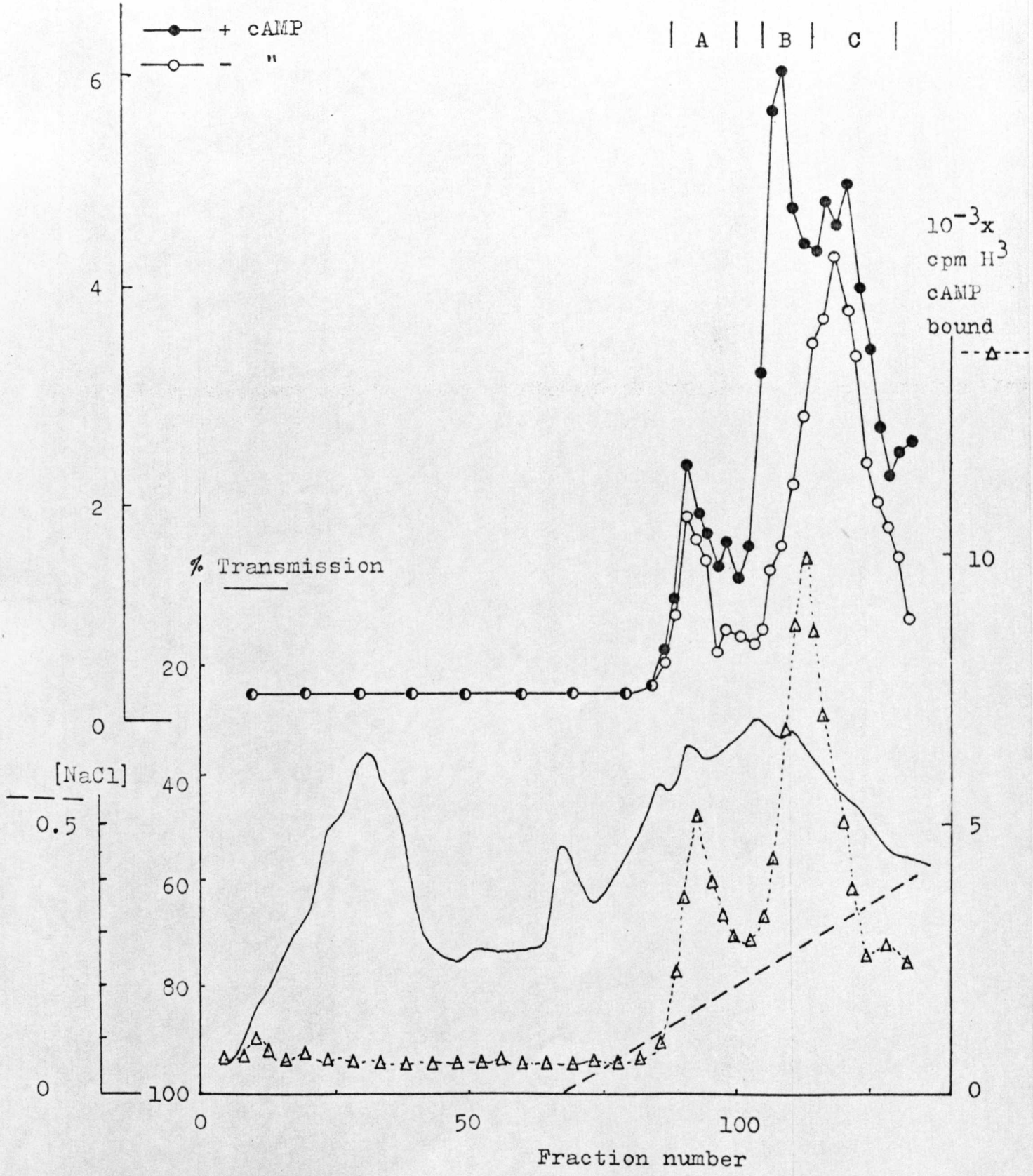


Figure 7.7

Dilution curves for the DEAE-cellulose III fractions A, B and C.

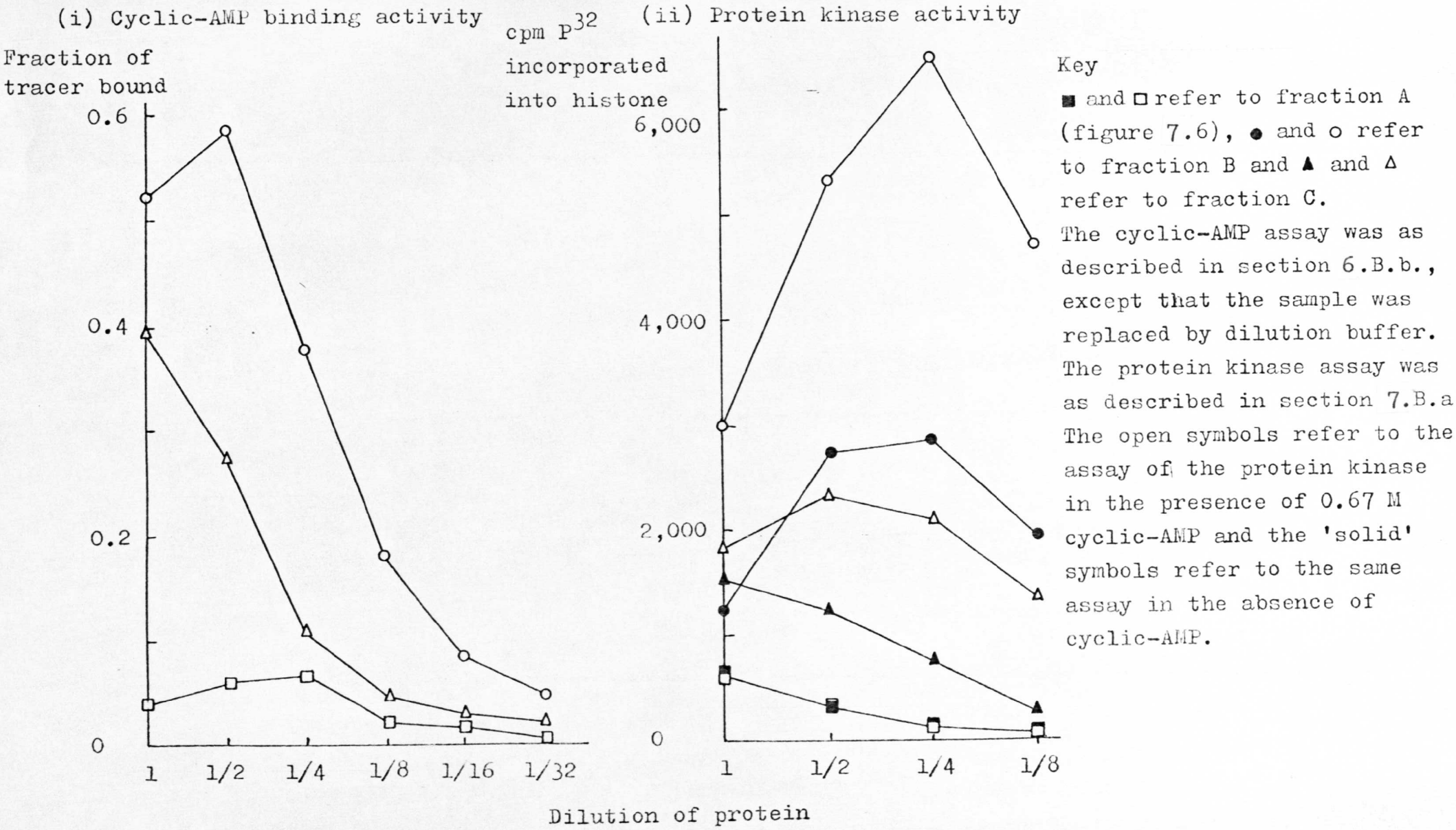


Table 7.3

Purification of the cyclic-AMP binding protein by DEAE-cellulose chromatography

I

Fraction	Volume ml	Protein mg/ml	C-AMP bound cpm (10nM)	C-AMP bound* cpm (20nM)
A	14.5	4.2	500	564
B	5.7	2.8	3400	4850
C	8.5	1.7	2900	2520
D	5.8	1.1	2300	1290

* the second assay using 20nM cyclic-AMP was performed after leaving the fractions for 1 week.

II

Fraction	Volume ml	Protein mg/ml	K_d nM	Comments
A	49	8.9	1.4	Expected larger value for K_d.
B	55	7.0	14	

III

Fraction	Volume ml	Binding activity		Protein kinase activity		
		dil^n	r	dil^n	+c-AMP	-c-AMP
A	170	1 in 4	0.07	1	650	650
B	195	1 in 2	0.59	1 in 4	2850	6500
C	160	1	0.40	1 in 2	1250	2350

The volumes and protein concentrations in part I relate to the fractions after treatment with Aquacide to reduce the volumes. The normal cyclic-AMP binding assay was used with the concentrations of tracer as shown in the table (see section 6.B.b. for the assay). In part III of the table the cyclic-AMP binding activity and the protein kinase activity are given for the dilution which gave the maximum activity in each case: r is the fraction of tracer bound and the protein kinase activity was measured in the presence and absence of 0.67μM cyclic-AMP as described in section 7.B.a..

with a 0 - 60% saturation ammonium sulphate cut at pH 6.0. The precipitate was resuspended in about 30ml of the column equilibration buffer and centrifuged at 38,000 rpm using a 50Ti head in the Beckman L-2 ultracentrifuge.

The clarified solution was applied to the column. Every third fraction was assayed for both cyclic-AMP binding activity and protein kinase activity (as described in sections 6.B.b. and 7.B.a. respectively). Small peaks of both activities were present (figure 7.8). However, assays carried out the following day on the fractions containing the peaks were not able to detect either activity.

It was concluded that as a result of the use of the Sephadex column the enzyme became unstable. It was noted that other workers using Sephadex gels to purify protein kinases use 10% glycerol to stabilise the enzyme.

7.C.e. Sucrose density gradient centrifugation

Samples taken from the DEAE-cellulose column I (7.C.c.) were applied to sucrose gradients and centrifuged as described in section 7.B.b.. The sedimentation pattern is shown in figure 7.9. The gradients were sampled by inserting a long needle down the tube and withdrawing the contents using a peristaltic pump at low speed. The fractions were measured by counting the drops. Equal aliqots from each fraction were assayed for cyclic-AMP binding activity.

A control in which increasing concentrations of sucrose were added to three of the DEAE-cellulose fractions showed that sucrose did not affect the binding of cyclic-AMP to the protein at concentrations below 0.2M. Above this concentration the binding is decreased. The molarity of the sucrose in the gradient was between 0.146M (5%) and 0.585M (20%). The samples taken from the gradients were all diluted 1 in 4 in the assay so the concentration of sucrose was less than 0.2M in every assay.

7.C.f. Polyacrylamide gel isoelectric focusing

A sample taken from the pH 7.4 cellulose phosphate column with high cyclic-AMP binding activity was applied

Figure 7.8

Sephadex G-100 gel filtration

The elution profile of cyclic-AMP binding activity and protein kinase activity from a 4.5 x 55cm Sephadex G-100 column. The details are given in the text (7.C.d.). Elution was with the equilibration buffer. The K_{av} values were calculated by using a standard equation (Reiland: 1971). The void volume was obtained using blue dextran.

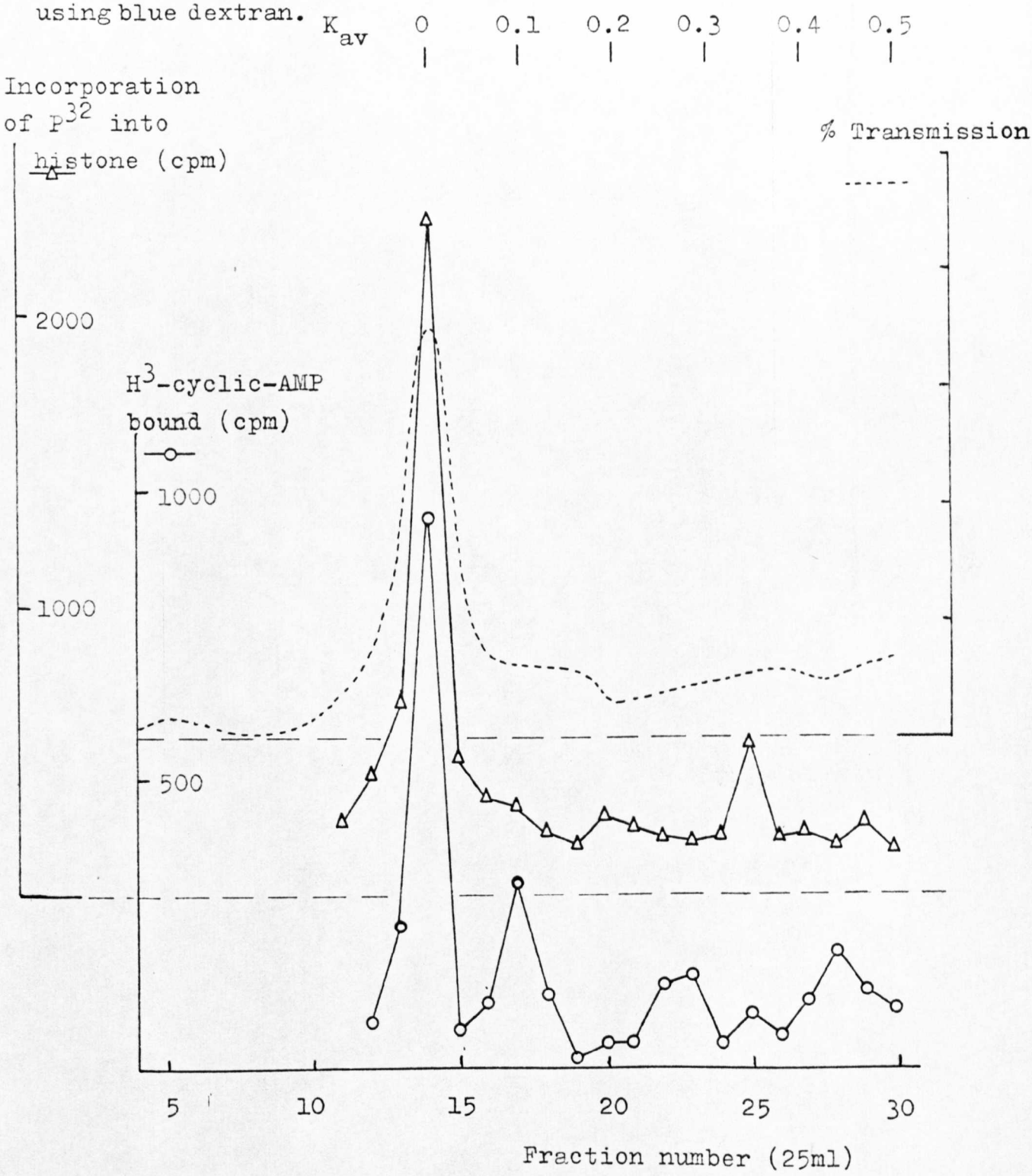


Figure 7.9

Sucrose density gradient centrifugation

(For details see 7.C.e.)

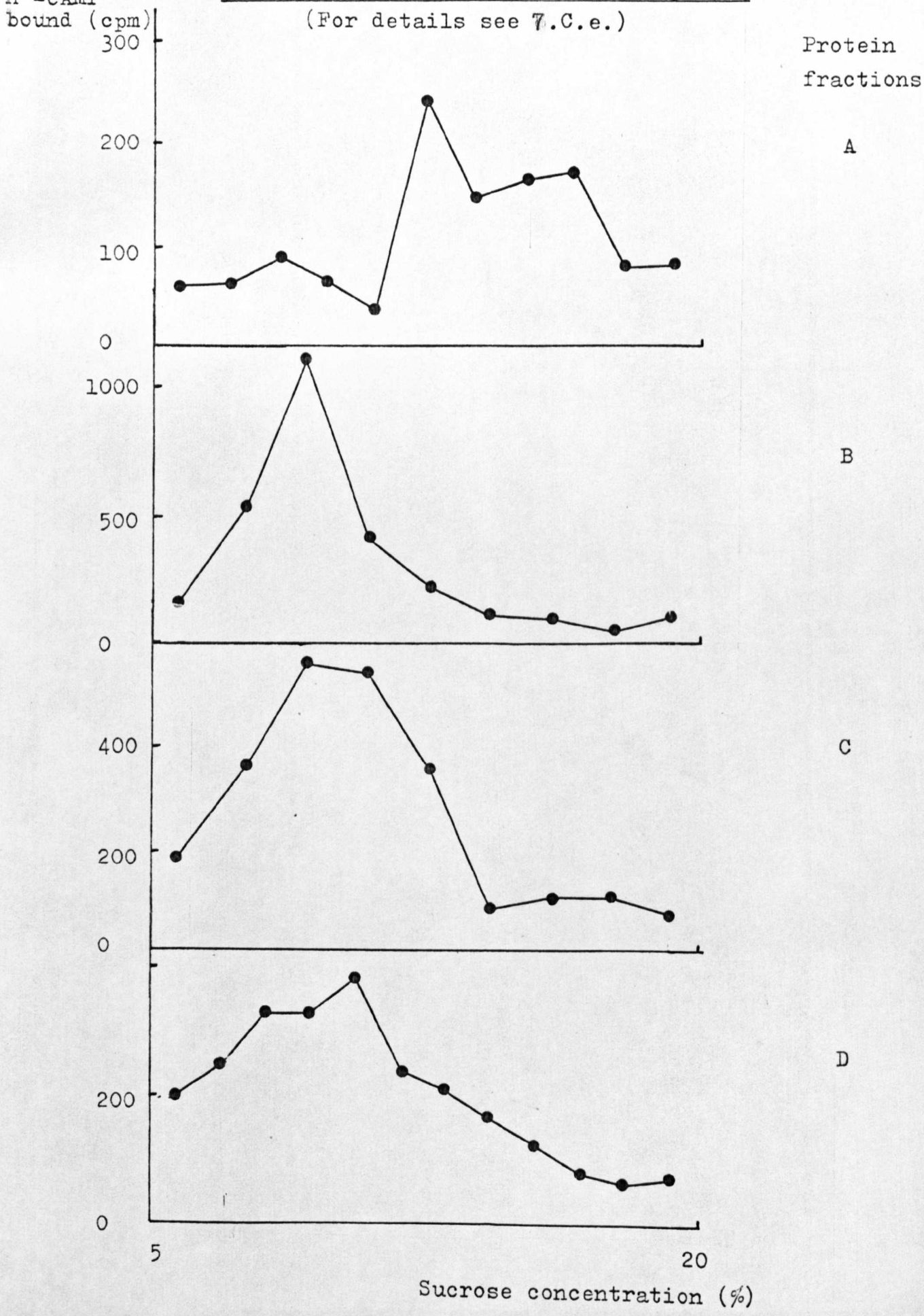
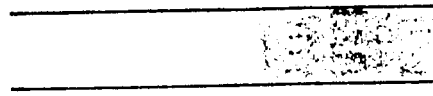


Figure 7.10

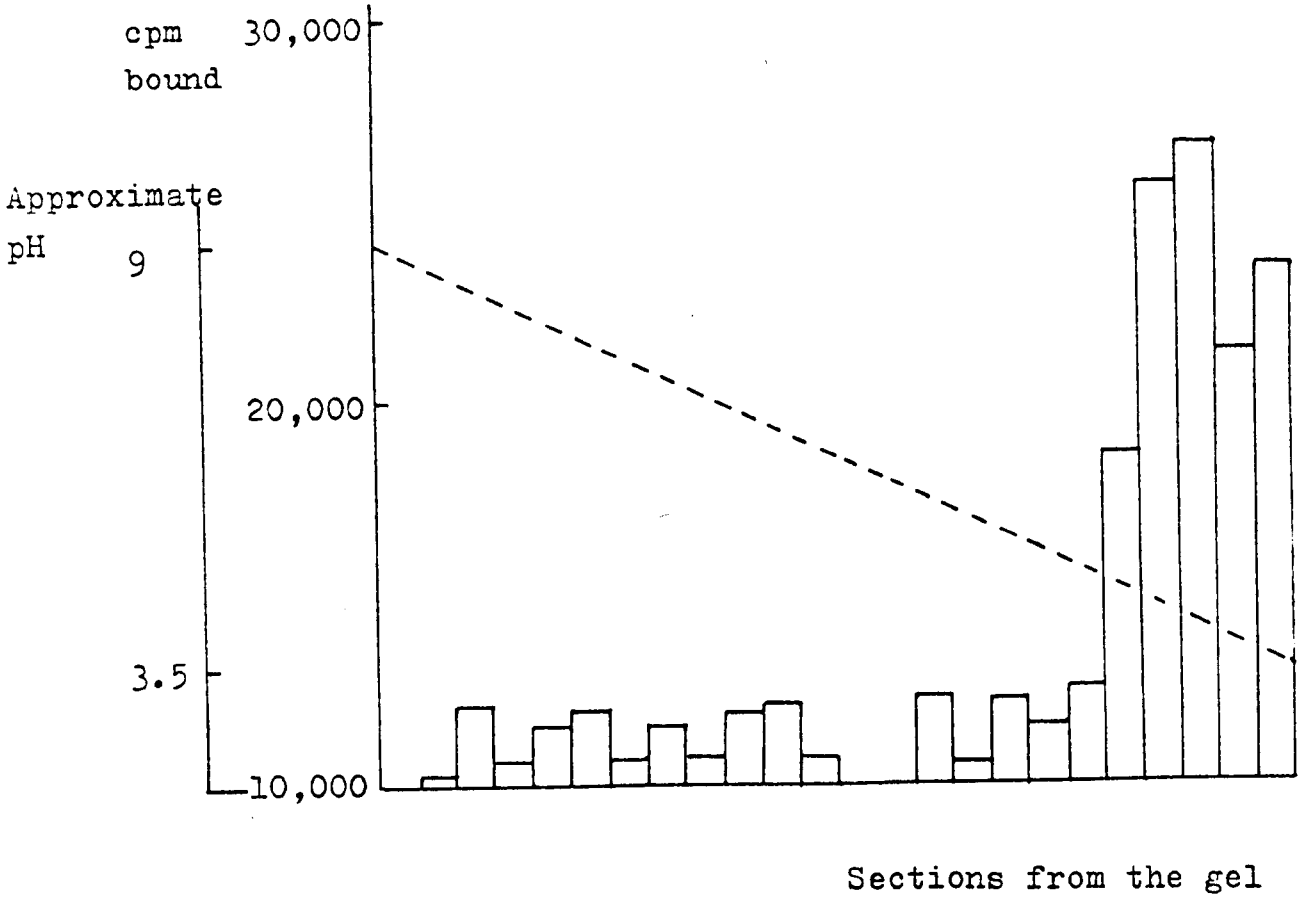
Polyacrylamide gel isoelectric focusing

(For details see 7.C.f.)

1) Impression of gel stained with Coomassie blue.



2) H^3-cyclic-AMP bound to protein in the gel.



to the polyacrylamide gel (see sections 7.B.c. and 7.C.b.). Figure 7.10 shows the migration pattern of the proteins in the sample and the position of the binding activity. The pH range across the gel was pH 3.5 to pH 9.0. The cyclic-AMP binding region was in the low pH region corresponding to pH 4.0 - 4.5. Gill and Garren (1971) found that the isoelectric point of the protein kinases from the bovine adrenal cortex was pH 4.75.

7.D. Discussion

The protein kinase assay was a combination of a standard protein kinase assay incubation (Kuo et al. 1971) and a batch method for preparing the assay samples for counting. This enabled the assay of large numbers of samples with good reproducibility.

A number of methods were used to obtain partial purifications of the cyclic-AMP binding protein and the protein kinase. These are discussed separately below.

(i) Ammonium sulphate fractionation A consistent finding in the ammonium sulphate fractionations was the presence of two protein fractions containing binding proteins with low dissociation constants ($K_d < 5$) and one with a higher dissociation constant ($K_d > 20$). The two protein fractions containing the proteins with low dissociation constants precipitated at about 20% and 40 - 50% ammonium sulphate saturation. The protein with the higher dissociation constant precipitated between these two.

(ii) Cellulose phosphate column chromatography Two peaks of cyclic-AMP binding activity were eluted from cellulose phosphate columns at pH 7.4 and pH 5.1 when a sample of the 5,000 x g supernatant was applied to the column. The two peaks, which probably correspond with the protein kinase holo-enzyme and the cyclic-AMP binding regulatory subunit, were not well resolved. So further characterisation was not attempted.

(iii) DEAE-cellulose column chromatography The results from these columns has been discussed a little in the relevant text. The first column described followed the pattern of Gill and Garren's (1970). The differences

between the results of the two columns has been commented on.

A 30 - 65% ammonium sulphate precipitated protein fraction was added to the second column (II). Two peaks of cyclic-AMP binding activity were eluted from this column. The first peak to be eluted was detected with 100nM cyclic-AMP, the second peak was more apparent with 10nM cyclic-AMP. The implication appeared to be that the second peak contained a protein with a low dissociation constant for the binding of cyclic-AMP and that the first peak contained a protein which bound cyclic-AMP more weakly. This is the reverse of what was actually found (S.D.c.) after the fractions had been stored in a frozen state. This is discussed more in the following chapter.

The third DEAE-cellulose column (III) was used to fractionate a 0 - 50% ammonium sulphate precipitated protein fraction. The eluate was assayed for both cyclic-AMP binding activity and protein kinase activity. Two peaks of cyclic-AMP binding activity were eluted. Cyclic-AMP independent protein kinase activity was associated with the first peak. The pattern of protein kinase activity around the second cyclic-AMP binding fraction was rather more complicated. A peak of cyclic-AMP dependent protein kinase activity just preceded the peak of cyclic-AMP binding activity. Immediately following the peak of binding activity was a peak of cyclic-AMP independent protein kinase activity. The peaks of activity were pooled as shown in figure 7.6. The cyclic-AMP binding and protein kinase activities of these pooled fractions was shown in figure 7.7. Both of these activities are increased by dilution in the case of fraction B and in the case of fraction C the cyclic-AMP dependent protein kinase activity is also increased by dilution. A similar effect has been noticed with the cyclic-AMP binding activity of the protein precipitated between 35 and 50% saturation of ammonium sulphate.

This behaviour may be due to some further equilibrium

being imposed on the cyclic-AMP binding equilibrium and controlling the amount of protein kinase holo-enzyme available for activation. The possible reasons for this are discussed further in the following chapter.

(iv) Sephadex G-100 gel filtration The exclusion limit for this gel prohibits its use to detect aggregates of the protein kinase. The conditions under which the column was used made the enzyme unstable.

(v) Sucrose density gradient centrifugation The protein fractions from the DEAE-cellulose column I were applied to sucrose gradients. The resulting sedimentation pattern was similar to that obtained by Gill and Garren (1971). Fraction A, however, in addition to a peak corresponding to Gill's 7S protein kinase holo-enzyme also had a broad shoulder peaking in the 10S region. The other fractions gave peaks in the region of 4S only. The 10S peak may represent a dimeric form of the protein kinase as found in bovine brain (Miyamoto et al.: 1971). A simple equation relating sedimentation coefficients, S, to molecular weight M is

$$\left|\frac{S_1}{S_2}\right|^{\frac{3}{2}} = \frac{M_1}{M_2} .$$

The equation is only approximate but if the values S_1=10 and S_2=7 are taken the ratio of molecular weights is 1.7:1.

(vi) Polyacrylamide gel isoelectricfocusing The result of the iso-electric focusing demonstrated the impurity of the enzyme eluted from the cellulose phosphate column. The isoelectric point corresponds to that found by other workers.

The various techniques applied here to differentiate between different forms of the cyclic-AMP binding protein and protein kinase could be employed jointly to purify one or more forms of these proteins. This work represents an extension of existing work. For example, ammonium sulphate precipitation has been used in most preparations of the cyclic-AMP dependent protein kinase but only as a concentration step and not as a fractionation (see Reimann et al.: 1971 and Yamamura et al.: 1971).

The results of these purification steps and the binding studies carried out on the different preparations strongly indicates that the binding of cyclic-AMP to the protein kinase is not just a simple one to one binding equilibrium as has been generally assumed up till now.

CHAPTER 8

SOME PROPERTIES OF THE CYCLIC-AMP BINDING PROTEIN

8.A. Introduction

The limiting sensitivity of the Brown assay for cyclic-AMP is partially determined by the dissociation constant for the binding of cyclic-AMP to the protein kinase (Ekins and Newman: 1970).

This chapter deals with some binding studies carried out on various preparations and fractions of the cyclic-AMP binding protein from the bovine adrenal cortex. These studies were carried out because in the course of this work it was found that some of the binding curves were non-linear. Such binding studies are highly relevant to the study of how the protein kinase functions as a link in the second messenger role of cyclic-AMP.

Cyclic-etheno-AMP, a fluorescent analogue of cyclic-AMP, was prepared. Its use as a probe for investigating the binding of cyclic-AMP to the protein kinase was evaluated.

8.B. Materials and methods

All the materials and methods used in the work presented in this chapter have been described elsewhere in the thesis except for the preparation of cyclic-ethenoAMP which is dealt with separately in section 8.F.a.

The assay used to measure cyclic-AMP binding was as described for the assay of chapter 6 (6.B.b.), but the concentration of cyclic-AMP was varied.

The preparations of binding protein which were used were the 5,000 x g supernatant (6.B.a.), ammonium sulphate fractions of the same (7.C.a.) and DEAE-cellulose column fractions (7.C.c.).

8.C. Analysis of multicomponent binding curves

The binding curves constructed in this work were based on the equilibrium relationship for one to one binding in which there are no interactions between the binding sites:

$$K_d = \frac{[\text{Protein kinase}].[\text{cyclic-AMP}]}{[\text{Protein kinase: cyclic-AMP complex}]}$$

Substituting B for the concentration of complex, F for the free cyclic-AMP concentration and P for the total binding site concentration, the equation rearranges to

$$B = P - K_d \cdot \frac{B}{F}.$$

A graph with B as the ordinate and B/F as the abscissa will be a straight line if the binding species is as described above.

A curved line with increasing gradient will result if there are more than one species of binding protein present which have different dissociation constants for the binding of cyclic-AMP. The curvature may also arise from negative cooperativity or binding protein subunit association/dissociation phenomena affecting either the stoichiometry of the binding process or the dissociation constant for the binding of cyclic-AMP.

If it is assumed that such curvature is due to the presence of more than one binding species, each of which has a single non-interacting one to one binding site for cyclic-AMP, the curve can be analysed to give the dissociation constants and binding site concentrations for the different species. In practice, it is only possible to do so with two binding species or three if the data is sufficiently accurate.

A graphical method of fitting curves to two binding species was used (see appendix) which was adapted from the work of Rosenthal (1967). Graphically the curve is the radial sum of the binding curves for the component species.

8.D. Cyclic-AMP binding studies.

8.D.a. Cyclic-AMP binding curves for the 5,000 x g supernatant

The shape of the binding curve and the constants derived from it were found to vary with the protein concentration used (figure 8.1).

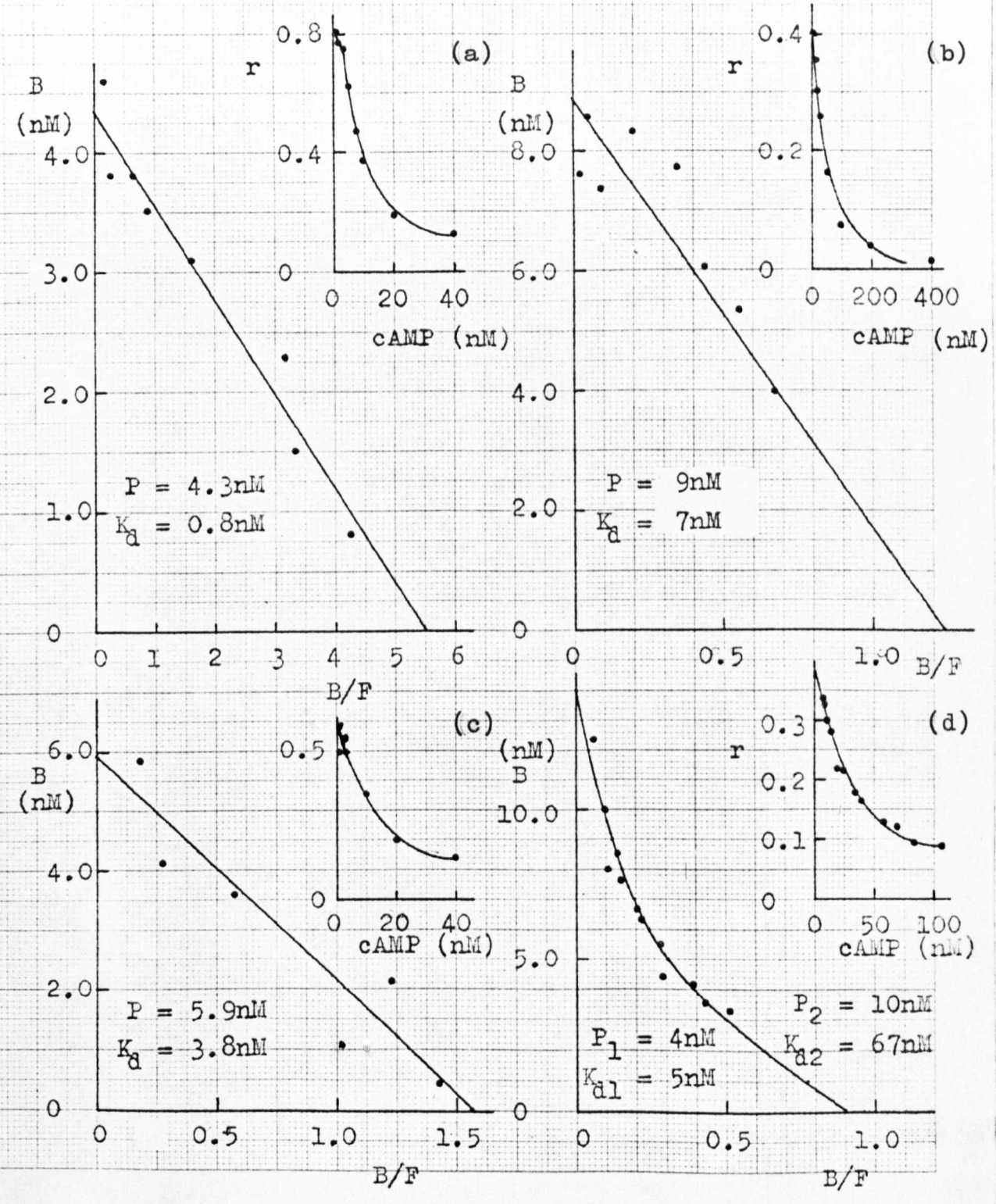
The binding curve (B against B/F) is best used for determining the dissociation constant of a binding

Figure 8.1

Cyclic-AMP binding curves for the 5,000 x g supernatant

a) 5,000 x g supernatant undiluted, cyclic-AMP 1 - 40nM
b) " " " " , " 10 - 400nM
c) " " " diluted 1 in 8, cyclic-AMP 1 - 40nM
d) " " " " 1 in 4, " 10 - 400nM

r = fraction of cyclic-AMP bound, B = concentration of bound fraction and F = concentration of free cyclic-AMP.



process when the binding site concentration is of the order of the dissociation constant or lower ($K_d \geq P$) and the cyclic-AMP concentration varies from approximately $K_d/2$ to $4K_d$. This is because the B/F ratio will be most precisely determined under those conditions in which both B and F can be accurately measured and this is when the ratio is about unity. If P is much less than K_d, the concentrations of B and F may be reduced to a low level difficult to determine accurately.

However, in the case of this cyclic-AMP binding protein it appeared that the dilution of the protein increased the dissociation constant. Also, given a sufficiently high concentration of cyclic-AMP (>40nM), the binding curves displayed a curvature characteristic of mixtures of binding proteins. This and the other possible explanations for the curvature are discussed more fully at the end of the chapter.

8.D.b. Cyclic-AMP binding to protein samples prepared by ammonium sulphate fractionation

The ammonium sulphate fractions containing the majority of the cyclic-AMP binding activity are shown in figure 8.2.. It appeared from these results that the protein which precipitated between 20% and 40% saturation had a slightly higher dissociation constant than that which precipitated at higher or lower concentrations of ammonium sulphate.

The protein precipitated at 15% saturation, and the residue remaining after the removal of protein precipitating at 65% saturation, both had negligible binding capacity for cyclic-AMP.

8.D.c. Binding of cyclic-AMP by protein fractions from a DEAE-cellulose column

The dissociation constants for the binding of cyclic-AMP by the protein fractions obtained from the DEAE-cellulose column II (7.C.c.) were obtained.

Fraction A was not detected by the assay of the column fractions in which 10nM H^3-cyclic-AMP was used, but was detected in the assay in which 100nM H^3-cyclic-AMP was used. It was therefore considered that it was

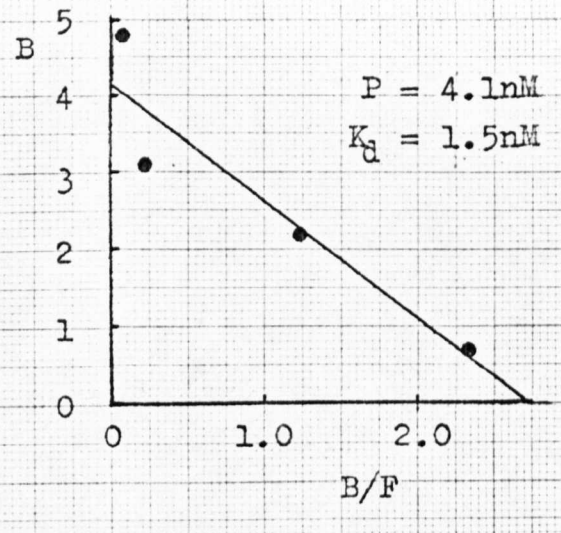
Figure 8.2

Cyclic-AMP binding curves for the protein precipitated by ammonium sulphate fractionation

(i) Cyclic-AMP binding curves for the protein fractions collected between 0 and 20%; 20 and 40%; 40 and 60% ammonium sulphate saturation, and the 5,000 x g supernatant from which the fractions were taken.

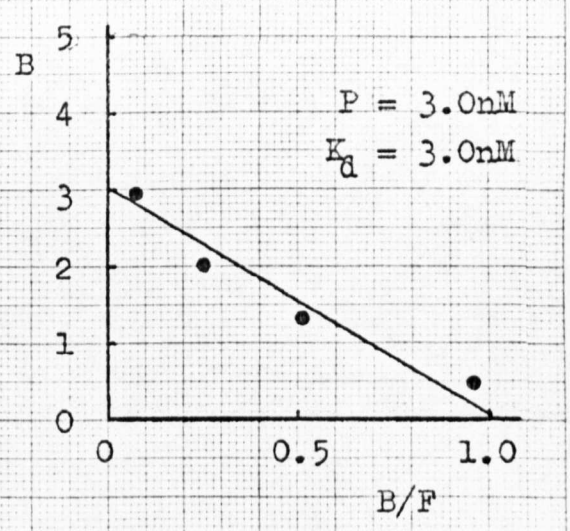
5,000 x g supernatant

(1 - 40nM cyclic-AMP)



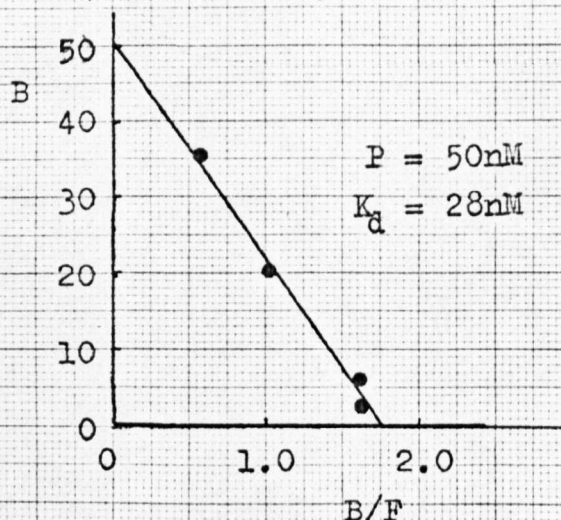
0 - 20% precipitate

(1 - 40nM cyclic-AMP)



20 - 40% precipitate

(4 - 100nM cyclic-AMP)



40 - 60% precipitate

(1 - 40nM cyclic-AMP)

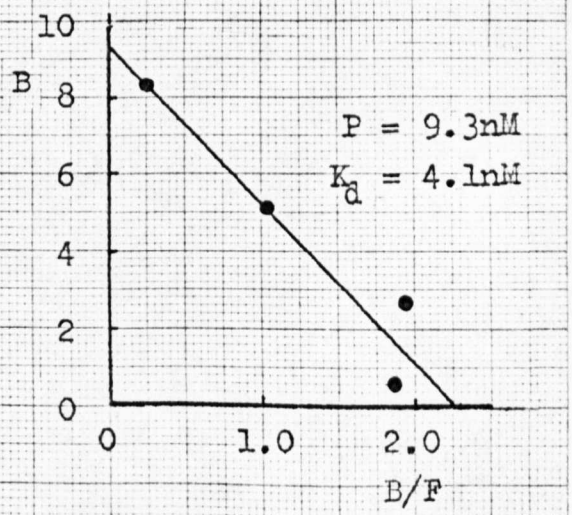
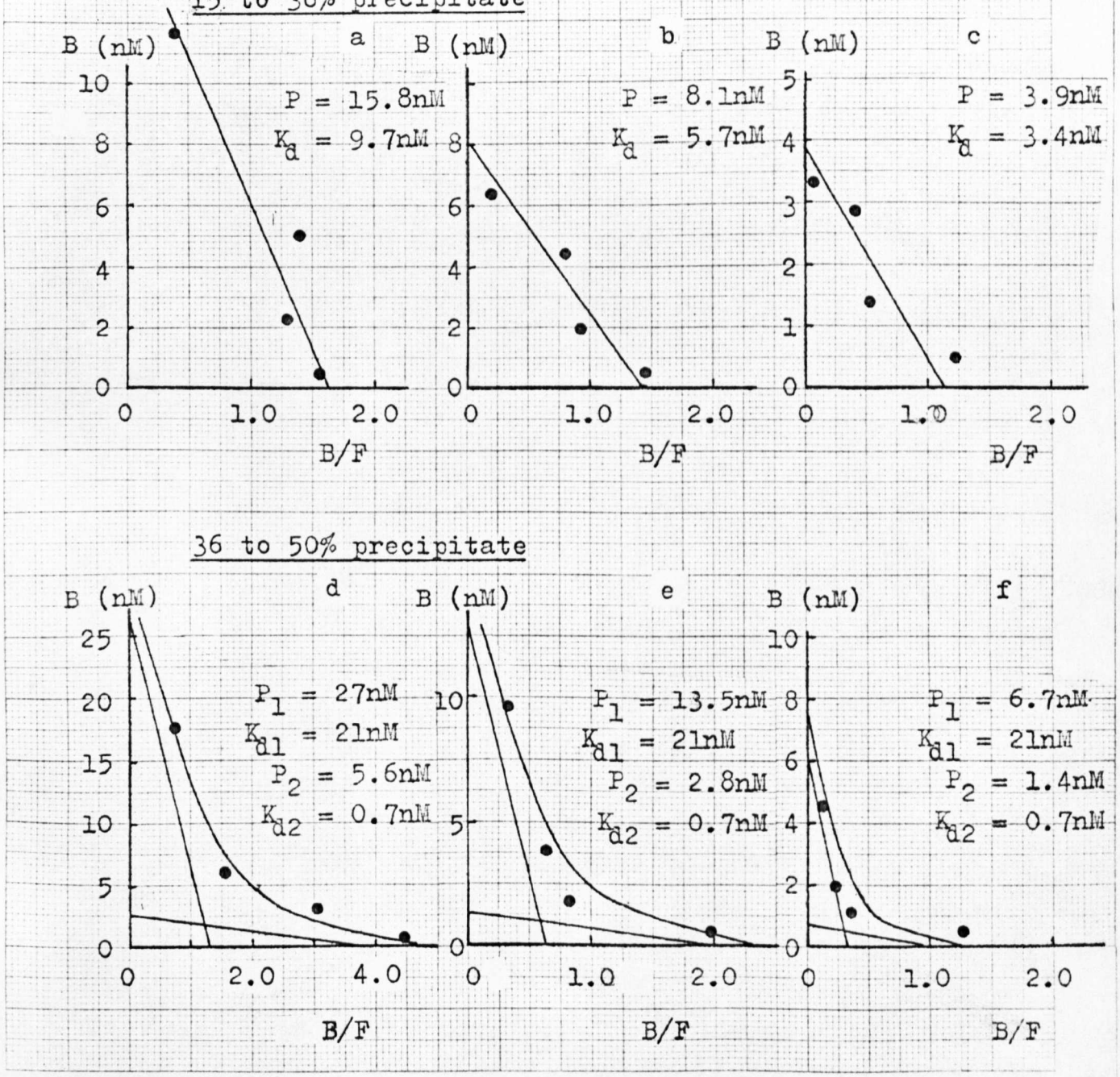


Figure 8.2 (continued)

(ii) Cyclic-AMP binding curves for the protein fractions collected between 15 and 36%, and 36 and 50% ammonium sulphate saturation. Curves a, b, and c are for the 15 to 36% ammonium sulphate precipitate diluted 1 in 4, 1 in 8 and 1 in 16 respectively. Curves d, e and f are for the 36 to 50% precipitate diluted 1 in 8, 1 in 16 and 1 in 32 respectively. The binding of cyclic-AMP was at concentrations from 1 to 40nM using the Brown assay (6.B.b.).



a weaker binding protein for cyclic-AMP than fraction B which was detected in the presence of 10nM H^3-cyclic-AMP. The binding study carried out after the fractions had been frozen and thawed gave the opposite result. Fraction A was determined to have a dissociation constant of 1.9nM and a binding site concentration of 8.9nM. Fraction B had a dissociation constant of 14nM and a binding site concentration of 7nM. The binding curves are shown in figure 8.3. This remains an anomalous result and no attempt was made to repeat it. The two fractions were mixed with other protein fractions from the same DEAE-cellulose column and assayed for binding activity.(8.E.c.). The binding of the fractions A and B did not appear to be affected by ethanol at 5% concentration in the cyclic-AMP assay (see 8.E.b.). These results are discussed at the end of the chapter.

8.E. Factors effecting the binding of cyclic-AMP to its binding protein.

8.E.a. The effect of cyclic-GMP on the binding of cyclic-AMP

The effect of cyclic-GMP on the binding of cyclic-AMP by the 5,000 x g supernatant protein and the protein from two ammonium sulphate fractionations and from DEAE-cellulose column I was determined.

The effect of cyclic-GMPon the 5,000 x g supernatant, and the 15 - 36% and 36 - 50% ammonium sulphate fractions is shown in figure 8.4. The cyclic-AMP binding assay was done in the presence and absence of 1µM cyclic-GMP (see section 6.B.b. for the assay).

Cyclic-GMP seems, at first sight, to be competitive with cyclic-AMP for the binding by the 5,000 x g supernatant protein. However, the line veers from the apparent intercept on the ordinate. It appears, therefore that the dissociation constant and the number of binding sites are both increased. A similar effect was observed with the 15 - 36% ammonium sulphate precipitate. In the absence of cyclic-GMP the 36 - 50% ammonium sulphate precipitate had a biphasic binding curve. On the addition of cyclic-GMP the strongly binding component

Figure 8.3

Cyclic-AMP binding curves for the DEAE-cellulose column II peaks A and B

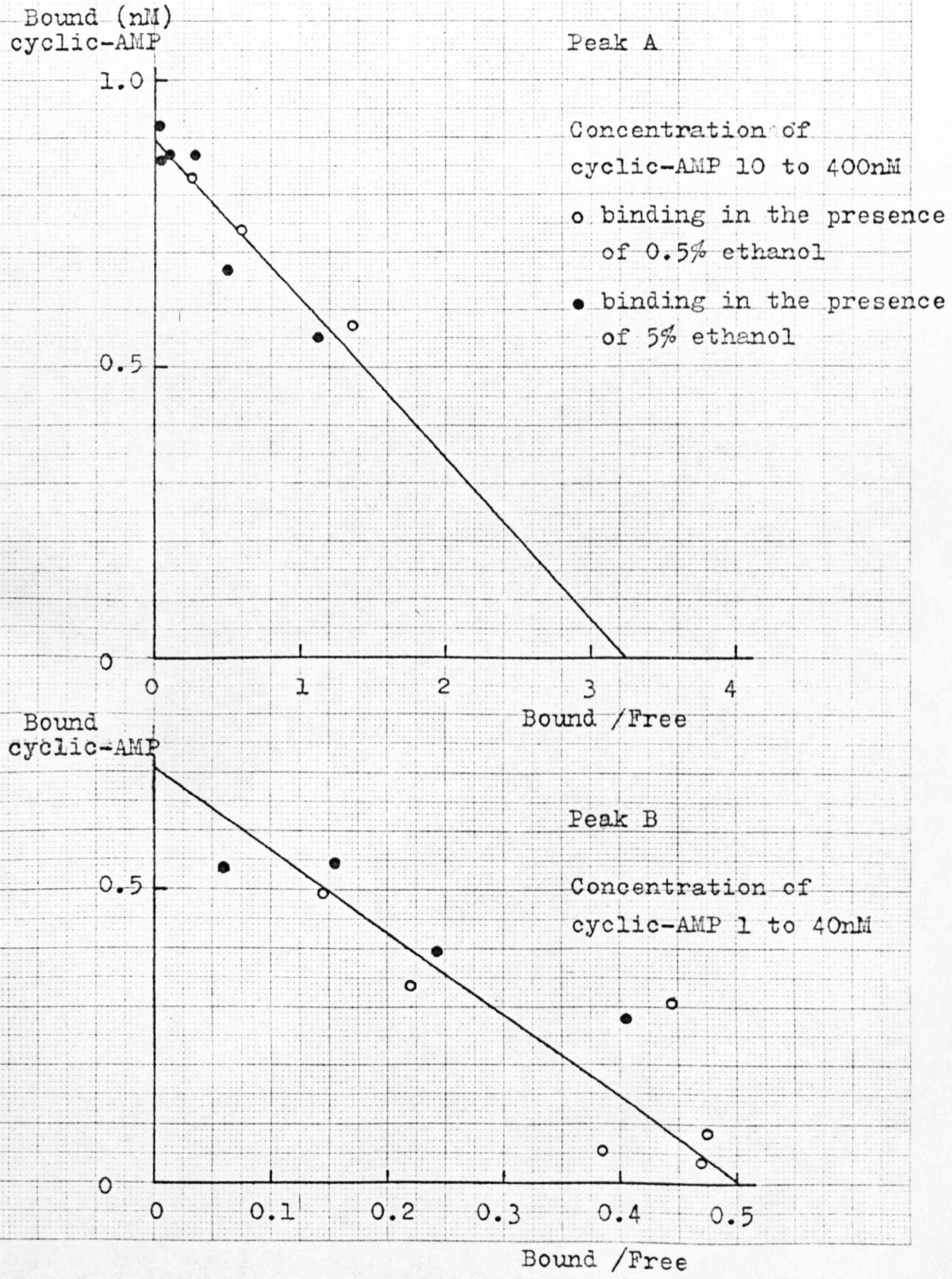
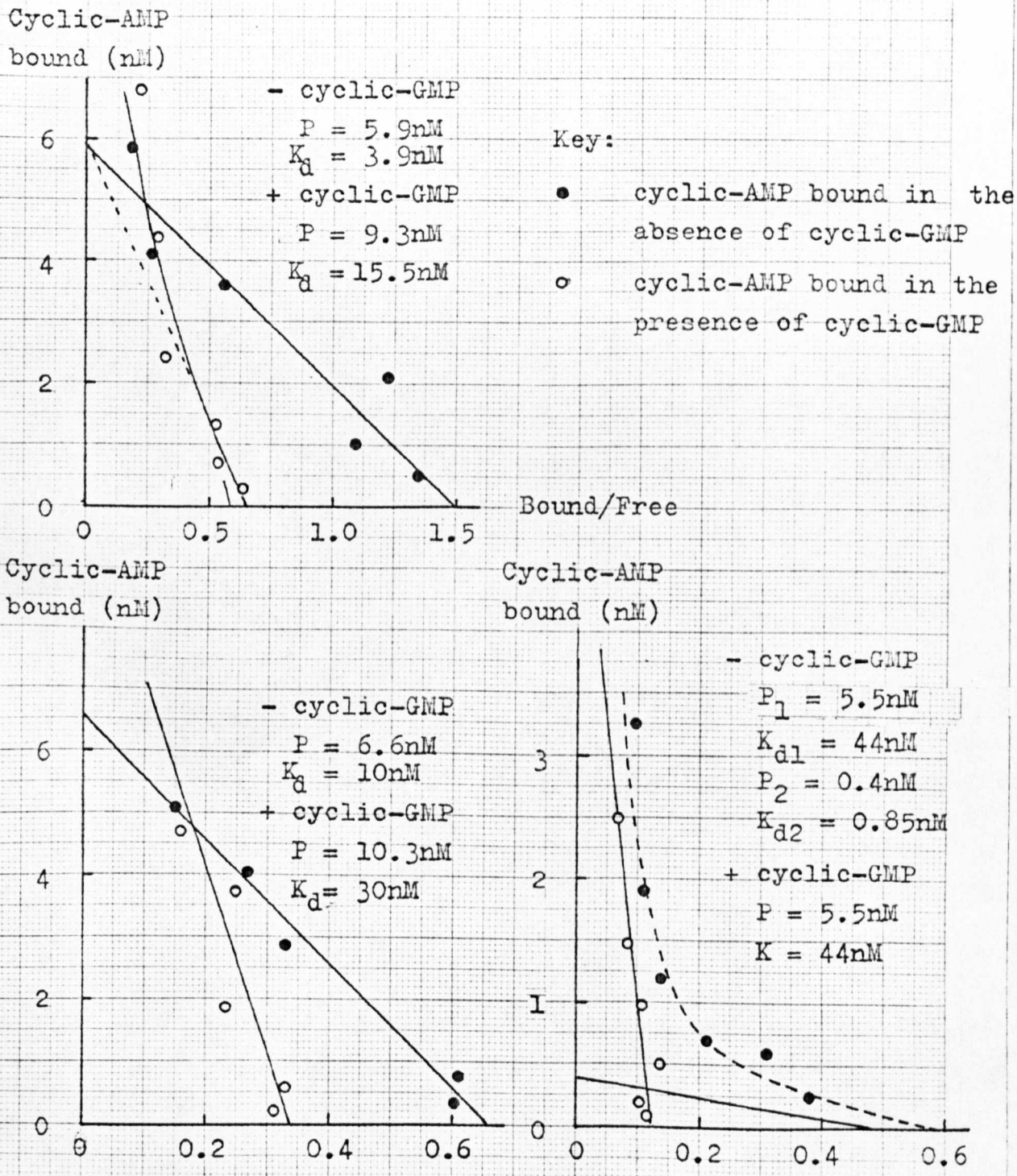


Figure 8.4

<u>The effect of cyclic-GMP on the binding of cyclic-AMP</u> by the binding protein in the 5,000 x g supernatant and in two ammonium sulphate fractions of it. The cyclic-AMP concentration varied from 1 to 40nM and the binding was carried out in the absence and in the presence of 1μM cyclic-GMP.



was not detected. These effects are discussed at the end of the chapter.

The binding of H^3-cyclic-AMP (10nM) by the fractions A to D from the DEAE-cellulose column I was determined with the following concentrations of cyclic-GMP present 10, 50, 250, and 500nM.

Increasing cyclic-GMP concentrations resulted in decreasing amounts of cyclic-AMP being bound. However, the amount of cyclic-AMP bound in the presence of 10nM cyclic-GMP was consistently greater than with no cyclic-GMP (figure 8.5).

8.E.b. The effect of ethanol on the binding of cyclic-AMP

The stock solutions of tritiated cyclic-AMP were made up in 50% ethanol to reduce the tritium exchange between the cyclic-AMP and the solvent water. As these solutions were not evaporated to dryness but were diluted before use the tracer solutions contained a small amount (about 0.3% in the assay) of ethanol. Therefore since the tracer used in the binding studies contained varying amounts of ethanol the effect of ethanol on the binding of cyclic-AMP to the binding protein was examined.

Two effects were observed. Firstly, with the 35-50% ammonium sulphate fraction the cyclic-AMP bound by the protein was increased by ethanol concentrations up to 5% by volume.(see figure 8.6). Secondly, at concentrations above 5% ethanol the amount of cyclic-AMP bound decreases as shown by binding curves for the 5,000 x g supernatant. (figure 8.6).

8.E.c. The effect of DEAE-cellulose column fractions on the binding of cyclic-AMP

An anomalous result was obtained for the dissociation constants of the two cyclic-AMP binding protein fractions from the DEAE-cellulose column II (7.C.c. and 8.D.c.). This led to an investigation of whether the other column fractions might contain effectors which might regulate the cyclic-AMP binding activity of one or both binding protein fractions.

Equal aliquots from each fraction were mixed with the same volume of the two binding protein fractions.

Figure 8.5

The effect of cyclic-GMP on the binding of cyclic-AMP by the fractions of binding protein from DEAE-cellulose column I.

The standard Brown assay was used with 10nM cyclic-AMP and the concentrations of cyclic-GMP indicated. The fractions are described in section 7.C.c.. The bars on the histogramme show the average deviation of four separate measurements.

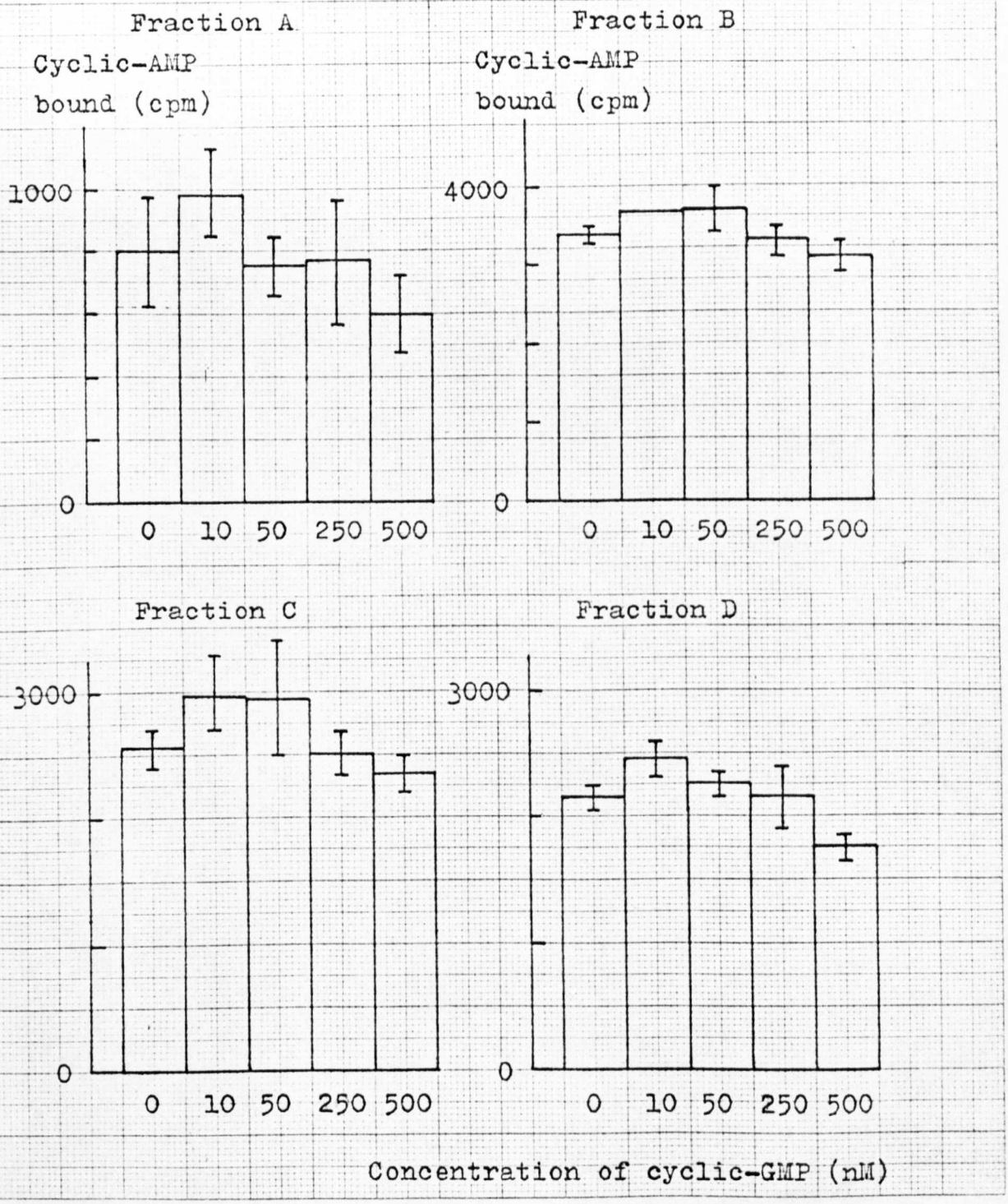
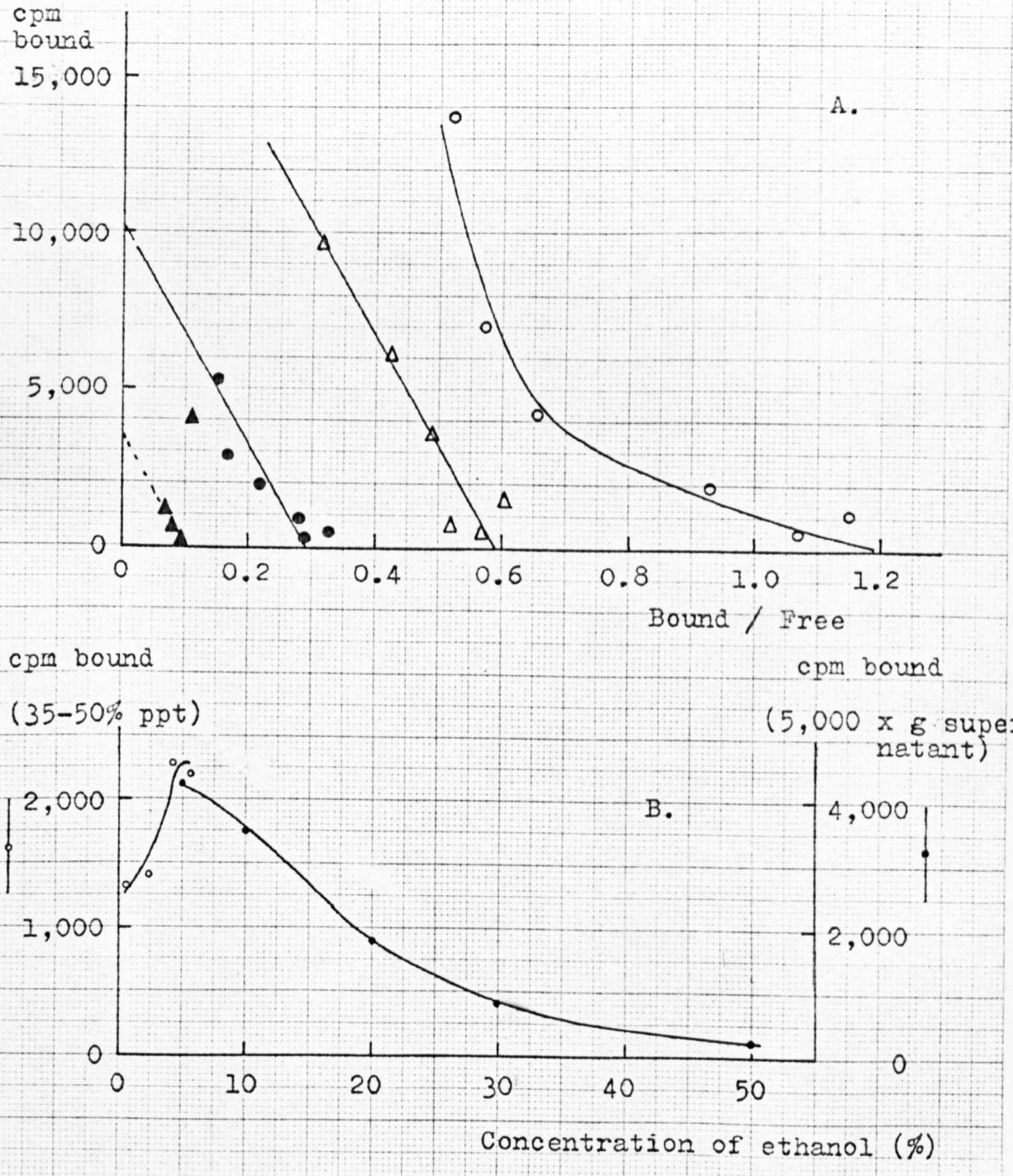


Figure 8.6

The effect of ethanol on the binding of cyclic-AMP

A. Binding curves for the 5,000 x g supernatant in the presence of the following concentrations of ethanol: o, 5%; Δ, 10%; •, 20%; and ▲, 30%. The cyclic-AMP concentration varied from 1 to 40nM.

B. The cyclic-AMP (cpm) bound by the protein from: —•— the 5,000 x g supernatant and —o— the 35-50% ammonium sulphate precipitate. The cyclic-AMP concentration was 10nM.



After leaving at 0°C for one week the mixtures were assayed for cyclic-AMP binding activity in the presence of 10nM H^3-cyclic-AMP. The results of this are shown in figure 8.7. There was a reduction of over 40% of the binding of cyclic-AMP by fraction B in the presence of column fractions 28 to 40 relative to the cyclic-AMP bound by the other mixtures. Following the week during which the mixtures were left the counts/minute bound by the mixtures of fraction B with fractions out of the range 20 to 48 was 1880±150. This compares with the counts/minute bound at the start of the week of 2900 by the same amount of protein from fraction B.

The average counts/minute bound by mixtures of fraction A with other fractions was 3700±350. The cyclic-AMP binding activity of the fraction A binding protein was not altered by mixing with the fractions in the range 28 to 40 but the counts/minute bound in all the mixtures was considerably less than the counts/minute bound by fraction A itself (6620cpm).

These results are discussed at the end of the chapter.

8.E.d. <u>The effect of pH on the binding of cyclic-AMP</u>

The effect of pH on the binding of cyclic-AMP was tested by diluting the 5,000 x g supernatant in six volumes of 0.1M citrate-phosphate buffers at different pH. The buffers each contained 8mM theophylline and 6mM 2-mercaptoethanol. The various protein solutions were centrifuged at 10,000 x g for 30 minutes to remove insoluble protein. The supernatants were removed and assayed for cyclic-AMP binding activity using the procedure of section 8.B.b. but at the pH of each supernatant and not at pH 7.4. The precipitates were resuspended in the dilution buffer (section 6.B.b.) and assayed at pH 7.4 following the procedure of section 6.B.b. in order to determine the amount of active binding protein that had been precipitated. The results are shown in figure 8.8.

Figure 8.7

Effect of other DEAE-cellulose column fractions on the cyclic-AMP bound by DEAE-cellulose column II fractions A and B

The cyclic-AMP binding was determined by the Brown assay described in section 6.B.b.. The details for this experiment are in the text.

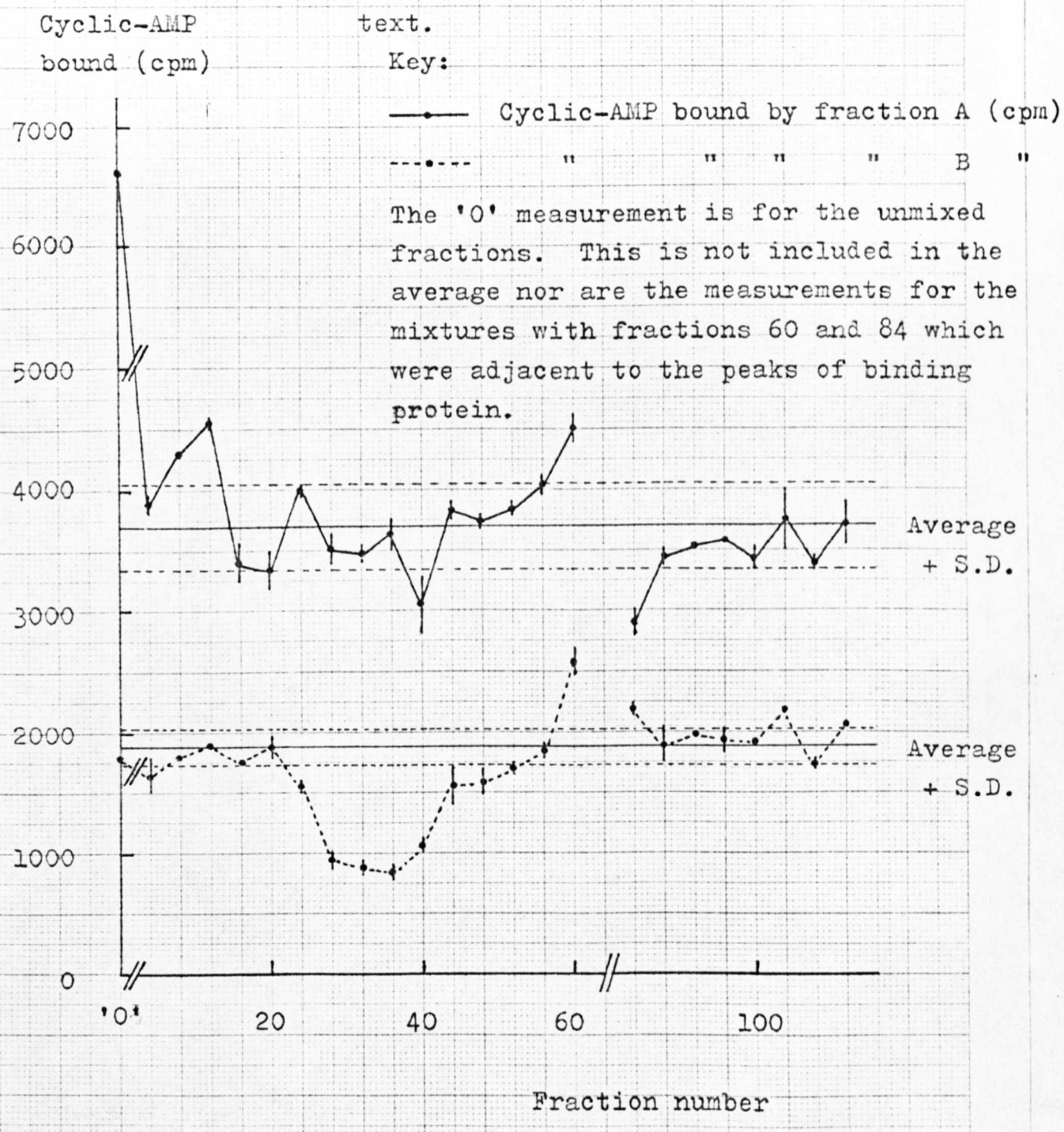
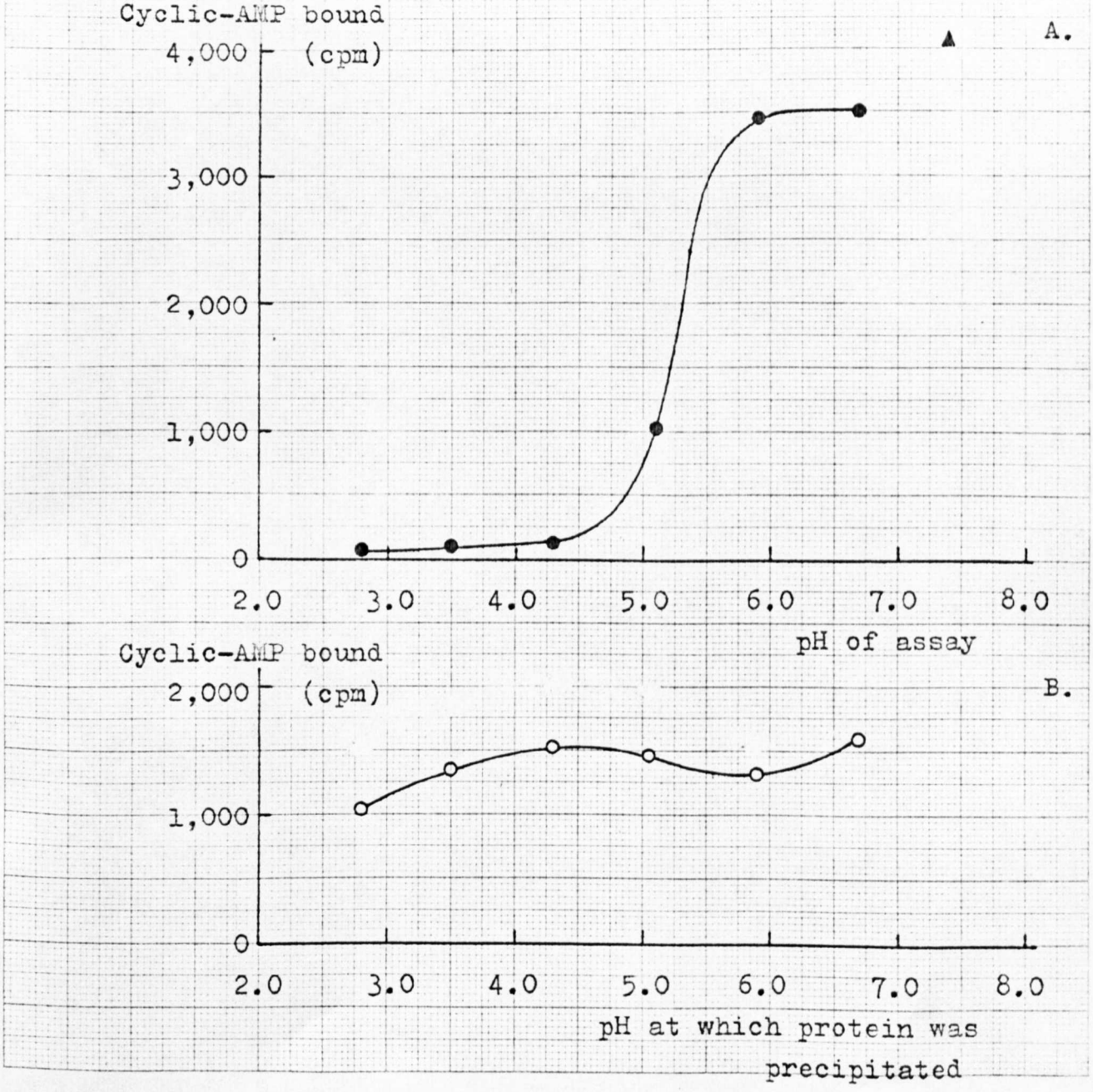


Figure 8.8

The effect of pH on the binding of cyclic-AMP

The 5,000 x g supernatant was diluted in phosphate citrate buffers at the pH shown below. The buffers were made up from 0.1M solutions of citric acid and sodium phosphate containing 8mM theophylline and 6mM 2-mercaptoethanol. The protein precipitated at each pH was removed by centrifugation and resuspended in dilution buffer (6.B.b.). The binding of cyclic-AMP (10nM) by the supernatants at the different pHs and of the resuspended precipitates at pH 7.4 are shown below. in parts A and B respectively. (▲ is the binding in Tris.HCl)



8.F.a. Preparation of cyclic-ethenoAMP

Aqueous chloroacetaldehyde at pH 5.0 was redistilled. The fraction collected between 92° and 98°C was used for the reaction. Cyclic-AMP (12mg) was mixed with 20ml of the distilled chloroacetaldehyde and placed in a shaking water bath at 37°C for 24 hours. The reaction contents were checked by tlc to estimate the extent of the reaction. The tlc system is described below. After 24 hours the reaction was almost complete and most of the aqueous chloroacetaldehyde was removed by freeze drying overnight. The remaining chloroacetaldehyde was removed by adding 5ml of distilled water and ether extracting twice. U.V. absorption at λ225 to 350nm showed that the aqueous layer was free of chloroacetaldehyde and that the ether layer was free of nucleotide. The volume of the solution was reduced by rotary evaporation.

The cyclic-ethenoAMP was purified by applying the solution to a 0.8 x 23cm DEAE-cellulose column equilibrated with 50mM ammonium acetate buffer at pH 4.0. The nucleotides were eluted with a convex gradient of the same buffer increasing in concentration to 0.5M. Two major peaks of U.V. absorbing material were eluted (figure 8.9). The U.V. spectrum of peak 1 showed a single maximum at λ276nm and a minimum at λ248nm. The second peak (2) also had a minimum at λ248nm but had two maxima at λ266nm and λ275nm with shoulders at λ253nm and λ300nm. Peak 2 corresponds to the spectrum of ethenoadenosine derivatives prepared by Secrist et al. (1972a) (see figure 8.10). The compound in peak 2 also gave a single fluorescent spot on cellulose tlc using isobutyric acid: NH_4OH: H_2O (75: 1: 24 by volume) as used by Secrist et al. (1972b).

8.F.b. The fluorescence of cyclic-ethenoAMP and the cyclic-AMP binding protein

The fluorescence spectra of the cyclic-ethenoAMP and a sample of protein from the DEAE-cellulose column III, fraction B, are shown in figure 8.11. The protein had an excitation maximum at λ295nm and a fluorescence

Figure 8.9

The purification of cyclic-ethenoAMP using a DEAE-cellulose column

5ml crude cyclic-ethenoAMP was added to a 0.8 x 23cm column of DEAE-cellulose equilibrated with 50mM ammonium acetate buffer pH 4.0. The sample was eluted by applying a convex gradient of buffer to the column. The gradient was made using a 100ml conical flask containing 100ml of the 50mM ammonium acetate and a 250ml beaker containing about 110ml 0.5M ammonium acetate buffer pH 4.0. The containers were linked by a siphon tube and the column was fed from the conical flask. All the U.V. absorbing material was eluted by the first 100ml of buffer.

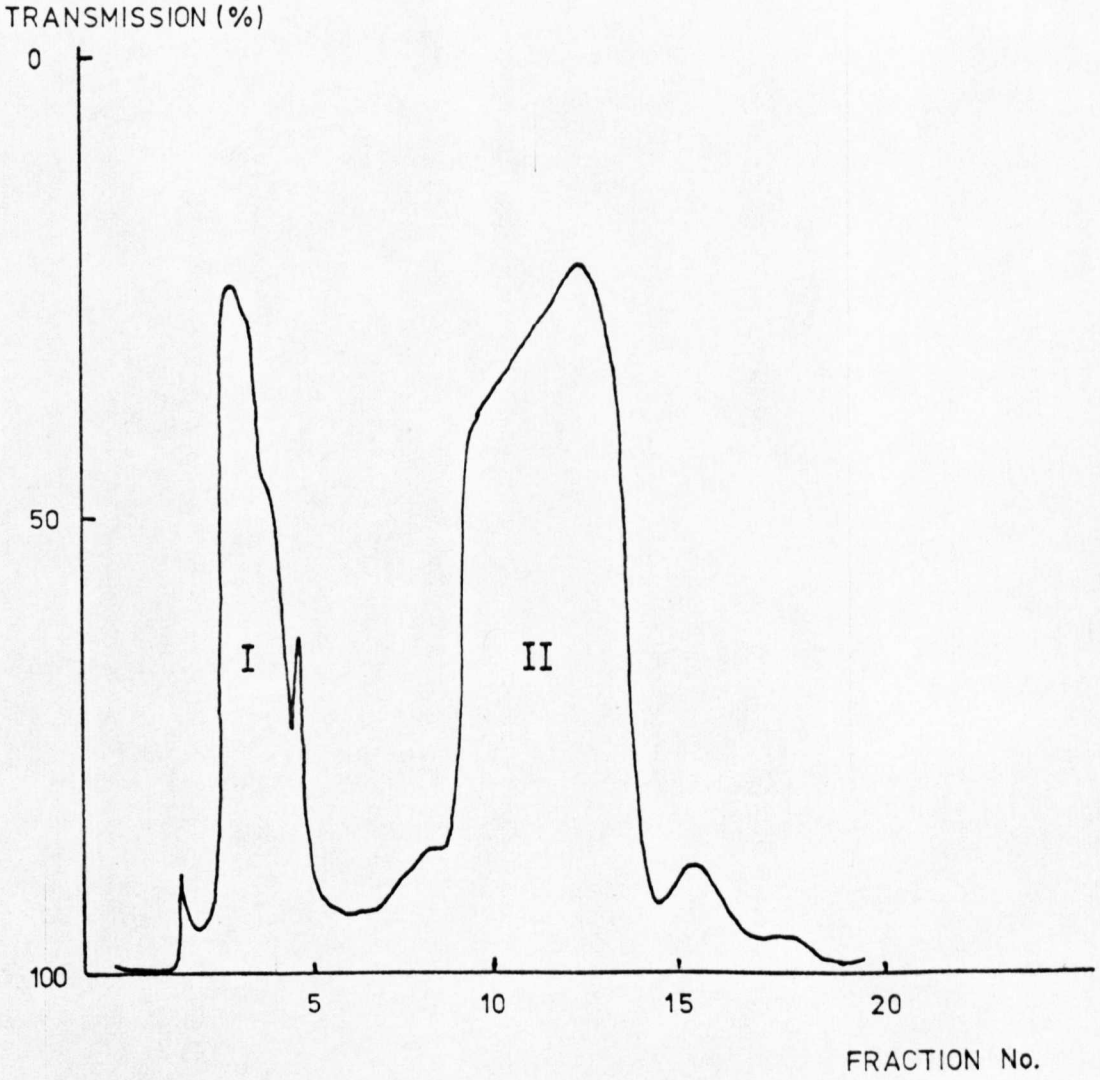


Figure 8.10

The U.V. spectrum of cyclic-ethenoAMP

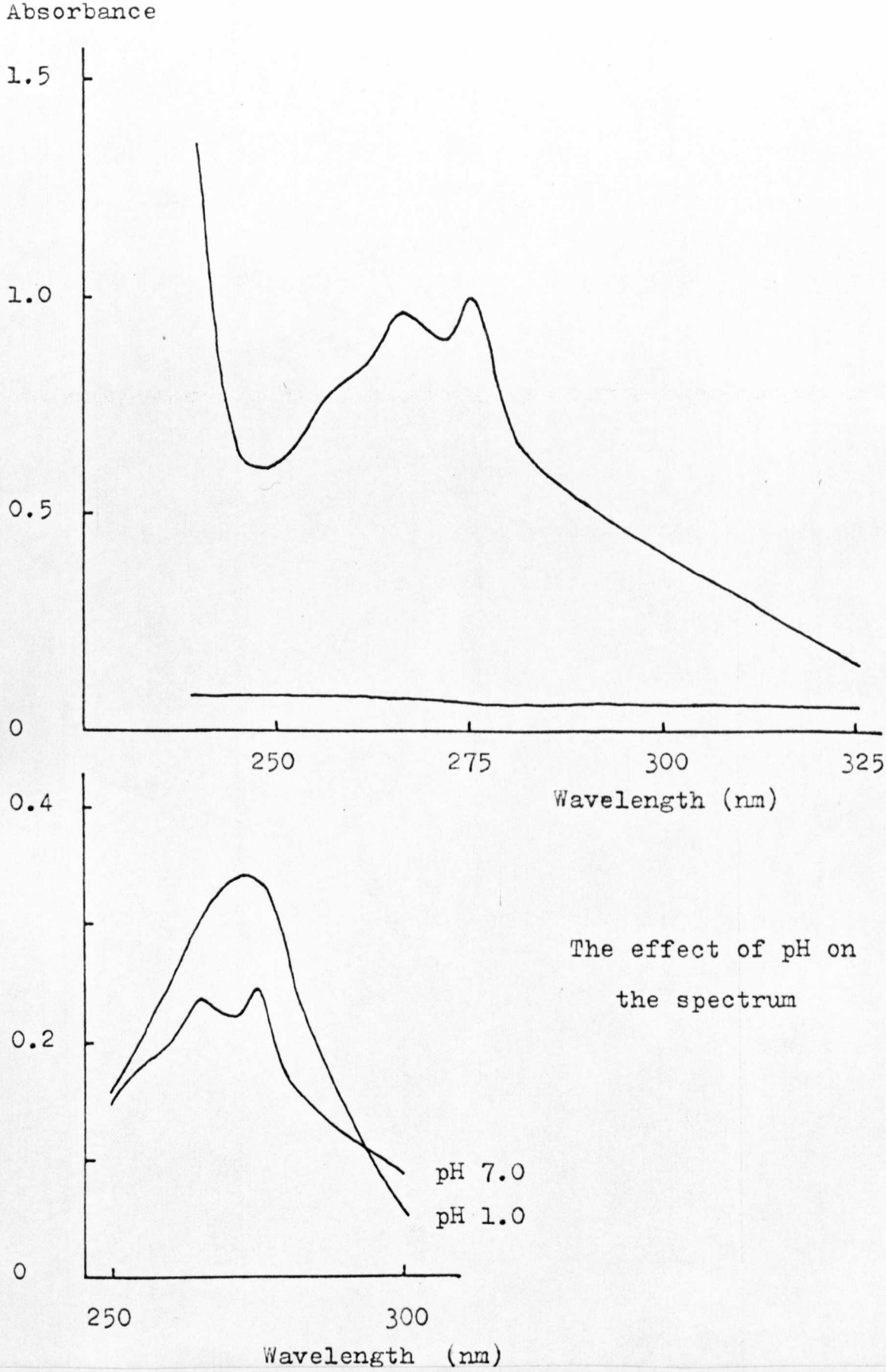
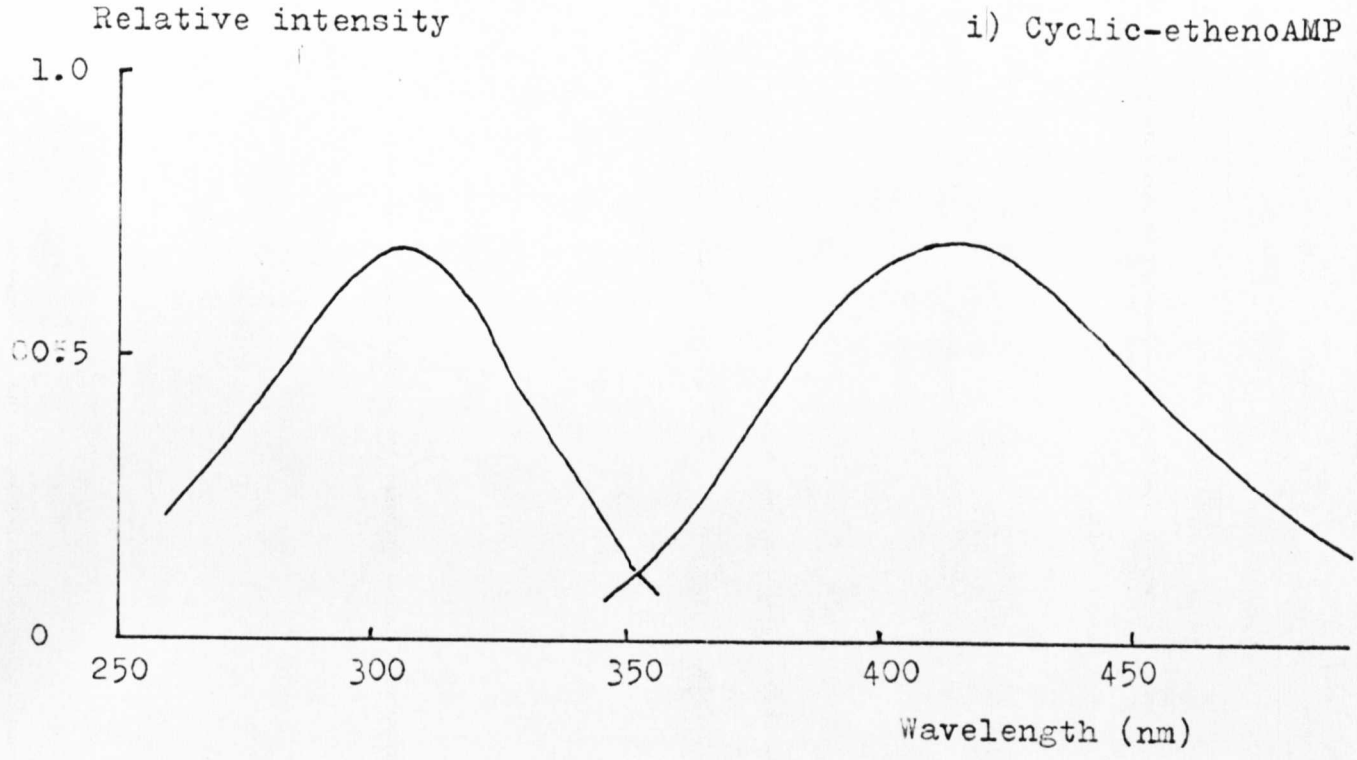
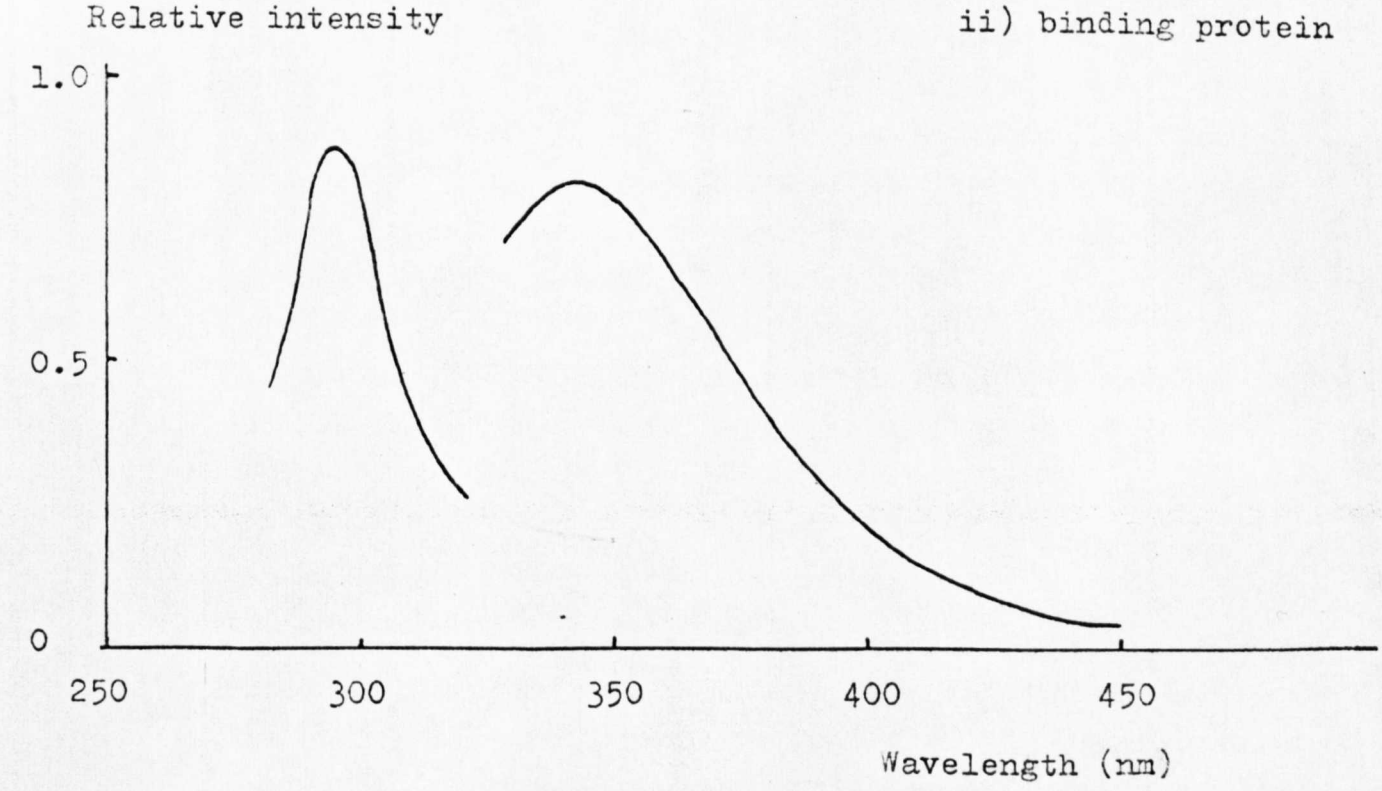


Figure 8.11

Fluorescence spectra of cyclic-ethenoAMP and the cyclic-AMP binding protein from DEAE-cellulose column III





maximum at λ 350nm. This corresponds to the fluorescence of tryptophan (excitation λ_{max} 287nm, fluorescence λ_{max} 348nm) rather than to the fluorescence of tyrosine (excitation λ_{max} 275nm, fluorescence λ_{max} 303nm). Cyclic-ethenoAMP had an excitation λ_{max} of 310nm and a fluorescence λ_{max} of 420nm.

Changes in the polarity of the medium do not affect the fluorescence spectra of the ethenoadenine derivatives except for a slight shift to shorter wavelengths (Secrist et al.: 1972a). As the binding of cyclic-AMP to the protein kinase is very firm it was thought that changes in the polarisation of fluorescence would enable a study of the binding process to be carried out.

The polarisation (p) of fluorescence is given by the following equation:

$$p = \frac{Vv - K.Hv}{Vv + K.Hv}$$

where the symbols V and H refer to the settings of the polarising filter placed immediately before the sample in the excitation light and the symbols v and h refer to the settings of the polarising filter placed between the sample and the analysing monochromator in the Farand fluorometer. In the settings V and v the electrical vector of the polarised light is vertical to the plane of incidence and observation and similarly H and h refer to the settings in which this vector is horizontal to the same plane. K is a correction factor for the non-ideal transmission of polarised light through the analysing monochromator. It is given by:

$$K = Hv/Hh.$$

The fluorescence spectrum of the protein solution partially overlapped that of the cyclic-ethenoAMP. Therefore a wavelength was chosen at which the contribution made by the protein fluorescence to the total fluorescence was minimised (table 8.1).

8.F.c. The binding of cyclic-ethenoAMP to the binding protein

The protein used for this work was the same as that used in the fluorescence measurements. (fraction B from DEAE-cellulose column III). An aliquot of 100μl of the binding protein diluted to 300μl in the presence of

100nM H^3-cyclic-AMP bound 10.5pmol of cyclic-AMP. Therefore the concentration of binding sites (assuming that they were saturated with cyclic-AMP) is about 100nM.

The relative fluorescence and polarisation of fluorescence of the binding protein, a solution of 130nM cyclic-ethenoAMP and a mixture of the two were measured. The spectral data for the solution of cyclic-ethenoAMP were obtained by adding 1 µl of a 0.38mM solution of cyclic-ethenoAMP to 3.0ml of clean distilled water using a 1 µl capillary tube (Microcaps). The cyclic-ethenoAMP was added to the protein solution in the same way. The results are also shown in table 8.1

Table 8.1

a) The relative fluorescence intensity of cyclic-ethenoAMP and the binding protein solution at various wavelengths.

Excitation wavelength	Fluorescence wavelength	Cyclic-ethenoAMP		Protein kinase	
nm	nm	I_f (rel)	p	I_f (rel)	p
310	415	16.5	0.005	1.94	0.319
315	425	14.6		1.20	
320	440	11.2	0.009	0.84	0.478
325	450	8.0		0.75	

b) The relative fluorescence and polarisation of cyclic-ethenoAMP and the binding protein solution. (Excitation λ320nm, fluorescence λ440nm)

Solution	Time after mixing	I_f (rel)	p
130nM cyclic-ethenoAMP	-	0.020	0.036
Binding protein	-	0.138	0.470
" " + c- AMP	3 min	0.131	0.471
" " "	6 "	0.127	0.480
" " "	16 h	0.126	0.494

It was clear that the protein fluorescence was too high and masked the cyclic-ethenoAMP fluorescence, therefore further purification of the binding protein was

required. However, there was a 5% increase in polarisation over the period of 16 hours, though most of this change occurred relatively quickly (2% in 6 minutes). Sanborn et al. (1973) have shown that the rate of association between cyclic-AMP and endometrial protein kinase is high and that a significant degree of binding occurs in a few minutes.

8.G. Discussion

It was noticed that the data obtained from binding curves for the concentration of binding sites and the dissociation constant for the binding of cyclic-AMP to the protein kinase varied with the concentration of the protein and the cyclic-AMP used. This is a fact that has been noted in passing by other workers (Wunderwald et al.: 1974) but which is of vital importance to an understanding of how the protein kinases function in response to cyclic-AMP.

As shown in figure 8.1 it is possible to get curved or linear binding plots for a 5,000 x g supernatant preparation depending on the dilution of the protein and the concentration range for the cyclic-AMP.

There are a number of possible reasons for curved binding plots.

(i) Multiple binding species: by which is meant the presence of two or more unrelated proteins capable of binding cyclic-AMP.

(ii) Association/dissociation of binding proteins: by which is meant that the protein kinase RC complex is able to dimerise to give R_2C_2 and perhaps higher aggregates or to dissociate to R and C spontaneously. It is assumed under this heading that the associated or dissociated forms of the protein kinase have different binding constants from the RC complex.

(iii) Allosteric regulation: which is taken to include co-operative binding and regulation by effectors.

The data from the binding studies on the 5,000 x g supernatant, the ammonium sulphate fractions and the DEAE-cellulose column fractions is tabulated below (table 8.2).

Table 8.2

Dissociation constants obtained for various preparations of the cyclic-AMP binding protein (nM)

5,000g supernatant	Ammonium sulphate fractions % saturation							DEAE-cellulose fractions	
	0	10	20	30	40	50	60	A	B
					- 0.7 -d3				
					- 0.7 -d3				
					- 0.7 -d3				
0.8 d1					- 0.8 -				
1.5 d1									
								1.9	
	-	3.0	-						
			- 3.4 d2-						
3.9 d1									
						- 4.1	-		
			- 5.7 d2-						
7.0 d1									
			- 9.7 d2-						
			- 10.0	-					
									14
15.5 c									
					- 21 d3-				
					- 21 d3-				
					- 21 d3-				
			-	28	-				
			- 30	c	-				
					- 44	-			
					- 44 c	-			
67 d1									

The data was obtained as described in the text: c indicates results with cyclic-GMP present, d1, d2.... are data for different dilutions of fractions 1,2... .

The dissociation constants fall broadly into three categories.

(a) Dissociation constants of the order $K < 2$ which are not changed by dilution but are sensitive to cyclic-GMP. The preparations giving binding constants of this type are the 5,000 x g supernatant when undiluted, the 36 - 50 percent saturation ammonium sulphate fraction and the DEAE-cellulose fraction A (see sections 6.B.a., 7.C.a. and 7.C.c. II respectively for the preparations and figures 8.1, 8.2, 8.3 and 8.4 respectively for the results of the binding studies).

(b) Dissociation constants of the order $2 < K < 10$ which are dependent on the dilution of the binding protein and are also affected by cyclic-GMP. The preparations giving this type of dissociation constant are the 5,000 x g supernatant when undiluted if high concentrations of cyclic-AMP are used to determine the constant, and diluted if low concentrations of cyclic-AMP are used in the binding study. The ammonium sulphate fractions precipitating between 0 and 36% saturation also fall into this category. The results may be seen in the sections mentioned above.

(c) Dissociation constants $K > 10$. The dissociation constants in the range were observed with the diluted 5,000 x g supernatant with high concentrations of cyclic-AMP and as a component of the 36 - 50% ammonium sulphate fraction. Similar high dissociation constants were observed for the 20 - 40% ammonium sulphate fraction and the samples in category (b) which were examined for the binding of cyclic-AMP in the presence of cyclic-GMP.

These results may arise from a protein subunit association/dissociation phenomenan. Miyamoto et al. (1971) have shown by sucrose density gradient centrifugation that there are three interconvertible protein kinase activities in a preparation from bovine brain. They have molecular weights of 140,000, 80,000 and 40,000. Only the larger two are cyclic-AMP dependent and they are also activated by histone. This gave rise to the postulate that there was an R_2C_2 complex, where

R refers to the regulatory subunit of the protein kinase, which was capable of dissociating to 2RC. This may have been occurring here although the molecular weight studies necessary to confirm it were not carried out. However, this phenomenon itself is insufficient to account for all the results, for example the dilution curve (figure 7.7).

The sole sucrose density gradient work was done with the binding protein fractions from DEAE-cellulose column I (section 8.C.e., figure 8.9). A binding activity at high molecular weight appeared as a shoulder on the main peak of binding activity from fraction A, which is thought to be the protein kinase holoenzyme.

Another possible explanation for the anomalous binding of cyclic-AMP by the DEAE-cellulose fractions (section 7.C.c. II and 8.D.c.), though it does not exclude the protein subunit association/dissociation hypothesis, is that there is an effector molecule present in the 5,000 x g supernatant. Walsh et al. (1971) demonstrated the presence of a heat stable protein which inhibited skeletal muscle protein kinase and increased the cyclic-AMP binding activity. In section 9.E.c. it was shown that a fraction from the DEAE-cellulose column II, separate from the cyclic-AMP binding fractions, was capable of reducing the cyclic-AMP bound by fraction B. The protein applied to the column was a 30 - 65% ammonium sulphate precipitate which had been extensively dialysed against 20mM Tris.HCl buffer pH 7.3 containing 6mM 2-mercaptoethanol. The effector was therefore not dialysable but no further characterisation was carried out.

Either of these effects may be the cause of the curvature of the binding plots observed for the 5,000 x g supernatant and the 36 - 50% ammonium sulphate fraction. However, this does not rule out the possibility of multiple binding species. Indeed there is some evidence in favour of them. The two protein fractions from the DEAE-cellulose column II which had different dissociation constants for the binding of cyclic-AMP and which

responded differently when they were mixed with the other column fractions may be interpreted in this way. The different binding proteins would not be expected to show the same response on being mixed with an inhibitor or other effector.

Cyclic-ethenoAMP was prepared because of its potential as a fluorescent probe in the study of the protein kinase activation by the binding of cyclic-AMP. The results showed that although the binding of the probe to the regulatory subunit of the protein kinase did not produce a significant change in the wavelength of the fluorescence and excitation spectra there was a change in the polarisation of the fluorescence. However, a further purification of the enzyme was required for more detailed studies.

The fluorescence technique would be a powerful one when applied to the determination of kinetic constants for the activation of the protein kinase by cyclic-AMP. No other existing assay is really adequate for kinetic studies of this process (Sanborn et al. 1973).

CHAPTER 9

DISCUSSION

9.A. The work presented in this thesis arose out of an interest in the controls necessary for the direction of metabolic reactions into pathways of synthesis and degradation which are essential for life. At first particular interest was centred upon the purine pathways which provide an intricate network of reactions requiring a high degree of control. When the work on the purines drew to a close, interest was directed towards the control exercised by cyclic-AMP on metabolism, in particular from the aspect of the activation of the protein kinase by cyclic-AMP.

9.B. The investigation into purine flux was intended to shed light on the factors controlling the adenine metabolism of the red cell and to see under what conditions the catabolism of ATP took place.

Approaches were made at two levels: firstly, to assay enzyme activities in the red cell and secondly, to study the fluxes in the pathway using radioisotopic tracers.

Work at the first level was conducted on AMP deaminase, an enzyme critically placed in the purine pathway. It is involved in the conversion of ATP into GTP and the catabolism of ATP. It is found to be controlled by a number of inhibitors, the most important of which is 2,3-DPG. The enzyme was partially purified and assayed spectrophotometrically.

Work at the second level was conducted on the metabolism of purine bases by using radio-isotopic tracers. The metabolic products were separated by thin layer chromatography and the radio-activity of the products was determined by liquid scintillation counting. The radio-activity of any product is a function of the flux in the pathway and of the isotopic dilution of its precursors. The results obtained agreed qualitatively with those of other workers but the technical problems

prevented the refinement of the technique necessary for quantitative results. A brief study of the transport of purine bases into the red cell showed that their influx into the cell was diffusion limited. The rate was not affected by adrenalin, a compound known to alter the properties of the red cell's membrane.

A hypothesis coupling the action of AMP deaminase with haemoglobin was formed. They are linked by the common effector 2,3-DPG. However, there does not appear to be a straightforward relationship between the oxygenation of the cell and AMP deaminase activity (section 1.E.). An alternative role for AMP deaminase in addition to its accepted role in the interconversion pathway for purine nucleotides may be in the cell aging process. This hypothesis can be stated as follows: the red cell requires ATP for the maintenance of its osmotic balance and cell shape. Gomperts (1969) estimated that the red cell utilises over 50% of its ATP supply for this purpose. The reaction of AMP kinase is near equilibrium because of its high activity in red cells so the following equation applies:

$$K_{eq} = \text{ADP}\ \ \text{Mg.ADP} / \text{AMP}\ \ \text{Mg.ATP}\ .$$

The ATP is formed from ADP by glycolysis and this normally ensures a high concentration of ATP and a low concentration of AMP in the cell. It has been observed that in old cells the rate of glycolysis declines. The thermal denaturation of hexokinase may be largely responsible for this (Gomperts: 1969). As a result the 2,3-DPG from the pool within the red cell is used as an energy source and its level declines (Haidas et al.: 1971). The decline in the level of 2,3-DPG makes haemoglobin a less efficient oxygen donor and also releases AMP deaminase inhibition. The net result may be a self destructive mechanism for old red cells which have inefficient oxygen carrying eapacity.

This hypothesis should be tested further by separation of the cells according to their age and examination of the relevant enzyme activities and

fluxes within the cells. Since red cells have densities which vary according to their age, isopycnic centrifugation can be used to accomplish a separation of cells of different ages.(Danon and Marikovsky: 1964).

9.C. During the preliminary studies of the binding protein for the assay of cyclic-AMP it was noticed that binding curves obtained for the protein varied from one preparation to the next. This observation led to the idea that binding equilibrium for the activation of the protein kinase by cyclic-AMP may be more complicated than is commonly assumed. The occurrence of non-linear binding curves has also been reported in passing by Gill and Garren (1969) and Wunderwald et al. (1974). In this discussion some of the possible causes of such curvature are considered. The derivation of the equations given in the discussion are to be found in the appendix. Firstly, the different types of equilibria which have been considered are listed together with the equilibrium equations. The properties of each of the equations are discussed here though the detailed treatment is to be found in the appendix. Secondly, the theoretical treatment of the equations is compared with the experimental results.

9.D. Binding equilibria.

1. Single site binding protein:

$$R + \text{c-AMP} \overset{K_1}{=} R.\text{c-AMP}$$

The equilibrium equation is of the form:

$$B = P - K_1.(B/F).$$

A plot of 'bound*' against 'bound/free*' is a straight line with gradient K_1 and 'bound' intercept P, the concentration of binding sites.

2. Mixture of type 1 binding proteins.

$$R_i + \text{c-AMP} \overset{K_i}{=} R_i.\text{c-AMP}$$

The equilibrium equation is of the form:

$$B = \sum_i B_i = \sum_i P_i - \sum_i K_i.B_i/F.$$

* Bound and free refer to concentrations of ligand.

This equation may also be expressed in radial co-ordinates (r, θ), where $B = r.\sin\theta$ and $B/F = r.\cos\theta$:

$$R = \sum_i r_i = \sum_i \frac{P_i}{K_i.\cos\theta - \sin\theta} .$$

It can be seen more clearly from this equation that R is the radial sum of the lines

$$r_i = P_i/(K_i.\cos\theta - \sin\theta).$$

This means that the shape of the curve depends on the dissociation constants (K_i) for the binding proteins constituting the mixture and their relative concentration. Dilution of the mixture of binding proteins will not affect this.

3. Single site binding protein which dissociates on cyclic-AMP binding.

$$RC + \text{c-AMP} \overset{K_3}{=} R.\text{c-AMP} + C$$

The equilibrium equation is of the form:

$$B = P - K_3.(B^2/F).$$

This equation may be rearranged to give:

$$B = \frac{P}{1 + K_3.(B/F).}$$

So a plot of B against B^2/F will be a straight line, but a plot of B against B/F will be a hyperbola in which the B/F axis is an asymptote.

4. Single site binding protein with subunit dissociation not dependent on the binding of cyclic-AMP.

$$RC \overset{K_s}{=} R + C$$

$$R + \text{c-AMP} \overset{K_{ss}}{=} R.\text{c-AMP}$$

$$(RC + \text{c-AMP} = R.\text{c-AMP} + C$$

$$K_{ss} = K_s/K_s)$$

The equilibrium equation is of the form:

$$B = \frac{P}{1 + \frac{K_{ss}}{K_1}.(B/F)} - K_{ss}.(B/F)$$

If $K_s \gg K_{ss}$ then the equation is approximately of the form:

$$B = P - K_{ss}.(B/F)$$

and the plot of B against B/F is a straight line. However, if $K_1 \ll K_2$ then the equation cannot be simplified. The plot of B against B/F gives a curve. The B/F intercept of this curve varies according to the concentration of the protein. If $P \gg K_1$ then the intercept is proportional to $\sqrt{P}$ and if $P \ll K_1$ then the intercept is proportional to P. So the apparent binding data obtained from the plot will vary with protein concentration.

5. Dimeric protein which dissociates to produce two type 3 binding proteins.

$$R_2C_2 \overset{K_1}{=} 2RC$$

$$RC + c\text{-}AMP \overset{K_2}{=} R.c\text{-}AMP + C$$

The equilibrium equation for this is of the form:

$$B = P - K_2.(B^2/F)\left(1 + \frac{K_2}{K_1}.(B^2/F)\right)$$

The equation cannot be reduced to a form in which B is a function of B/F only. If $K_1 \gg K_2$ then the equation is of the form found in 3.

6. Dimeric protein which dissociates to produce two type 4 binding proteins.

$$R_2C_2 \overset{K_1}{=} 2RC$$

$$RC \overset{K_2}{=} R + C$$

$$R + c\text{-}AMP \overset{K_3}{=} R.c\text{-}AMP$$

The variables in the equilibrium equation cannot be separated in this case, but in the limiting case when K_1 is infinite the equation reverts to type 4. As shown in the appendix the shape of the curve can be dependent on the protein concentration.

Of these six examples of binding equilibria only the first two give binding curves of the form B against B/F which are not dependent on the protein concentration.

9.E. This theoretical analysis of the possible binding equilibria and the binding curves which arise from them can be applied to the experimental results. Firstly, none of the binding curves (shown in chapter 9) of 'bound' against 'bound/free' ligand are hyperbolic. They all have clear intercepts with the 'bound/free' axis. This rules out the form of binding in section 9.D. types 3 and 5. The first of these is of the form

$$RC + cAMP = R.cAMP + C$$

which is commonly assumed to be the equilibrium governing the activation of the protein kinase.

Secondly, the theoretical analysis shows that changes in the binding curve brought about by using different concentrations of the binding protein are a criterion for distinguishing different binding equilibria. This is exemplified by reference to the binding curves for two protein fractions prepared by ammonium sulphate precipitation from the bovine adrenal cortex 5,000 x g supernatant (see figure 8.2). The protein fraction precipitating between 15 and 36% ammonium sulphate saturation gave a binding curve which was approximately linear and the slope of which increased with protein concentration (apparent dissociation constant decreases). The ratio between the protein concentration (binding sites) as given by the 'bound' intercept and the 'bound/free' intercept for the three dilutions of binding protein used was 9.9:1, 5.8:1 and 3.5:1 respectively. This ratio is not constant. However, the ratio between the square root of the binding site concentration and the 'bound/free' intercept for each of the three dilutions was 2.5:1, 2.0:1 and 1.8:1 respectively. These ratios are more nearly constant and are in accord with the predicted behaviour for the binding of cyclic-AMP by a protein kinase which dissociates into the catalytic and regulatory subunits before binding cyclic-AMP (type 4 binding protein: see 9.D. and the appendix). The condition required by the theory for this to be true is that both the protein binding site concentration and K_{∞} are greater than K_1. (K_1 and K_{∞} are defined in 9.D.).

Sanborn et al. (1973) proposed that uterine protein kinase bound cyclic-AMP in a similar manner.

The second fraction precipitated by ammonium sulphate between 36 and 50% saturation gave curved binding plots. The shape of these plots did not vary on dilution and was consistent with them being the radial sum of two straight lines. This behaviour is predicted for a mixture of single site binding proteins which have no subunit dissociation (type 1: 9.D. and appendix).

9.F. The 5,000 x g supernatant of the bovine adrenal cortex was fractionated by a number of methods. Binding studies were carried out on the different fractions. According to the theoretical criteria shown in the appendix the binding species in some of the fractions were identified. The binding studies showed that the fractionation procedure had to be carefully controlled in order to obtain consistent results.

The result of the binding studies carried out on the protein fractions obtained by ammonium sulphate fractionation indicates that this technique alone is sufficient for separating the protein kinase from the regulatory subunit. The protein kinase was identified by the binding equilibrium which it exhibited. It is usually taken that cyclic-AMP initiates the dissociation of the protein kinase into the regulatory and active catalytic subunits. However, the results of this binding study imply that there is a significant dissociation of the protein kinase into its subunits prior to the binding of cyclic-AMP.

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ABBREVIATIONS

Abbreviation	Meaning
a	atto (10^{-18})
A	absorbance, $\log_{10}(I_o/I)$
ade	adenine
adenosine deaminase	adenosine aminohydrolase (EC 3.5.4.4.)
adenosine kinase	ATP: adenosine phosphotransferase (EC 2.7.1.20.)
ado	adenosine
ADP	" -5'-diphosphate
AMP	" -5'-monophosphate
AMP deaminase	AMP aminohydrolase (EC 3.5.4.6.)
AMP kinase	ATP: AMP phosphotransferase (EC 2.7.4.3.)
AMP-S	N^6-succinyladenosine-5'-monophosphate
APRT	AMP:PP_i phosphoribosyltransferase (EC 2.4.2.7.)
ATP	adenosine-5'-triphosphate
B	concentration (or cpm) of bound fraction in an (equilibrium) binding process.
B.S.A.	bovine serum albumin (fraction V unless specified as otherwise)
c	concentration
c.a.	circa
cAMP	cyclic adenosine-3',5'-monophosphate
C_i	Curie
C_o/C_i	Radioimmunoassay nomenclature of Hales and Randle (1963). Ratio between 'zero' and 'sample' count rate.
cpm	count rate, counts per minute.
cyclic-AMP	as cAMP
cyclic-ethenoAMP	3H-Imidazo [2,1-i] purine, 3-(3,5-O-phosphinico-β-D-ribofuranosyl) Chemical Abstracts nomenclature
d	optical depth
D	toluene excimer
DEAE-cellulose	diethylaminoethyl-cellulose
dil^n	dilution
DNA	deoxyribonucleic acid
2,3-DPG	2,3-diphosphoglyceric acid

EDTA	ethylenediaminetetraacetic acid
2-EE	2-ethoxyethanol
equm	equilibrium
E_s	external standard reading for the Packard series 2450 liquid scintillation counter
F	concentration (or cpm) of free ligand fraction in an (equilibrium) binding process
g	gram, acceleration due to gravity
G	Gibb's free energy
GDP	guanosine-5'-diphosphate
gln	glutamine
glu	glutamic acid
GMP	guanosine-5'-monophosphate
GMP kinase	ATP: GMP phosphotransferase (EC 2.7.4.8.)
GMP reductase	reduced NADP: GMP oxidoreductase (deaminating) (EC 1.6.6.8.)
GMP synthetase	XMP:ammonia ligase (AMP) (EC 6.3.4.1.)
GTP	guanosine-5'-triphosphate
gua	guanine
guanase	guanine aminohydrolase (EC 3.5.4.3.)
guo	guanosine
h	hour, Planck's constant
Hb	haemoglobin
HGPRT	IMP: PP_i phosphoribosyltransferase (EC 2.4.2.8.)
hyp	hypoxanthine
I_o	intensity of incident light, or radiation
I_{rel}	relative intensity (of fluorescence)
IMP	inosine-5'-monophosphate
ino	inosine
K,k	equilibrium constant, rate constant
K_i	inhibitor constant
K_m	Michaelis constant
l	litre
m	milli, 10^{-3}
M	molar
min	minute
mol	gram molecule

n	neutrino, nano, 10^{-9}
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced NAD^+
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced $NADP^+$
5'-nucleotidase	5'-ribonucleotide phosphohydrolase (EC 3.1.3.5.)
p	polarisation (degree of)
PEI-cellulose	polyethyeneimine-cellulose
pH	$-\log_{10}$(hydrogen ion concentration)
P_i	orthophosphate
PNPlase	purine nucleoside: P_i ribosyltransferase (EC 2.4.2.1.)
POPOP	1,4-bis-(2-(5-phenyloxazolyl))-benzene
PP_i	pyrophosphate
PPO	2,5-diphenyloxazole
PRPP	α-D-ribose-1-pyrophosphate-5-phosphate
pyr	pyridoxal
pyr-P	pyridoxal phosphate
r	fraction of tracer bound to a specific binding protein
R	gas constant, response parameter (nomenclature of Ekins et al. 1968)
S.D.	standard deviation
S.D.S.	sodium dodecyl sulphate
S.G.	specific gravity
T	temperature (Kelvin), toluene
TCA	trichloroacetic acid
tlc	thin layer chromatography
U.V.	ultra violet
v	rate (of reaction)
V_{max}	maximum rate for an enzyme catalysed reaction
xan	xanthine
xanthine oxidase	xanthine: O_2 oxidoreductase (EC 1.2.3.2.)
xao	xanthosine
XMP	" -5'-monophosphate

β	β-particle
∂/∂	partial differential
Δ	change (in)
ε	extinction coefficient,
μ	micro, 10^{-6}
ν	frequency
λ	wavelength
λ_{max}	" at maximum absorption
λ_{excit}	" at which excitation of fluorescence is measured
λ_{fluor}	" at which fluorescence is measured
ϕ_f	fluorescence quantum yield
∞	infinity

APPENDIX

A theoretical discussion relating to binding curves

In this appendix the equilibria governing the shape of binding curves are considered. The same symbols will be used throughout the discussion.

B	bound fraction concentration (of cyclic-AMP)
F	free fraction " (" ")
P	binding sites "
P_i	
K	equilibrium or dissociation constant
RC	protein kinase
R	" " regulatory subunit
C	" " catalytic "
r	concentration of R
c	" " C

1. **Protein with a single binding site and no interacting subunits.**

Equilibrium: $$R + c\text{-}AMP \overset{K_1}{\rightleftharpoons} R.c\text{-}AMP \qquad (i)$$

Equilibrium relationship:

$$K_1 = \frac{(P - B).F}{B} \qquad (ii)$$

This equation rearranges simply to give:

$$B = P - K_1.(B/F). \qquad (III)$$

The plot of bound against bound/free (B against B/F) is a straight line with gradient $-K_1$ and intercept on the B axis of P.

2. **Mixture of type 1 binding proteins.**

Equilibrium: $$R_i + c\text{-}AMP \overset{K_i}{\rightleftharpoons} R_i c\text{-}AMP \qquad (iv)$$

The same equation applies for all components (i) of the mixture.

Equilibrium relationship:

$$K_i = \frac{(P_i - B_i).F}{B_i} \qquad (v)$$

which rearranges to give the relationship:

$$B_i = P_i - K_i.(B_i/F). \qquad \text{(vi)}$$

Again the same equation holds for all the components of the mixture. The total bound fraction will simply be the sum of the different components' bound fractions.

$$B = \sum B_i \qquad \text{(vii)}$$

The result of this summation is not easy to see. However, if the equations for the different components (vi) are rewritten in the perpendicular form for radial co-ordinates, we have:

$$\rho_i = \frac{P_i}{K_i.\cos\theta - \sin\theta} \qquad \text{(viii)}$$

These equations summed over all the components clearly gives the radial sum of the straight lines which represent the contribution of each component of the mixture of binding proteins to the total cyclic-AMP bound.

$$\mathcal{R} = \sum \rho_i = \sum \frac{P_i}{K_i.\cos\theta - \sin\theta} . \qquad \text{(ix)}$$

Some examples of such curves are shown in figure A.1

3. Protein with a single binding site with subunit dissociation on binding of cyclic-AMP.

Equilibrium: $RC + c\text{-}AMP \overset{K_3}{=} R.c\text{-}AMP + C$ (x)

Equilibrium relationship:

$$K_3 = \frac{(P - B).F}{B^2} \qquad \text{(xi)}$$

This equation rearranges to give:

$$B = P - K_3.(B^2/F). \qquad \text{(xii)}$$

A plot of B against B^2/F is a straight line. The equation may be rearranged to give a relationship between B and B/F:

$$B = \frac{P}{(1 + K_3.(B/F))}. \qquad \text{(xiii)}$$

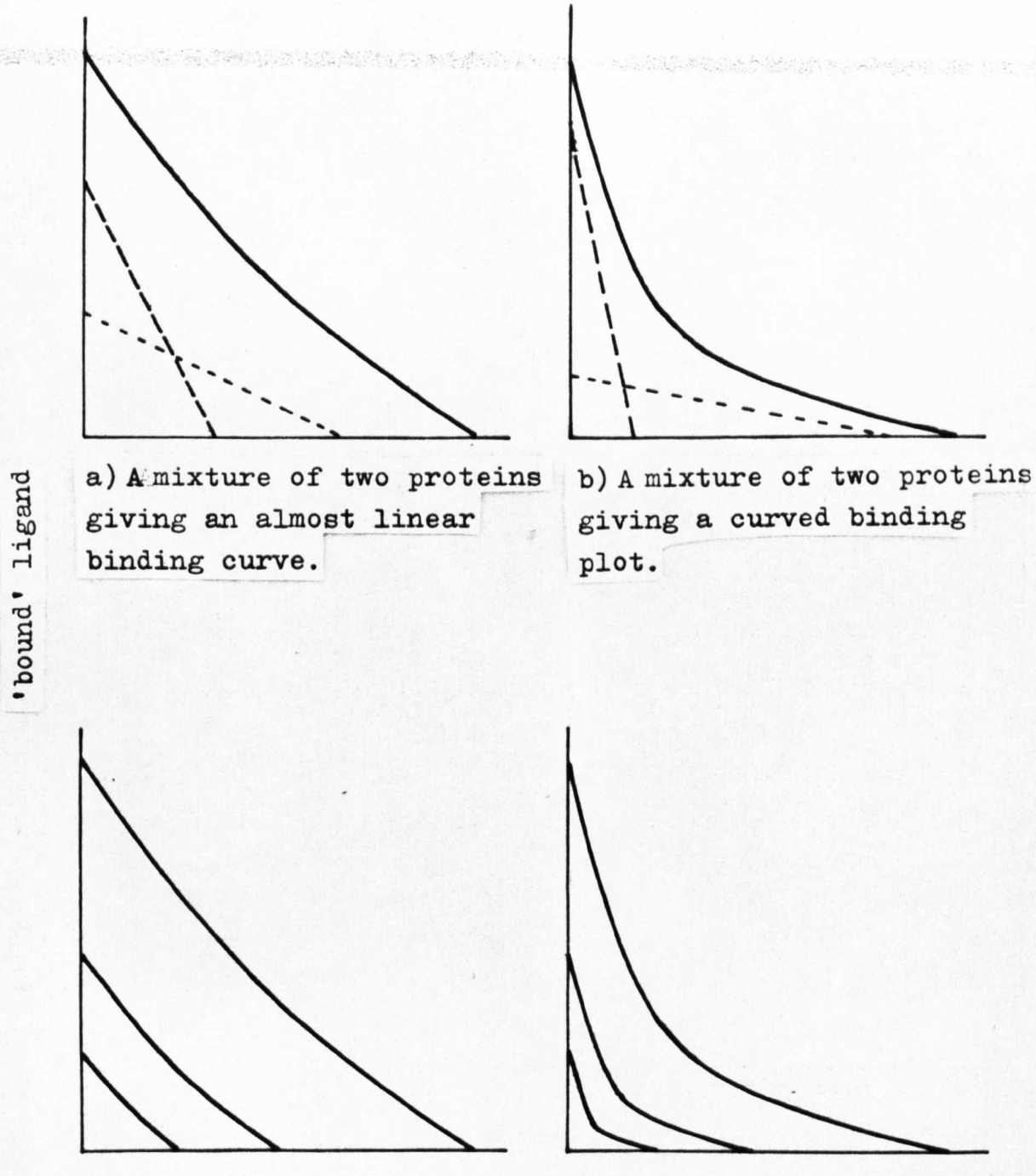
This is the equation of a hyperbola. The intercept on the B axis is P. Differentiating (xiii) with respect to B/F we get:

$$\frac{dB}{d(B/F)} = \frac{-P.K_3}{(1 + K_3.(B/F))^2} \qquad \text{(xiv)}$$

Figure A.1

Theoretical Binding Curves for a Mixture of Type 1 Binding Proteins

The figure shows how curved or almost linear binding plots may arise from a mixture of binding proteins. The effect of dilution on these curves is also shown.



a) A mixture of two proteins giving an almost linear binding curve.

b) A mixture of two proteins giving a curved binding plot.

c) Change in curve a) on 1 in 2 dilution twice.

d) Change in curve b) on 1 in 2 dilution twice.

The gradient of the line at the point of interception on the B axis is therefore $-P.K_3$. The initial gradient of the line is dependent on the protein concentration. The B/F axis is an asymptote of the hyperbola. Examples of this type of curve are shown in figure A.2.

4. Single binding site protein with subunit dissociation before cyclic-AMP binding.

Equilibrium:

$$RC \overset{K_1}{=} R + C \qquad \text{(xv)}$$

$$R + c\text{-}AMP \overset{K_{11}}{=} R.c\text{-}AMP \qquad \text{(xvi)}$$

The two equilibrium relationships are:

$$K_1 = \frac{(r - B).c}{(P - r)} \qquad \text{(xvii)}$$

$$K_{11} = \frac{(r - B).F}{B} \qquad \text{(xviii)}$$

From (xviii) $r = K_{11}.(B/F) + B$ (xix)

and substituting for r in (xvii) and putting c = r we get:

$$K_1.(P - K_{11}.\frac{B}{F} - B) = K_{11}.\frac{B}{F}.(K_{11}.\frac{B}{F} + B) \qquad \text{(xx)}$$

Rearranging (xx) becomes:

$$-B.(K_1 + K_{11}.\frac{B}{F}) = K_{11}.\frac{B}{F}.(K_1 + K_{11}.\frac{B}{F}) - K_1.P \qquad \text{(xxi)}$$

Dividing (xxi) by $-(K_1 + K_{11}.\frac{B}{F})$ we get:

$$B = \frac{P}{1 + \frac{K_{11}}{K_1}.\frac{B}{F}} - K_{11}.\frac{B}{F} \qquad \text{(xxii)}$$

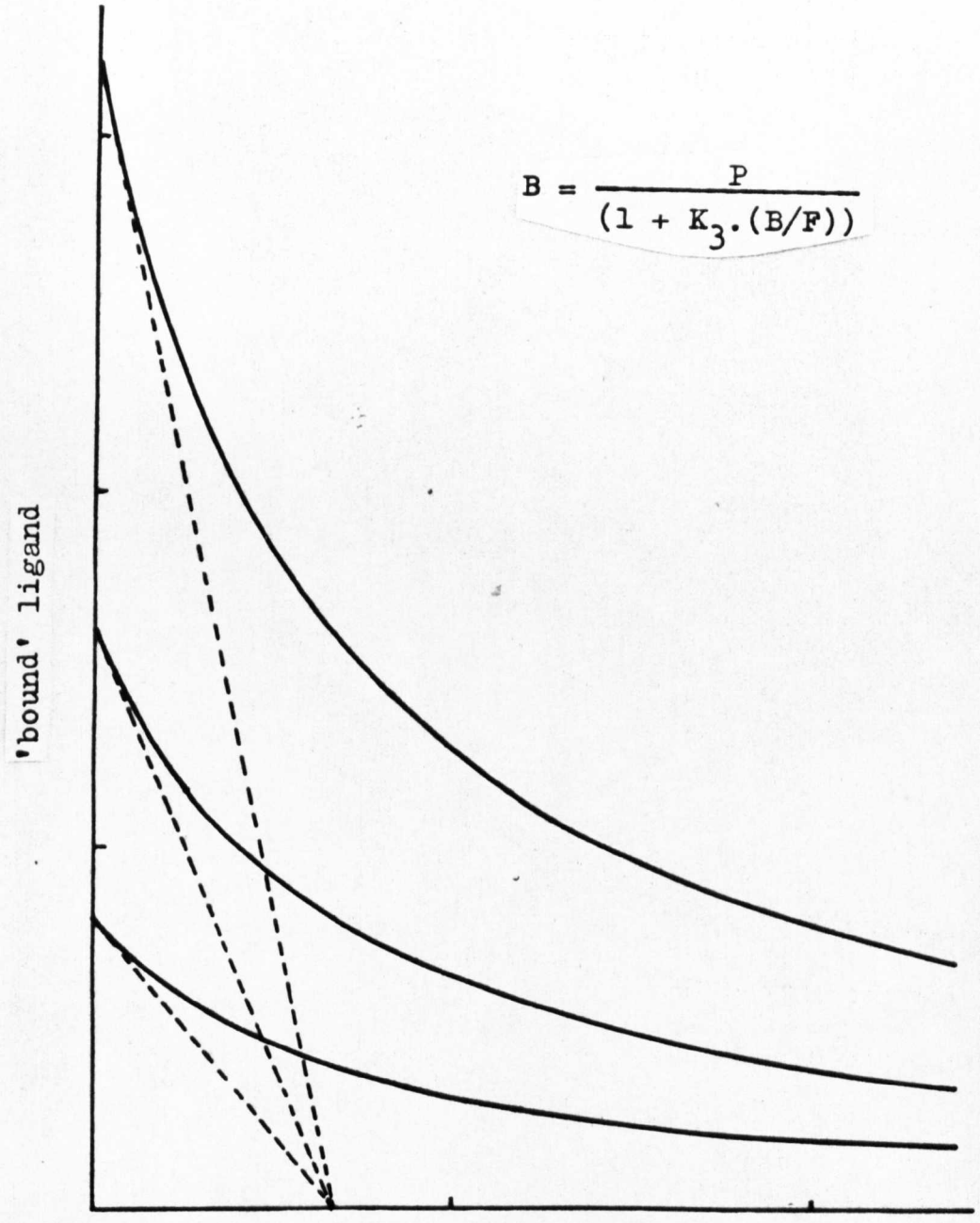
Now if $K_{11} \ll K_1$, then the equation will be approximately of the form seen for equation (iii), and the plot of B against B/F will be very nearly a straight line. If $K_{11} \gg K_1$, then this simplification does not apply, however, we can find the intercepts of the curve with the axes and differentiate to find the gradient of the line.

The intercept on the B axis is clearly P.

Putting B = 0 and rearranging we can get a quadratic equation the positive root of which is the intercept on the B/F axis, $(B/F)_0$.

Figure A.2

Theoretical Binding Curves for Type 3 Binding Proteins



The three curves represent binding plots for three serial dilutions of a type 3 binding protein. The dotted lines which meet on the abscissa show the intial gradient of the lines to be proportional to the concentration of binding sites.

$$\frac{(K_{11})^2}{K_1}(B/F)_0^2 + K_{11}.(B/F)_0 - P = 0 \qquad \text{(xxiii)}$$

The positive root of this equation is

$$(B/F)_0 = \frac{K_1.\sqrt{1 + 4P/K_1} - K_1}{2K_{11}} \qquad \text{(xxiv)}$$

If $P \gg K_1$ then $\sqrt{1 + 4P/K_1}$ is approximately $2.\sqrt{P/K_1}$. and the intercept $(B/F)_0$ is proportional to $\sqrt{P}$.
If $4P \ll K_1$ then $\sqrt{1 + 4P/K_1}$ is approximately $(1 + 2P/K_1)$ by the binomial expansion and the intercept $(B/F)_0$ is proportional to P.
Differentiating (xxii) with respect to (B/F) we get:

$$\frac{dB}{d(B/F)} = \frac{-P.K_{11}/K_1}{(1 + \frac{K_{11}}{K_1}.\frac{B}{F})^2} - K_{11} \qquad \text{(xxv)}$$

As (B/F) increases the gradient increases and approaches the value of $-K_{11}$. The gradient of this line is always an overestimate of K_{11}.
The gradient of the line at the point of interception with the B axis is given by:

$$\frac{dB}{d(B/F)} = -K_{11}.(1 + P/K_1) \qquad \text{(xxvi)}$$

The gradient here therefore depends on the protein concentration. The change of gradient at this point with protein concentration is greater when P is large in comparison with K_1.
Some examples of these results are found in figure A.3.

5. Dimeric protein kinase dissociating to give type 3 binding proteins.

Equilibria: $$R_2C_2 \overset{K_1}{\underset{K_{11}}{=}} 2RC \qquad \text{(xxvii)}$$

$$R_2C_2 + c\text{-AMP} = R.c\text{-AMP} + C \qquad \text{(xxviii)}$$

The two equilibrium relationships are:

$$K_1 = \frac{(P_1 - B)^2}{P_{11} - P_1} \qquad \text{(xxix)}$$

$$K_{11} = \frac{(P_1 - B).F}{B^2} \qquad \text{(xxx)}$$

Figure A.3
(i)

Theoretical Binding Curves For Type 4 Binding Proteins

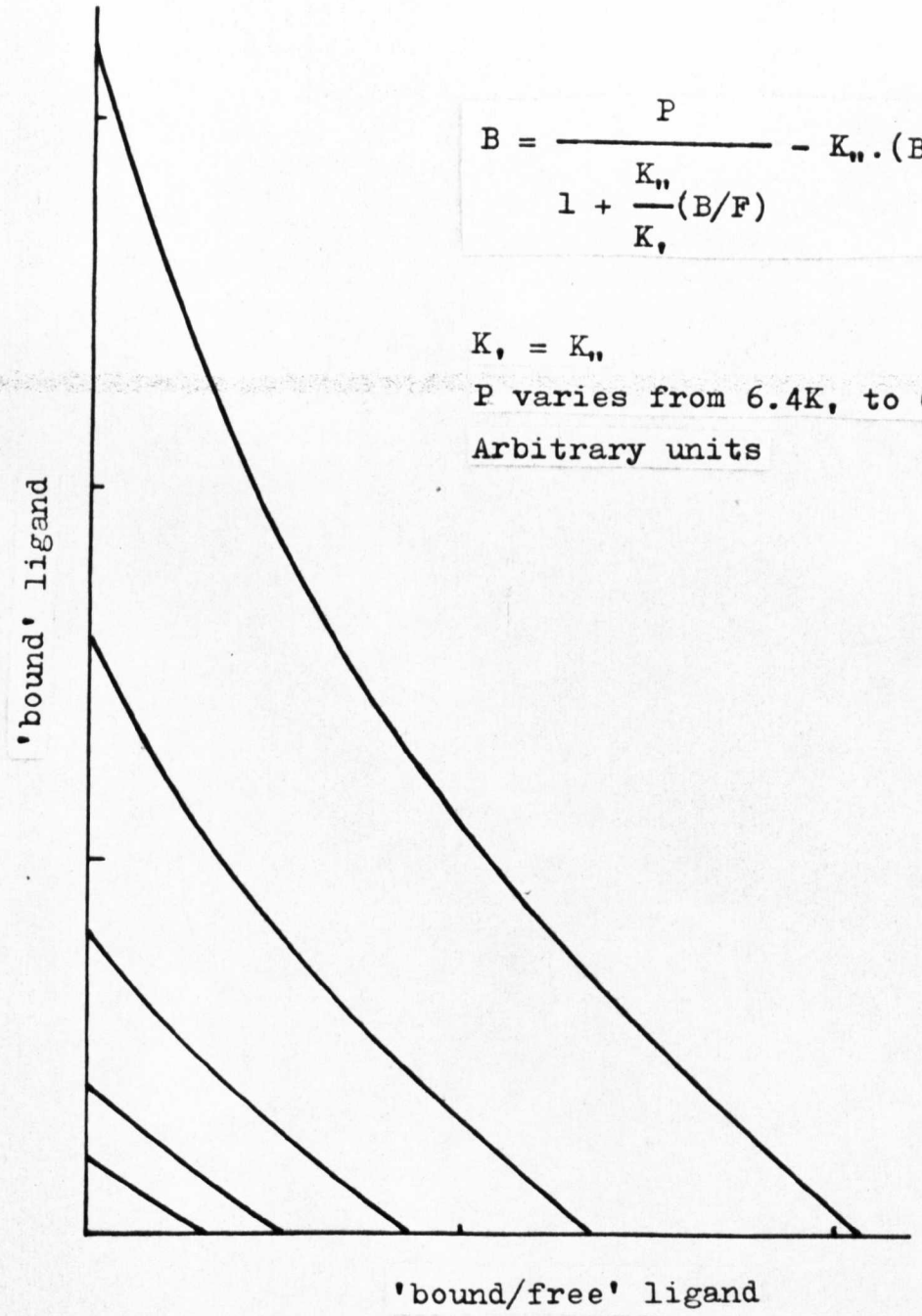
Variation in curve with protein concentration.

$$B = \frac{P}{1 + \frac{K_{,,}}{K_{,}}(B/F)} - K_{,,}.(B/F)$$

$K_{,} = K_{,,}$

P varies from 6.4K, to 0.4K,

Arbitrary units

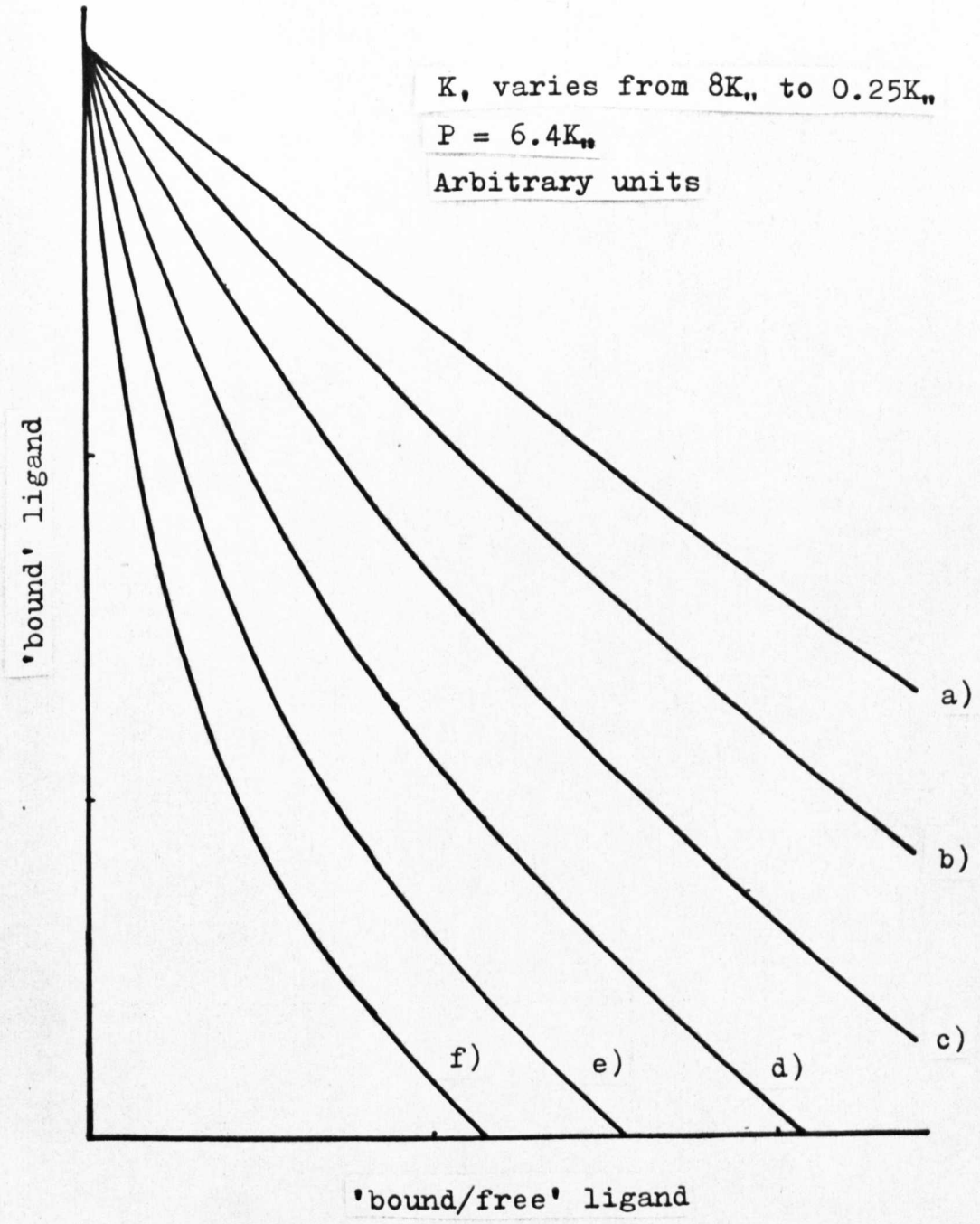


The figure shows the variation of the binding curve with the concentration of binding sites and with the variation of binding constants.

Figure A.3
(ii)

Theoretical Binding Curves for Type 4 Binding Proteins

Variation of curve with the constant K_1.



Values of K_1: a) 8, b) 4, c) 2, d) 1, e) $\frac{1}{2}$ and f) $\frac{1}{4}$ x K_n

From (xxx) it can be seen that:

$$P_1 = K_{II}\frac{B^2}{F} + B \qquad \text{(xxxi)}$$

Substituting for P_1 in (xxix) we get:

$$K_I.(P_{II} - K_{II}.\frac{B^2}{F} - B) = (K_{II}.\frac{B^2}{F})^2 \qquad \text{(xxxii)}$$

Dividing both sides by K_1 and rearranging:

$$B = P_{II} - K_{II}.\frac{B^2}{F}.(1 + \frac{K_{II}}{K_I}.\frac{B^2}{F}). \qquad \text{(xxxiii)}$$

The equation (xxxiii) cannot be treated in the form B as a function of B/F without becoming obscure because of its complexity. However, it can be seen that (xxxiii) will revert to the form of (xii) if $K_1 \gg K_{II}$.
The relationship between B and B/F is:

$$B = \frac{(K_{II}.B/F + 1).\left(\sqrt{1 + \frac{4P.(K_{II}.B/F)^2/K_I}{(K_{II}.B/F + 1)^2}} - 1\right)}{2(K_{II}.B/F)^2/K_I} \qquad \text{(xxxiv)}$$

As with equation (xxxiii) this one simplifies when $K_1 \gg K_{II}$ to give (xiii) by expanding the square root term binomially and ignoring second order and higher terms.

6. Dimeric protein kinase dissociating to give type 4 binding proteins.

Equilibria:

$$R_2C_2 \overset{K_I}{=} 2RC \qquad \text{(xxxv)}$$

$$RC \overset{K_{II}}{=} R + C \qquad \text{(xxxvi)}$$

$$R + \text{c-AMP} \overset{K_{III}}{=} R.\text{c-AMP} \qquad \text{(xxxvii)}$$

Equilibrium equations:

$$K_I = \frac{(P_I - r)^2}{P_{II} - P_I} \qquad \text{(xxxviii)}$$

$$K_{II} = \frac{r.(r - B)}{P_I - r} \qquad \text{(xxxix)}$$

$$K_{III} = \frac{(r - B).F}{B} \qquad \text{(xl)}$$

From (xl) $$r = K_{III}.B/F + B. \qquad \text{(xli)}$$

Substituting for r in (xxxix) and rearranging

$$P_I = (K_{III}.\frac{B}{F} + B).(\frac{K_{III}}{K_{II}}.\frac{B}{F} + 1) \qquad \text{(xlii)}$$

Substituting for r and P_t in (xxxviii) using (xli and xlii) we get:

$$K_t = \frac{\left(\frac{K_m \cdot B}{K_n} \cdot \frac{B}{F} \cdot \left(\frac{K_m \cdot B}{F} + B\right)\right)^2}{P_n - \left(\frac{K_m \cdot B}{F} + B\right) \cdot \left(\frac{K_m}{K_n} \cdot \frac{B}{F} + 1\right)} \qquad \text{(xliii)}$$

The variables B and B/F or B and B^2/F cannot be separated simply in this equation with out resorting to taking roots as in 5. The limiting case when K_t approaches infinity will occur if the denominator in (xliii) approaches zero. If the denominator is equated with zero it can be arranged to give equation (xxii).
The equation for B in terms of B/F is:

$$B = \frac{\sqrt{\left(\frac{K_m \cdot B}{K_n \ F} + 1\right)^2 + \frac{4K_m \cdot B \cdot P_n}{K_t \cdot K_n^2 \cdot F} - \frac{2K_m^3 \cdot B^3}{K_t \cdot K_n^2 \cdot F^3}} - \frac{K_m \cdot B}{K_n \ F} - 1}{\frac{2K_m^2 \cdot B^2}{K_t \cdot K_n^2 \cdot F^2}} \qquad \text{(xliv)}$$

This equation reduces to (xxii) if the square root term is expanded binomially and second order and higher terms are ignored. This procedure is valid if K_t is very large or if P_n is very small.