MECHANISM OF ACTION OF CHOLERA TOXIN

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PREFACE

The work reported in this thesis was carried out between 1st October 1976 and 30th September 1979 under the supervision of Dr. Simon van Heyningen at the Department of Biochemistry, University of Edinburgh Medical School. Unless stated otherwise, all of the material presented was the sole work of the author.

A part of the thesis has already been published independently in a paper entitled, 'The Adenylate Cyclase Activating Activity of Cholera Toxin is not Associated with a Nicotinamide Adenine Dinucleotide Glycohydrolase Activity', which appeared in Biochemical Journal 174, 1059-1062 (1978). A reprint of the above publication is included at the rear of this thesis.

DEDICATION

The present work is dedicated to my wife, Tricia, for all her help and understanding during the preparation of this manuscript.

I also gratefully acknowledge the helpful guidance of my supervisor, Dr. Simon van Heyningen.

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ABSTRACT

Cholera toxin, obtained as crude culture filtrates of the bacterium Vibrio cholerae, was purified by a combination of ammonium sulphate fractionation, ion-exchange chromatography on DEAE-cellulose, and gel filtration on Ultrogel AcA-44. The purified toxin migrated as a homogeneous protein on SDS and native polyacrylamide gels, and activated adenylate cyclase in rat liver homogenates. Activation of rat liver adenylate cyclase by cholera toxin was strictly dependent upon the presence of NAD^+. In the absence of exogenous NAD^+, homogenates or membrane preparations were refractory to the toxin as a result of high levels of endogenous NAD^+ - utilising activities. Under conditions in which competition for NAD^+ by endogenous enzymes was minimised, the activity of cholera toxin as a stimulator of adenylate cyclase was markedly enhanced and activation of the enzyme occurred with a detectable toxin-specific incorporation of $[^{14}C]$ into TCA-precipitable material from [adenine U-^{14}C]-NAD^+. The results were consistent with the hypothesis that cholera toxin acts by catalysing the ADP-ribosylation of an intracellular membrane-bound acceptor protein, and that this modification is responsible for the toxin-induced stimulation of adenylate cyclase activity.

Results are also presented which argue against the validity of extrapolating from studies of the NADase activity of cholera toxin to the mechanism of toxin action on adenylate cyclase. Under a variety of conditions, NADase activity appeared to proceed independently of the physiologically important ADP-ribosyltransferase activity. Furthermore, culture filtrates of V. cholerae were shown to contain a highly active NADase distinct from cholera toxin, and the possibilities of NADase contamination, even in apparently pure toxin preparations, are emphasised.

Chapter 1. Introduction

1.1. Definition of clinical cholera

Cholera is an acute infectious disease usually occurring in epidemic form and characterised by severe diarrhoeal dehydration of the body. Choleraic symptoms appear rapidly and victims can be reduced from a state of normal health to one of extreme illness in a matter of three to four hours, a fact which prompted Magendie (quoted in De, 1961) to describe cholera as 'a disease which begins where other diseases end, with death'. The disease state is the result of intestinal colonisation by the bacterium *Vibrio cholerae*.

1.2. Clinical course of cholera

The early stages of cholera are characterised by copious diarrhoea and vomiting, when fluid losses from the gut can often exceed 1 litre per hour. The fluid itself, which is alkaline and has a typical 'rice-water' appearance, is usually devoid of faecal matter and contains characteristic quantities of sodium, potassium, bicarbonate and chloride (Carpenter, 1971). As a result of choleraic dehydration the victim enters a 'stage of collapse', in which rapid drop in blood pressure, suspension of urinary excretion, muscular cramps and laboured respiration are the major symptoms. Survivors from the stage of collapse then display a gradual restoration of normal functions and urinary excretion is re-established, although in many cases the latter is again suspended and a state of post-choleraic uraemia develops. This final stage of the disease is

responsible for death in a small proportion of cases, but mortality in cholera, which can approach 80% in the absence of effective treatment, is greatest during the stage of collapse when circulatory failure is a common cause of death (see De, 1961). The detailed internal and external pathological changes associated with the various stages of cholera have been described by De (1961).

1.3. Early events in the history of cholera.

Cholera is often prefixed with the term 'Asiatic' to indicate the supposed geographical origin of the disease, although from a detailed historical study, De (1961) concluded that a primary Asiatic origin of cholera could not be confirmed with certainty. Regardless of its origin however cholera, on many occasions, has demonstrated a remarkable ability to spread rapidly from its endemic confinement in the Indian Sub-Continent to encircle the rest of the world in pandemic out-breaks. The first well-recorded cholera pandemic in 1817-1822 left Europe largely unscathed but in some of the subsequent outbreaks of 1831-1837, 1848-1858, 1865-1875, 1883-1885 and 1892-1894, heavy mortalities were sustained throughout Europe. Amid great confusion as to the nature and epidemiology of cholera during these earlier periods, the work and insight of John Snow (1813-1858) were of fundamental importance in establishing a sound basis for future progress. Snow's connection between impure water supplies and choleraic disease, and his (then) revolutionary concept that the spread of infection in cholera was directly from the alimentary canal of one patient to that of another (which he established during the 1848-1858 epidemic in Britain: see Snow, 1849), provided the first major clue

as to rational preventative measures during cholera outbreaks: namely improved sanitation, and isolation of impure water supplies. Since Snow had also pin-pointed the mucous membranes of the intestine as being the tissue in which 'the mischief of cholera was at first confined', his work undoubtedly contributed to the isolation and detailed description of the causative agent of cholera by Koch in 1884. Koch, leading the German contingent to study cholera during the epidemics in Egypt and India in 1883-1884, prepared mucous from the intestines of cholera victims as soon as possible after death and observed short, curved, comma-shaped bacilli. Although Koch is often credited with the first detailed description and characterisation of the 'comma-bacillus' of cholera, he was not in fact the first to observe the causative agent, and was himself unaware of the previous description of an organism termed Vibrio cholerae by Pacini in 1854 who had observed 'millions of vibriones' in the intestines of cholera patients. Even Pacini, whose nomenclature is accepted as the correct one today, may not have been the first to recognise the cholera vibrios. It seems likely that Pouchet in 1849 had already observed V. cholerae in the dejecta of cholera patients, while the earliest known claim to have seen the cholera bacillus, by Boehm in 1838, cannot be verified (see Pollitzer, 1965).

1.4. Taxonomy of vibrios and identification of choleragenic organisms

In the words of Smith and Goodner (1965), 'the vibrios form a vast sub-continent of the bacterial world - regions which are almost barren of exploration'. The lack of information on taxonomy of the vibrios is related to the great polymorphism found in this group,

making the task of classification a difficult and unpleasant one. However the choleragenic vibrios have been well characterised and De (1961) has summarised the criteria upon which identification of V. cholerae has been based. Apart from possessing the typical morphological features (the organism is a gram-negative, motile, curved rod), the accepted bacterium must be able to ferment mannose and saccharose but not arabinose, it should fail to haemolyse sheep or goat erythrocytes, and it should be agglutinated by O-Subgroup I serum.

However the classification of choleragenic vibrios has been complicated by the occurrence of several forms which do not obey all of the above criteria. In 1905 for example Gotslich (quoted in Pollitzer, 1965) isolated six strains of vibrio from the dead bodies of pilgrims returning from Mecca at the El Tor quarantine station in the Sinai peninsula. Although the vibrios possessed all the known features of V. cholerae, the subjects from which they were isolated had shown no signs of choleraic disease, and on closer examination of the 'El Tor vibrios' Kraus and Pribram (quoted in Pollitzer, 1965), found all of them to be strongly haemolytic for sheep and goat erythrocytes. The species name V.eltor was introduced for these organisms in 1933 by Pribram (see Hugh, 1965) although Hugh (1965) suggested that since V. eltor was so similar to classical V. cholerae, it should be included under the species name of the latter and be regarded as a natural variant or biotype of classical V. cholerae. The haemolytic properties of the El Tor vibrios have in any case, proved to be unstable and most of the V. eltor strains isolated during the 1962-1963 cholera epidemic in New Guinea were either only

weakly haemolytic or completely non-haemolytic (Mukerjee, 1965).

The classification scheme is further complicated by the occurrence of a diverse group of non-agglutinable vibrios (NAG-vibrios), so named because they are not agglutinated in cholera O-Subgroup I serum (see Finkelstein, 1973). Nevertheless these organisms are capable of eliciting a cholera-like disease in man. Because of the relative mildness and short duration of the diarrhöeagenic effects induced by NAG-vibrios, Finkelstein (1973) has suggested that they should be grouped together under the new species name '*Vibrio enteritidis*' in order to distinguish them from the more serious infections caused by the classical and El Tor biotypes of *V. cholerae*.

Among the many hundreds of non-cholera vibrios (NCVs) i.e. vibrios which are not associated with choleraic disease, several of those studied by Smith and Goodner (1965) were found to mutate spontaneously into highly choleragenic strains indistinguishable from *V. cholerae*, and the authors emphasized the possible importance of NCV mutations in the epidemiology of cholera.

1.5. Recognition of an exotoxin as the active principle in cholera.

After the isolation and characterisation of *V. cholerae* by Koch in 1884, numerous experiments were conducted to establish definitively that this organism was the causative agent of cholera, by attempting to reproduce the symptoms of the disease in a variety of experimental

animals after administration of live vibrios. Although some of these experiments (summarised by De,1961) did result in the induction of cholera-like symptoms, the lack of a consistently observed and easily measured response precluded any definite assessment of the results. Similarly, attempts to establish that 'toxicosis' was the result of a 'specific extracellular poison', as suggested by Koch in the 1880s, by administration of cell-free extracts of V. cholerae cultures, also gave rise to a great deal of confusion. There are probably many reasons for the inconsistency of results during this period. Different workers used different experimental animals and administered vibrios, or extracts therefrom, by different routes (intravenously, intraperitoneally, orally, intraintestinally, etc.) Methods of preparing V. cholerae cultures and of extracting cell-free substances also varied widely and undoubtedly, many of the diverse effects reported were the result of irrelevant, non-specific toxicities arising artefactually with the methods employed. It is surprising to note that few of these early studies investigated the effects of V. cholerae, or extracts of the organisms, on the intestine, and most workers concentrated on the systemic and lethal effects of toxic preparations after intravenous or intraperitoneal injection.

This was the growing state of confusion between 1884 and 1953, at the end of which period, cholera toxin was beginning to be described as an endotoxin. Then in 1953 a major advancement in cholera research was made by De and Chatterje (De and Chatterje, 1953) who noted that when live V. cholerae were injected into a loop of adult rabbit small intestine, isolated from the rest of the gut by two ligatures, the loop subsequently accumulated a large volume of fluid and became excessively

swollen. Although this model was originally conceived for diagnostic purposes, it was soon adapted and improved upon to enable detailed patho-physiological studies on cholera to be made (see for example, Schafer and Lewis, 1965). The fluid accumulated in the intestinal loops had all the gross characteristics of the typical cholera stool, and the technique provided the first reliable animal model for studies on choleragenesis.

Soon afterwards Dutta and Habbu (1955) were able to produce consistently, a fatal diarrhoeal disease in suckling rabbits, resembling cholera in man, after intraintestinal injection of live V. cholerae.

The great importance of these two developments can be appreciated by considering that only several years after their introduction, the exotoxic nature of the active principle in cholera was firmly established after 75 preceding years of confusion. In 1959 the laboratories of both De and Dutta reported that identical responses, in their respective model systems, to those observed with live suspensions of V. cholerae, could be produced upon administration of cell-free culture filtrates of the bacteria (De, 1959: Dutta et al, 1959).

When it was recognised that choleraic symptoms could be induced by a heat-labile exotoxin, produced and secreted by V. cholerae, progress was rapid and in 1969 purification of the active material, known synonymously as cholera toxin, cholera enterotoxin or choleragen, was reported (Finkelstein and LoSpalluto, 1969). The purification of cholera toxin by these workers is described fully in Ch. 4 of this work.

1.6. The structure of cholera toxin

The structure of cholera toxin purified by Finkelstein and LoSpalluto, (1969) has now been studied extensively and current knowledge on the subject has been reviewed recently by several authors (van Heyningen, 1977a; Gill, 1977; Finkelstein, 1976).

The complete toxin molecule is a protein of molecular weight 82,000 - 84,000 daltons as determined in the ultracentrifuge by several different groups (van Heyningen, 1976a; LoSpalluto and Finkelstein, 1972; Lai et al, 1976; Sattler et al, 1975), and the purified material contains no detectable quantities of either lipid or carbohydrate (LoSpalluto and Finkelstein, 1972). That the toxin possesses a subunit structure was first implied by the observation that, during purification, a non-toxic but immunologically related antigen was also recognised and purified (Finkelstein and LoSpalluto , 1969). This material, designated choleragenoid, was subsequently found to be a spontaneous degradation product (a natural toxoid) of cholera toxin (Finkelstein et al,1971) and the molecular weight of purified choleragenoid, determined in the ultracentrifuge, was 56,000 daltons (LoSpalluto and Finkelstein, 1972).

Subsequent studies on the subunit structure of cholera toxin have revealed the characteristics summarised in Fig. 1.1. It has been possible to generate a material identical to the purified choleragenoid of Finkelstein and LoSpalluto by chromatography or electrophoresis of cholera toxin in the presence of denaturing agents such as urea, guanidinium-HCl or SDS (van Heyningen, 1974a; Holmgren and Lonnroth, 1975;

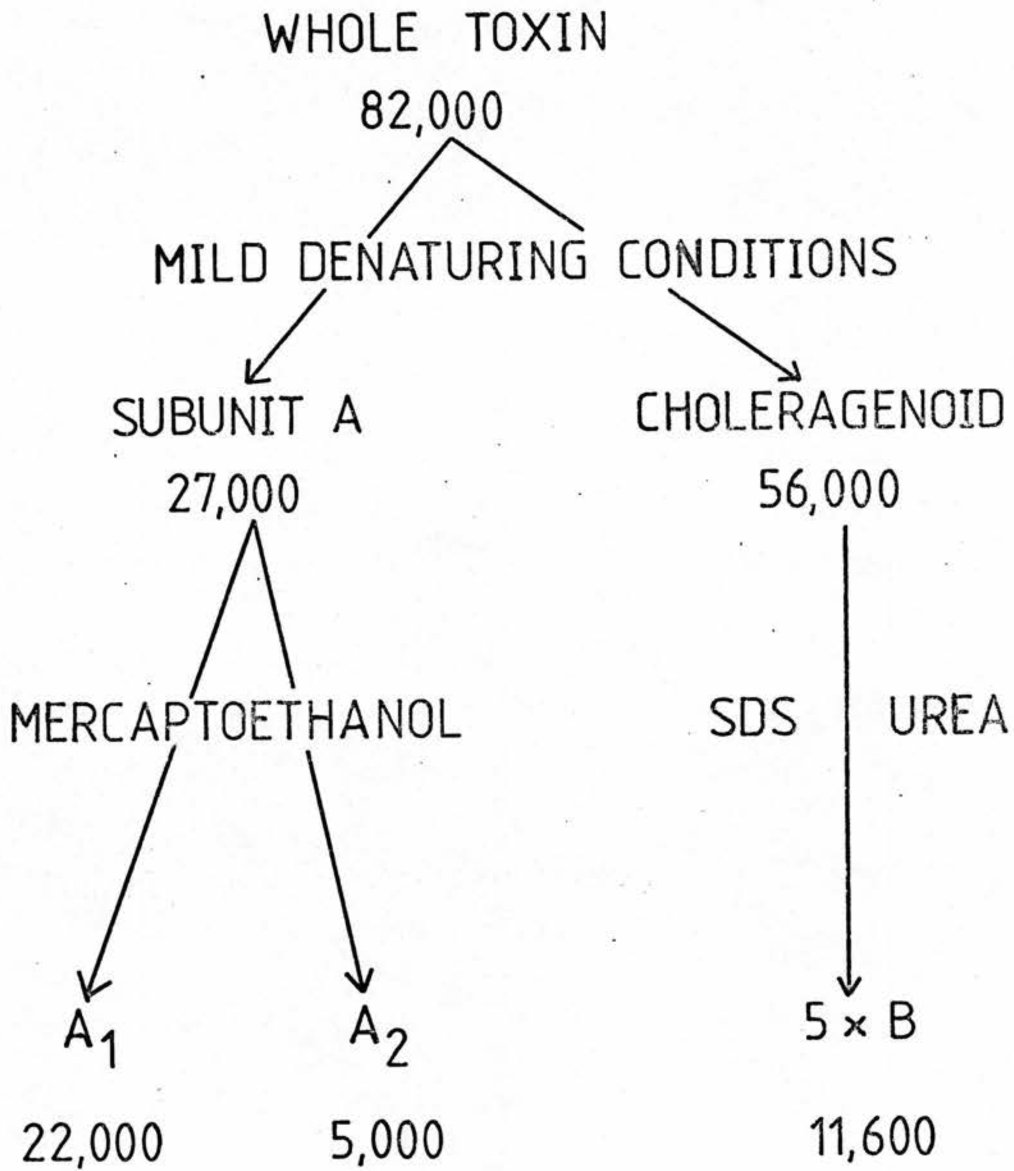


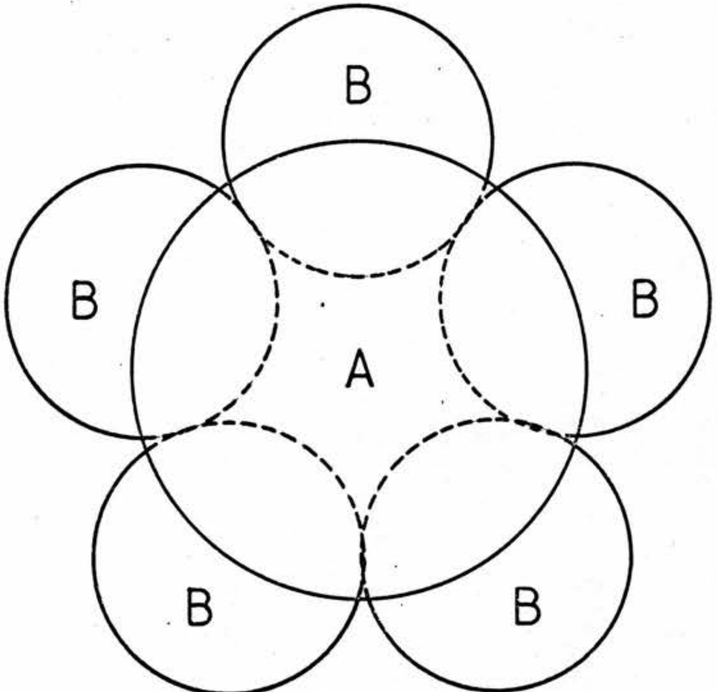
Figure 1.1. The subunit structure of cholera toxin.

van Heyningen, 1976a; Lai et al, 1976; Cuatrecasas et al, 1973; Sattler et al, 1975; Finkelstein et al, 1974). In the presence of these agents cholera toxin dissociates rapidly into two different types of subunits designated A and B. The B subunit prepared in this way (which is synonymous with choleragenoid) has a molecular weight, determined in the ultracentrifuge, of about 54,000 daltons (van Heyningen, 1976a), a value which is in good agreement with that obtained by LoSpalluto and Finkelstein referred to above, for the material they isolated during toxin purification. The toxin also dissociates slowly into its subunits at room temperature and neutral pH and the rate of dissociation is accelerated in buffers of low pH. Such buffers are commonly employed for preparation of the toxin subunits (van Heyningen, 1974a; 1976a). Subunit A of the toxin migrates on SDS-polyacrylamide gels with a molecular weight of about 28,000 while studies with the ultracentrifuge have yielded a value of 27,000 - 28,000 daltons (van Heyningen, 1976a) and therefore intact cholera toxin (82,000 - 84,000 daltons) is composed of Subunit A (27,000-28,000 daltons) plus choleragenoid (54,000-56,000 daltons).

Subunit A itself is made up of two polypeptide chains, termed peptides A_1 and A_2, joined by a single disulphide bridge and upon treatment with thiols and electrophoresis in SDS or urea a small 5,000-7,000 dalton fragment (A_2) is split off (van Heyningen, 1974a, 1976a, Lai et al, 1976; Sattler et al, 1975). Furthermore, when harsher conditions are employed to dissociate intact cholera toxin, such as boiling in 1%-SDS, the 56,000 dalton subunit is no longer visible on subsequent SDS gel electrophoresis, and is replaced by material of much lower molecular weight, the exact size of which was

in dispute for some time, with different values being reported in a variety of different analytical systems and even in the ultracentrifuge (van Heyningen, 1976a; Sattler et al, 1975). However the amino acid sequence of this low molecular weight polypeptide has now been published independently by three different groups, all showing good agreement, and all of which report a molecular weight of about 11,600 (Nakashima et al, 1976; Kurosky et al, 1977; Lai et al, 1977). It therefore appears that the complete B portion of cholera toxin (54,000-56,000 daltons) must be composed of 5 identical B subunits, a conclusion which was reluctantly accepted owing to the rare, if not unknown, occurrence of such a stoichiometry in other proteins (Klotz et al, 1975). However, the evidence in favour of such a structure is now compelling (see van Heyningen, 1977a; Gill, 1977). Each of the 5 B subunits contains one intrachain disulphide bridge (Klapper et al, 1976).

Intact cholera toxin therefore consists of Subunit A, comprising peptides A_1 and A_2 in covalent linkage, joined non-covalently to choleragenoid, comprising 5 identical B subunits all of which are in non-covalent association with each other. It is possible to envisage only one three-dimensional structure for this stoichiometry which would satisfy currently accepted principles of protein aggregation (Klotz et al, 1975), namely a heterologous ring of B subunits with subunit A in the centre as depicted in Fig. 1.2. Some electron micrographs have provided support for this model (Ohtomo et al, 1976) as have cross-linking experiments with bifunctional reagents, which indicate that links between B subunits are formed more readily than between subunit A and the B subunits, and links



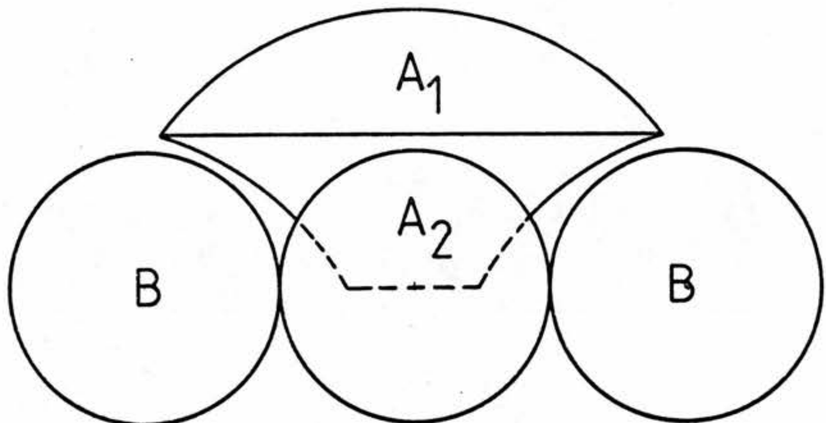


Figure 1.2. Proposed geometry of the cholera toxin subunits (1: Plan view; 2: Front view).

between the A_2 peptide and B subunits more readily than between A_1 and B subunits (van Heyningen, 1976a; 1977b; Gill, 1976a). The model also receives support from the observation that toxic protein can be reconstituted from the separated A and B subunits, but not from peptide A_1 alone and B subunits (Gill, 1976a) indicating that peptide A_2 is essential as an 'adaptor' between peptide A_1 and the B subunits. Finally mercaptoethanol, which cleaves disulphide bridges only, can release peptide A_1 from intact toxin leaving an A_2-5B complex behind (Sattler et al, 1975). Confirmation of this proposed structure will of course be dependent on X-ray diffraction studies. Crystals of cholera toxin and of choleragenoid were first produced by Finkelstein and LoSpalluto (1972) although these were unsuitable for such analysis. More recently Sigler et al (1977) have reported the production of crystals suitable for X-ray analysis (interestingly, these crystals support the unusual stoichiometry of cholera toxin, and are consistent with 5B subunits per molecule). To date however, no further work in this field has been reported.

1.7. Effects of cholera toxin on the small intestine and delineation of the primary choleragenic response.

The demonstration that cell-free culture filtrates of V. cholerae could induce fluid accumulation in ligated intestinal loops (De, 1959) and could elicit choleraic disease in suckling rabbits (Dutta et al, 1959), clearly indicated that a soluble exotoxin elaborated by the bacteria was responsible for the production of choleraic symptoms. Sack and Carpenter (1969) confirmed these findings by demonstrating that fluid losses similar to those seen

in the ligated rabbit ileal loop could also be induced in segments of canine small bowel by culture filtrates of *V. cholerae*. That these observations were of direct relevance to human cholera had been shown previously by the experiments of Benyajati (1966), who was able to produce the symptoms of cholera in human volunteers after oral administration of *V. cholerae* culture filtrates. Elucidation of the gross pathogenic mechanisms of fluid loss in cholera was achieved by a remarkable combination of *in vitro* and *in vivo* studies during the years 1960-1970.

Since all previous studies on cholera had isolated the small intestine as the primary site of action of the choleragenic principle, most of the studies described below were performed on this tissue. Direct confirmation of this assumption was not made until 1966 however, when Sack and coworkers demonstrated that only the small intestine contributes to the release of fluid during experimental canine cholera (Sack *et al*, 1966). Later the same conclusion was reached by Banwell *et al* (1970) from studies on convalescent and acutely ill cholera patients. Thus although *V. cholerae* is present in all regions of the gastrointestinal tract, from mouth to anus, in human cholera (Gorbach *et al*, 1970), it is only in the small intestine that the unique fluid secretory mechanisms are operative. Carpenter *et al* (1968) extended this observation and noted that fluid loss in the small intestine is greatest, per unit length, in the duodenum and least in the ileum.

Early workers had suggested that sloughing of the intestinal mucosa occurred during cholera (see references cited in Pierce _et al_ 1971a). It seems likely however that many of these observations were made during post-mortem examinations several hours, and possibly much longer in some cases, after death, when non-specific tissue degradation would have been a complicating factor. More recent studies (see Pierce _et al_ 1971a) suggested that the intestinal mucosa remained intact during cholera, when it was demonstrated that leakage of plasma proteins into the bowel did not occur during the course of the disease, indicating that no substantial denudation had occurred. The light and electron microscopic studies of Elliott _et al_ (1970), using intestinal mucosa from choleraic dogs showed only minor changes in intestinal morphology including slight capillary dilation and increased production and discharge of mucous from the goblet cells. As suggested by Carpenter (1972) these, and other observed minor alterations, probably represent functional changes only, and are not indicative of cell damage. The observations that several other functions of the small intestine are unaltered during cholera, such as glucose absorption and glucose-related enhancement of water and sodium absorption (Hirschhorn and Rosenberg, 1968; Pierce _et al_, 1968; Carpenter _et al_, 1968; Iber _et al_, 1969; Love, 1965) and absorption of glycine (Nalin _et al_, 1970), also argue against any significant amount of cell damage occurring during the disease.

The first clue as to the mechanism by which cholera toxin stimulates fluid secretion in the small intestine came with the observations of Field _et al_ (1968) who noted that when theophylline or dibutyryl cAMP was applied to pieces of intestinal mucosa, stripped

of muscularis and mounted in an Ussing Chamber, the normal transport of Na^+ from the mucosal to the serosal surface was inhibited, and that transport of Cl^-, also normally from mucosa to serosa, was reversed, resulting in active Cl^- secretion. Culture filtrates of V. cholerae were then shown to produce exactly the same alterations and the effect of theophylline was eliminated in tissues pretreated with V. cholerae culture filtrates (Field et al, 1969). In 1970, Al-Awqati using the same technique, obtained identical results with the highly purified cholera toxin which had recently become available (Al-Awqati et al, 1970a). Theophylline, an inhibitor of cAMP phosphodiesterase and dibutyryl cAMP, an analogue of cAMP which is resistant to hydrolysis by phosphodiesterase, both serve to effectively raise the intracellular concentration of cAMP when added to cells. Since both treatments mimicked the effects of cholera toxin on the small intestine, it was suggested that the toxin may act by altering the levels of cAMP in intestinal epithelial cells. Further support for this notion was provided by Al-Awqati et al (1970b) who showed that the same changes in Na^+ and Cl^- transport were induced by prostaglandin E_1, an agent known to increase cAMP levels via activation of adenylate cyclase. The in vivo studies of Pierce et al (1971b) also lent support when theophylline and several different prostaglandins were shown to induce fluid loss from canine small bowel after infusion into the mesenteric artery. The secreted fluid displayed the characteristics of the typical cholera stool.

Kimberg et al (1971) and Sharp and Hynie (1971) were the first workers to demonstrate a direct effect of cholera toxin on cAMP metabolism. Both groups reported activation of the membrane-bound

enzyme adenylate cyclase after cholera toxin treatment of gut mucosal cells, and the time courses of cyclase activation correlated well with the previously established time course of fluid accumulation in ligated intestinal loops (Carpenter et al, 1968). The toxin had no effect on phosphodiesterase activity and measurement of intracellular cAMP levels in mucosal cells after toxin treatment confirmed that marked elevation of the nucleotide concentration had occurred (Kimberg et al, 1971; Schafer et al, 1970). As a final confirmation of adenylate cyclase activation being the primary choleragenic mechanism, Chen et al (1972) demonstrated elevated adenylate cyclase activity in human intestinal mucosa during the course of naturally acquired cholera.

1.8. Other activities of cholera toxin

The recognition that the primary action of cholera toxin was activation of adenylate cyclase and therefore that the toxin's main effect was to increase intracellular concentrations of cAMP, provided an explanation for the many other activities of cholera toxin which were being reported concurrently with the work described in the previous section, and of additional activities reported subsequently. A glance at Table 1.1. indicates the great diversity of effects elicited by cholera toxin on many different cellular systems which had defied all attempts to be rationalised and related in a unifying hypothesis. However with the knowledge that cholera toxin can increase intracellular levels of cAMP, many of these observations can be readily explained. For example, lipolysis in

Table 1.1. Effects of cholera toxin on cells and tissues

Tissue or cell type	Effect	Reference
Ligated ileal loop	Fluid accumulation	De, 1953; 1959 Schafer and Lewis, 1965
Rabbit skin	Increase in vascular permeability	Craig, 1965
Rat/mouse foot pad	Oedema	Finkelstein et al, 1969
Fat cells	Lipolysis	Greenough et al, 1970.
Liver cells/platelets	Glycogenolysis	Zieve et al, 1971 Gorman and Bitensky, 1972
Y1 adrenal cells	Steroidogenesis altered morphology (Rounding)	Donta and King, 1973 Kwan and Wishnoy, 1974a Wolff et al, 1973
Chinese hamster ovary cells	Altered morphology (elongation)	Guerrant and Brunton, 1977 Guerrant et al 1974
Dog liver (after intravenous injection)	Increased serum levels of hepatic alkaline phosphatase	Pierce et al, 1972
Pituitary	Growth hormone release	Rappaport and Grant, 1974 Sundberg et al, 1974.
Pancreas	Insulin secretion	Hahn et al, 1974
Immune system	Various modulations of immune response	Northrup and Fauci, 1972 Chisari and Northrup, 1974 Cook et al, 1975 Sultzer and Craig, 1973 Strom et al, 1972 Warren et al, 1974 Bourne et al, 1974
Adenylate cyclase in almost all eucaryotic cells.	Stimulation of activity	see Van Heyningen, 1977a, Gill, 1977 Richards and Douglas, 1978

isolated fat cells is known to be under hormonal control and cAMP acts as the 'second messenger' in this system (Rodbell and Jones, 1966). Likewise glycogenolysis in liver cells depends on the activation of phosphorylase, which again is under the ultimate control of cAMP levels (Robison et al, 1971). Steroidogénesis in the Y1 adrenal cell line (derived from mouse adrenal cortex) is an event normally controlled by ACTH, also via modulations of adenylate cyclase activity and cAMP levels (Yasumura et al, 1966) and the alterations in cell morphology induced by cholera toxin, both in this system and in the Chinese Hamster Ovary (CHO) cell line, are also thought to be cAMP-mediated events, since altered morphology is seen only and always under conditions which result in increased intracellular levels of cAMP (Kwan and Wishnow, 1974a,b; Donta and King, 1973; Johnson and Pastan, 1972; Nozawa et al, 1975; Guerrant et al, 1974; Guerrant and Brunton, 1977).

Some of the other effects of cholera toxin such as increase in vascular skin permeability, induction of rat foot pad oedema, and increased serum levels of alkaline phosphatase after intravenous injection of toxin, are not sufficiently well understood to be related directly to an effect of cAMP but it seems likely that this will be the underlying cause of all cholera toxin effects. This contention is supported by the final entry in Table 1.1. which indicates that cholera toxin is capable of activating adenylate cyclase in virtually all eukaryotic cell types and tissues studied to date. No attempt has been made to list all of the systems in which toxin has been tested, examples of which can be found in most recent reviews (van Heyningen, 1977a; Gill, 1977; Finkelstein, 1976; Richards and Douglas, 1978). Suffice it to say that 'it has become almost axiomatic

that if a particular reaction under study is not affected by cholera enterotoxin, then the process is not cyclase/cAMP-mediated' (Finkelstein, 1976).

It is therefore evident that all of the various activities of cholera toxin are the result, not of differential effects on different cell types, but of different cells responding in different ways to the same basic signal, which is increased cAMP concentrations. In the small intestine, the only tissue which is normally exposed to cholera toxin in nature, this response is fluid secretion. In other cells specialised for different purposes, responses vary depending on the function and metabolic characteristics of the cell type in question, and invariably cholera toxin will activate those processes which are normally under the control of cyclase-directed hormones. There are of course exceptions; cholera toxin has no known effect on procaryotic adenylate cyclases, an observation which is perhaps not surprising since the enzyme from microorganisms bears little resemblance to eucaryotic cyclases (see for example, Peterkofsky et al, 1978; Ozer and Schelt, 1978). There is also one eucaryotic tissue, in which cAMP is known to be a mediator (toad bladder), on which cholera toxin has been reported to have no effect (Lief and Keusch, 1971). However the system has not been well studied and the lack of toxin-responsiveness may simply reflect an absence of the appropriate cholera toxin receptors on the cell surface (see following section).

Many of the activities listed in Table 1.1. have formed the basis of accurate and convenient assays for cholera toxin. These include rat foot pad oedema (Finkelstein et al, 1969), steroidogenesis in Y1 adrenal cells (Donta and King, 1973; Donta, 1974; Donta et al, 1976), CHO cell elongation (Guerrant et al, 1974; Guerrant and Brunton, 1977), lipolysis in fat cells (Greenough et al, 1970) and skin vascular permeability (Craig, 1965). A comparison of fat cell, ileal loop and rabbit skin assay systems by Curlin et al (1973) indicated good agreement among all three techniques.

1.9. The role of the cholera toxin subunits in activation of adenylate cyclase.

The vast majority of research effort since 1971 has been directed towards an understanding of the mechanisms involved in cholera toxin stimulation of adenylate cyclase. Primarily it was necessary to establish how the structure of the toxin was related to its action and to answer such questions as 'How is cholera toxin able to activate adenylate cyclase in almost all cells no matter how far removed they may be from clinical cholera?' Many of these questions have been resolved by studying the effects of cholera toxin on simple cell systems, of which pigeon erythrocytes have been especially popular. This system, introduced by Gill in 1974 (quoted in Gill, 1977), appeared to be ideally suited to a study of cholera toxin action. The erythrocytes are readily available and easily prepared, and are characterised by an extremely low basal activity of adenylate cyclase which can be stimulated 20-30 fold by cholera toxin (in other tissues with higher basal levels of

adenylate cyclase, the effects of cholera toxin, although easily observable, are much less dramatic). Although pigeon erythrocytes appear to be particularly remote from any relevance to clinical cholera, it has been generally assumed that activation of adenylate cyclase in the small intestine is not substantially different from activation in other tissues, and indeed there are no reasons to conclude otherwise, although there may be qualitative differences between tissues (see Ch. 6.). The choice of such simple cell systems is therefore justified and almost all of the current knowledge on cholera toxin action has been derived from studies in these systems. It should also be noted that, had pigeon erythrocytes not been chosen initially as a tissue of study, progress may well have been much less rapid. (The reasons for making this statement will become clear in the following section when the role of NAD^+ in toxin action is discussed.)

The years 1970-1976 saw the development of cholera research from the stage at which highly purified cholera toxin had only just become available, and when its effect on adenylate cyclase was recognised for the first time, to the elucidation of the gross mechanism of its action. The studies which were performed during this period have been reviewed recently in detail by van Heyningen (1977a) and Gill (1977) and only the major conclusions are summarised here.

Although cholera toxin can be regarded as having certain similarities with hormones, there are nevertheless important differences in the actions of these molecules, one of which is that cholera toxin,

unlike hormones which activate adenylate cyclase almost instantaneously, displays a lag phase between addition to a population of intact cells and the onset of cyclase activation. An early report of Carpenter et al (1968), who observed a 30 min. lag between the introduction of V. cholerae filtrates into canine jejunal loops and the commencement of fluid accumulation, has since been confirmed in all intact simple cell systems studied including fat cells (Vaughan et al,1970; Cuatrecasas, 1973a), pigeon erythrocytes (van Heyningen and King, 1975; Gill and King, 1975) and rat liver (Houslay and Elliott, 1979). Furthermore an unpublished observation of Carpenter and Sack (alluded to by Pierce et al, 1971a), that cholera toxin placed within a canine jejunal loop produces its full effect even if attempts are made to flush it out of the loop or to neutralise it with specific antibodies within 1 min. after its introduction, has also been confirmed in simpler cell systems, and extracellular antitoxin added to a population of intact cells after the addition of cholera toxin but before the onset of cyclase activation, cannot 'rescue' the cells from the subsequent effect of the toxin (Gill and King, 1975). These observations suggested that cholera toxin was bound rapidly to the surfaces of cells and was thereafter unavailable for neutralisation with antitoxin.

That the toxin does bind tightly to cells at specific receptor sites was the culmination of a series of experiments initiated by the observations of van Heyningen and co-workers in 1971, who noted that if cholera toxin was preincubated with a mixture of brain gangliosides, its subsequent effect on gut cells was prevented (van Heyningen, et al,1971).

Numerous studies performed subsequently have shown that cholera toxin exhibits a strong binding affinity for the monosialoganglioside Gm_1 (for reviews of ganglioside structure and function, see van Heyningen, 1974b; Fishman and Brady, 1976) and that, of all gangliosides, only Gm_1 significantly inhibits the action of cholera toxin on intact cells (van Heyningen et al, 1971; Holmgren et al, 1973; Staerk et al, 1974; King and van Heyningen, 1975; Cuatrecases et al, 1973; Sattler et al, 1977; King and van Heyningen, 1973, Cuatrecasas, 1973b; Wiegandt et al 1976.) Studies with ^{125}I-cholera toxin have revealed that the toxin also binds rapidly to cell surfaces with a dissociation constant in all cells studied of around $10^{-10}M$, indicative of very tight binding (Walker et al, 1974; Cuatrecasas, 1973a,b,c; Holmgren et al, 1974, 1975). Since gangliosides are ubiquitous components in the plasma membranes of cells (van Heyningen, 1974b; Fishman and Brady, 1976) these observations strongly suggested that cholera toxin binds to gangliosides and specifically Gm_1 on the surfaces of cells, and that Gm_1 is probably the natural in vivo receptor for the toxin. This hypothesis has now been thoroughly tested and most results are consistent with it. The Gm_1 content of plasma membranes can be altered in several ways and in all cases, any treatment which increases the Gm_1 content of a cell leads to an increase in the amount of cholera toxin which can be bound, and to an increase in the observed physiological response elicited by the toxin (Cuatrecasas, 1973b ; Gill and King, 1975; King et al, 1976; Holmgren et al, 1974; King and van Heyningen, 1973; Haksar et al, 1974; Staerk et al, 1974; Hollenberg et al, 1974; Moss et al, 1976a, Mullin et al, 1976; Fishman et al, 1976; Moss et al, 1977a). It should be noted however

that the increase in physiological response seen with these treatments is not always directly related to the amount of toxin which can be bound, and there is good evidence for a large amount of non-productive binding (but nevertheless specific binding), even in untreated cells (see for example, King et al, 1976). If Gm_1 is the natural cholera toxin receptor then the extraordinary promiscuity of cholera toxin referred to earlier, which enables it to affect all cells, may be explained, in that gangliosides, including Gm_1 are widely distributed in nature and are ubiquitous components of cellular plasma membranes, although the relative amounts of individual gangliosides vary from cell to cell (van Heyningen, 1974b, Fishman and Brady, 1976).

Many studies have also revealed that it is only Subunit B of the toxin molecule which shows affinity for Gm_1 ganglioside, and Subunit A displays no binding affinity for either gangliosides or cells (van Heyningen, 1974a; Cuatrecasas et al, 1973; Holmgren and Lonnroth, 1975; Sattler et al, 1975). Indeed choleragenoid, the natural toxoid of cholera toxin containing only the B-subunits, also binds to cells and to Gm_1 in an almost identical fashion to intact cholera toxin, with the important difference that the binding to cells is totally non-productive and causes no increase in adenylate cyclase activity (King and van Heyningen, 1975). This, and the observations that once choleragenoid is bound to cells, intact toxin can no longer bind and its action is prevented (Holmgren et al, 1974; Gill and King, 1975; Cuatrecasas 1973c; Pierce, 1973), indicated that both species must compete for the same Gm_1 binding sites, and since choleragenoid displayed no activity, Subunit A of the toxin must

necessarily be responsible for the activation of adenylate cyclase.

Direct evidence for this latter conclusion was first provided by van Heyningen and King (1975) who demonstrated activation of adenylate cyclase in pigeon erythrocytes by both intact toxin and by purified Subunit A. With intact cells, both species exhibited the characteristic lag phase before the onset of cyclase activation while in lysed cells, both induced immediate cyclase activation on addition. The results of Gill and King (1975) using the same system, were similar, and these workers also demonstrated activity of Subunit A but in this case, the subunit was active only in lysed cells and displayed no activity towards intact erythrocytes. Like the results of van Heyningen and King however, both Subunit A and intact toxin had an immediate effect in lysed cell systems, and intact toxin displayed its characteristic lag phase when tested on whole cells. In the same report Gill and King extended their observations and showed that peptide A_1 of the toxin alone was the active species.

The abolition of the lag phase in broken cell systems had been observed previously. Zieve et al (1970) first reported such an effect in sonicated human platelets and in rat liver homogenates, and the phenomenon is now known to be a general one in all broken cell systems studied. Only Cuatrecasas and his group have reported fundamentally different results to those described above. Bennett et al (1975) and Sahyoun and Cuatrecasas (1975), working with isolated fat cells, also demonstrated an activity of Subunit A but in contrast to other workers' results, the subunit in this system was found to

activate adenylate cyclase in intact cells with no lag phase, while intact cholera toxin exhibited its usual lag phase. The model proposed by these workers to account for their observations is described in the following section.

The results of this period can be conveniently summarised as follows. Binding of cholera toxin to cells is via Subunit B of the toxin interacting with the ganglioside Gm_1 on cell surfaces. There then follows a lag phase during which no cyclase activation occurs and in which extensive washing of the cells or addition of antitoxin cannot prevent the subsequent activation process. In broken cell systems the lag phase is abolished. Subunit A, and specifically peptide A_1, is the active moiety of the toxin which, in lysed cell systems, activates adenylate cyclase immediately upon addition. Addition of Subunit A to intact cells can result in cyclase activation although perhaps not in all cases. Subunit B of the toxin is devoid of activity, can inhibit the action of toxin on intact cells, and is required only for the initial attachment of cholera toxin to cell surfaces.

1.10. The nature of the lag phase and theories of toxin action.

Since there is no known instance of cholera toxin activation of adenylate cyclase in intact cells occurring without a lag phase, the length of which varies widely depending on cell type, this unique characteristic must be an integral part of any proposed theory for the mechanism of toxin action. (Even in the experiements referred to above in which subunit A of the toxin was reported to have an

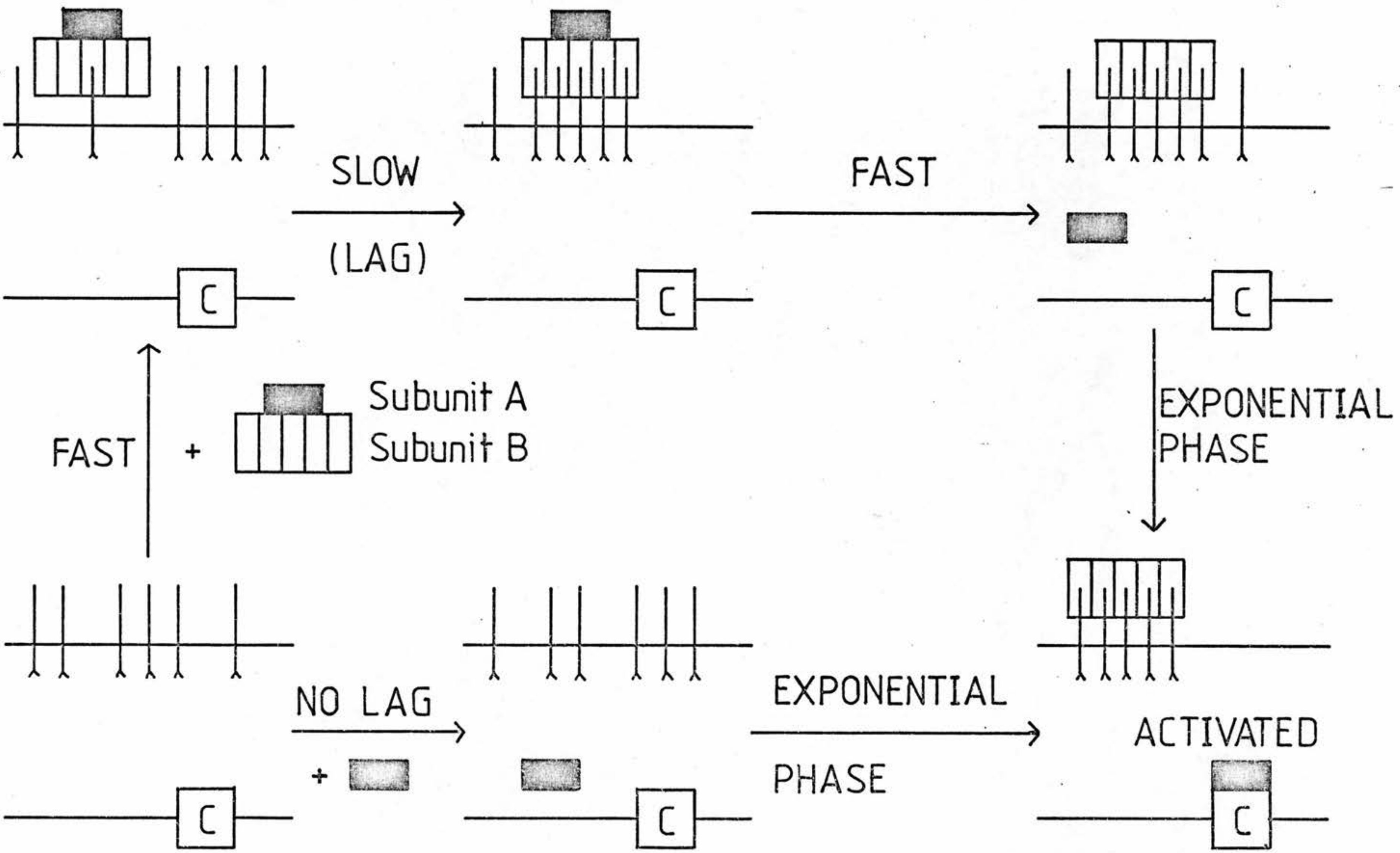
immediate effect on intact cells, the whole toxin still exhibited the usual lag phase).

Cuatrecasas and his group have developed a model to explain the action of cholera toxin, in which lateral mobility of toxin-receptor (Gm_1) complexes in the plane of the plasma membrane is the central idea (Sahyoun and Cuatrecasas, 1975). In this model, depicted in Fig. 1.3., cholera toxin is proposed to bind initially and rapidly through one of its B subunits to a molecule of Gm_1 on the cell surface, an event which results in the lateral movement of the toxin- Gm_1 complex in the plane of the plasma membrane. During this period of lateral mobility, further attachments between the toxin and the cell membrane are established through the remaining B subunits, by collisions with other ganglioside molecules. It was suggested that active subunit A of the toxin could be released only on the attainment of such multivalent attachment sites, the time taken to achieve this constituting the lag phase. The final establishment of the correct configuration then destabilises the toxin molecule resulting in the release of subunit A and its spontaneous incorporation into the lipid bilayer where it is free to interact directly with adenylate cyclase in a 1:1 association, causing activation of the enzyme. Their observation that subunit A of the toxin activated adenylate cyclase in intact fat cells without a lag phase, was accounted for in this model by supposing that the rather hydrophobic nature of the subunit allowed it to penetrate lipid bilayers spontaneously and unaided, causing immediate cyclase activation.

Figure 1.3. Binding model of cholera toxin action after Sahyoun and Cuatrecasas (1975)

C : Adenylate cyclase

| : Gm_1 Ganglioside


SLOW
(LAG)
FAST
C
Subunit A
Subunit B
FAST
+
EXPONENTIAL
PHASE
NO LAG
+
EXPONENTIAL
PHASE
ACTIVATED

Much evidence in support of this model was collected by these workers, and their results can be summarised as follows:

(i) Lateral mobility of toxin-receptor complexes was observed on the surfaces of rat lymphocytes using fluorescin-labelled cholera toxin and treatments which prevented this movement, also inhibited toxin action (Craig and Cuatrecasas, 1975).

(ii) Correlations were observed between the temperature dependence of toxin action and of membrane fluidity (Bennett and Cuatrecasas, 1975).

(iii) The duration of the lag phase was independent of the toxin concentration (Craig and Cuatrecasas, 1976).

(iv) Cyclase activation by cholera toxin occurred in the presence of various metabolic inhibitors and 'negligible' intracellular levels of the toxin were detected during the activation process (Bennett and Cuatrecasas, 1975).

(v) Adenylate cyclase activity could be precipitated with antibodies against subunit A of cholera toxin, provided that cyclase activation had occurred (Sahyoun and Cuatrecasas, 1975).

(vi) Solubilised adenylate cyclase comigrated with ^{125}I on gel filtration after activation by ^{125}I-cholera toxin, and adenylate cyclase activity could be adsorbed on agarose-subunit A derivatives (Bennett et al, 1975a).

(vii) The kinetics of the activation process, studied in intact toad erythrocytes, were considered to be indicative of a bimolecular association between subunit A and adenylate cyclase (Bennett and Cuatrecasas, 1975).

At the same time, a completely different theory of toxin action was proposed by Gill (1975) who suggested that subunit A of cholera toxin was an enzyme which catalysed an unknown intracellular reaction leading to adenylate cyclase activation. (This was analogous to the known action of diphtheria toxin which catalyses the NAD^+-dependent ADP-ribosylation of elongation factor 2 : see Ch. 6.1.). The theory received considerable support from the demonstration that NAD^+ was a necessary cofactor for the toxin-induced activation of adenylate cyclase in lysed pigeon erythrocytes (Gill, 1975), an observation which has subsequently been confirmed in many broken cell systems including rat liver (Martin et al, 1977: Flores et al, 1976), various cancer cells (Bitensky et al, 1975; Wheeler et al, 1976), thyroid membranes (Mullin et al, 1976) and fibroblast membranes (Moss et al, 1976a). NAD^+ by itself was not the only necessary factor required, and ATP as well as one or more unidentified macromolecular cytosolic factors, were also implicated (Gill, 1975; 1976b)

This demonstration of an NAD^+ requirement highlights the good fortune (alluded to in the previous section) of the choice of pigeon erythrocytes as a tissue of study. Most cells contain endogenous NADase activities which rapidly hydrolyse cellular NAD^+ on lysis, and such tissues quickly become refractory to cholera toxin. Zieve et al (1970) were the first workers to describe activation of adenylate cyclase by cholera toxin in a broken cell system (rat liver homogenate), but because the observed activation was only very slight - undoubtedly due to NAD^+ depletion (see Chs. 5 and 6)- the observation received little attention and was not investigated further. Pigeon erythrocytes, unlike most cells, completely lack endogenous NADase activities and intracellular levels of NAD^+ in these cells are sufficient to allow consistent effects of cholera toxin to be observed after lysis. In order to demonstrate the NAD^+ requirement, endogenous NAD^+ was first destroyed by addition of an insoluble NADase (Gill, 1975).

Further support for the enzymic theory of toxin action comes from the observed kinetics of adenylate cyclase activation in broken cell systems, which show typical enzymic characteristics of temperature dependence and linearity with time and with subunit A concentration (Gill, 1976b). Cyclase activation can also occur with extremely small amounts of cholera toxin, perhaps as little as 1 molecule per cell, and this in itself argues strongly against a 1:1 binding mechanism. In this respect the observations of Cuatrecasas and coworkers that negligible amounts of ^{125}I-cholera toxin are detected inside cells during cyclase activation, is of no consequence to an enzymic theory of toxin action, since the necessary quantities

of a catalytic subunit A to fully activate adenylate cyclase are probably too small to be detected with conventional techniques.

To a certain extent, both of these early theories can now be combined in a unifying hypothesis, the model of Cuatrecasas being used to explain the lag phase and that of Gill to explain the subsequent events occurring after penetration of the membrane by subunit A. All of the data reported by Cuatrecasas concerning membrane fluidity and the dependence on this of toxin action, can easily be incorporated into a theory in which the final event at the end of the lag phase is the penetration of subunit A to the cell interior where it catalyses an enzymic reaction, rather than penetration into the membrane and direct interaction with adenylate cyclase. Similarly, the kinetic results of Cuatrecasas, which he considered to be suggestive of a bimolecular association between toxin and adenylate cyclase, were obtained with intact toad erythrocytes in which the complex events leading to penetration of the membrane by subunit A may have masked the true kinetics of a toxin-catalysed process within the cell. The kinetics of the activation process in broken cell systems are undoubtedly characteristic of an enzyme (Gill, 1976b).

The evidence of Cuatrecasas in support of a direct association between subunit A and adenylate cyclase is perhaps more difficult to reconcile with this unifying hypothesis. However, in almost all cases, the effects observed in these experiments were extremely small and the hydrophobic nature of subunit A may have produced some non-specific adsorptive effects. Furthermore, if

subunit A is an enzyme, one of its substrates is presumably a part of the adenylate cyclase complex, and it is perhaps not surprising that some degree of affinity exists between subunit A and adenylate cyclase if this is analogous to the affinity displayed by an enzyme for its substrate.

In this unifying hypothesis therefore, the lag phase of cholera toxin action is accounted for by the time taken for the toxin to establish multivalent attachment sites on the cell surface favouring the release and penetration of subunit A through the membrane. In this process, lateral mobility of toxin - Gm_1 complexes in the plane of the membrane is probably an important consideration and low temperatures or inhibitors of membrane fluidity, by inhibiting lateral mobility, would prevent toxin action. Speculations as to the possible mechanisms by which subunit A penetrates the lipid bilayer have been discussed by Gill (1976a).

Once inside the cell, subunit A acts as an enzyme and in an unknown NAD^+-dependent process, catalyses activation of adenylate cyclase. The observations of Sahyoun and Cuatrecasas(1975) relating to the activity of subunit A in intact cells without a lag phase, cannot be explained by this theory. The phenomenon, which invokes serious teleological questions as to why V. cholerae should elaborate a toxin with an apparently useless B subunit (especially when the structure of this subunit is so unusual and perhaps unique), may be due to some peculiarity of fat cells or of the particular experimental conditions employed by these workers. Most researchers now agree that subunit B of cholera toxin has the important function

of concentrating at a cell surface (via tight binding to Gm_1 gangliosides) an active subunit A, thereby enabling an unlikely event (penetration of a large molecule through a lipid bilayer) to become more likely.

One important aspect of toxin action has recently come into some doubt. It has been considered probable (and has been assumed for convenience in the above discussion) that subunit A must dissociate from the rest of the toxin molecule before it can activate adenylate cyclase, an idea that has received support from the observation that treatments which favour dissociation of subunit A (and of the active species, peptide A_1), such as preincubation in the presence of low concentrations of SDS and thiols, enhance the subsequent ability of the toxin to activate adenylate cyclase in broken cell systems (van Heyningen, 1976a; Gill, 1976b). However, van Heyningen, using cross-linked cholera toxin in which all subunits of the toxin were attached covalently to each other in an A_1-A_2-5B complex (by treatment with the bifunctional reagent N, N' - bis (carboximidomethyl) tartaramide dimethyl ester dihydrochloride), demonstrated that this material was still able to activate adenylate cyclase, not only in broken cells, but also in intact pigeon erythrocytes (van Heyningen, 1977b). Activation proceeded with the same characteristics as seen with untreated toxin and in the experiments with intact cells, was shown not to be due to a small degree of cell lysis, or to a small amount of un-crosslinked toxin. The inevitable conclusion was that subunit A did not have to dissociate from the rest of the toxin before its activity could be expressed, and at first glance the results appear to contra-indicate

an enzymic mechanism of action for subunit A inside the cell. However, as suggested by the author, it may simply be sufficient that subunit A be exposed on the cytoplasmic face of the plasma membrane where it can still engage in a catalytic reaction while bound to the B subunits on the cell suface. Another possibility is that intracellular proteases may be able to cleave some or all of the subunit at the inner face of the membrane which could then dissociate from the rest of the molecule and enter the cytosol completely. This latter possibility is supported by the observation that at least one cell type, sarcoma 180 cells, can elaborate proteases which are capable of cleaving cholera toxin into fragments (many of which are smaller than subunit A itself) which are still able to activate adenylate cyclase (Matuo _et al_, 1976).

Investigations carried out concurrently with the work described in this report have now made an enzymic mechanism of toxin action almost undeniable and the major conclusions arising from these studies are summarised in Ch. 1.12. Before describing these results however, it is helpful to consider some aspects of the enzymology of adenylate cyclase.

1.11. Enzymology of adenylate cyclase

Adenylate cyclase, a complex regulatory enzyme occurring as an integral protein in cellular plasma membranes, is responsible for the formation of cAMP from ATP:

$$ATP \rightleftharpoons cAMP + PPi$$

The true substrate is in fact ATP-Mg^{++} (Perkins; 1973) and some of the many techniques which have been employed for the difficult assay of this enzyme are discussed in Ch. 3.3.

Adenylate cyclase normally displays low levels of activity and in some tissues, such as pigeon erythrocytes, these levels are only just detectable with sensitive assay methods. The activity of the enzyme under these conditions is usually referred to as 'basal activity'. This activity can be stimulated by a variety of agents including many of the catecholamine, peptide and glycopeptide hormones, prostaglandins, and cholera toxin. Additionally other agents, such as fluoride and other halide anions, and guanylnucleotides, are also capable of stimulating basal adenylate cyclase activity. The latter group of compounds, which are becoming increasingly implicated in the regulatory mechanisms of adenylate cyclase, appear to play a central role in mediating the action of many stimulatory agents on the enzyme.

Guanylnucleotides were first implicated as regulators of adenylate cyclase activity by Rodbell _et al_ (1971a; 1971b), when they were reported to enhance glucagon-stimulated cyclase activity

in liver plasma membranes. Soon afterwards, many hormone-responsive adenylate cyclase systems were found to be under the regulatory control of these nucleotides (Goldfine et al, 1972; Krishna et al, 1972; Wolff and Cook, 1973; Bockaert et al, 1972; LeRay et al, 1972; Harwood et al, 1973).

In a report published in 1974, Londos and coworkers introduced an analogue of GTP in which the terminal -P-O-P- bonds were replaced by -P-N-P, and this compound, 5'-guanylylimidodiphosphate (Gpp (NH)p), was shown to be a potent activator of adenylate cyclase systems (Londos et al 1974). Gpp(NH)p was studied further by Pfeuffer and Helmreich (1975), along with two other guanyl-nucleotide analogues:

(i) 5'-guanylylmethylenediphosphonate (Gpp(CH_2)p)- with a terminal -P-C-P- grouping.

(ii) Guanosine 5'-O-(3-thiotriphosphate) ($GTP_{\gamma}S$)- with a terminal sulphur on the γ-phosphorus atom.

All three analogues, which are characterised by their relative resistance to hydrolysis by intracellular GTPases, caused marked elevation of adenylate cyclase activity when added by themselves, and induced enhanced responsiveness to isoprotorenol when substituted for GTP in incubations with the hormone (Pfeuffer and Helmreich, 1975). These actions of the GTP analogues, which closely resemble those of cholera toxin (see following section), have since been confirmed in most cell systems studied (Lefkowitz, 1974; Lereau et al, 1978; Spiegel et al, 1977; Palmer and Palmer, 1978; Ignarro and Grass, 1978; Young and Stansfield, 1978; Fischer and

Sharp, 1978). The fact that the GTP analogues were not metabolised or bound covalently to adenylate cyclase (Pfeuffer and Helmreich, 1975) ruled out any possibility of phosphorylation or guanylylation of the cyclase as a regulatory mechanism. Later, Spiegel et al (1977), using another GTP analogue - Guanosine-5'-α,β-methylenetriphosphate (in which a $-CH_2-$ group occurs between the α and β phosphorus atoms) - also ruled out pyrophosphorylation as a possible mechanism in guanylnucleotide action.

Pfeuffer and Helmreich (1975) also reported the separation of a guanylnucleotide binding protein from solubilised preparations of pigeon erythrocyte membranes, and presented evidence for the importance of this fraction in conferring hormone responsiveness to adenylate cyclase. The authors speculated that adenylate cyclase in the membrane is normally in an inhibited state and that inhibition is released by the interaction of guanylnucleotides with a regulatory subunit. This latter entity would correspond to the 'transducer' element postulated earlier by Rodbell's group as being an intergral component of hormone-receptor-adenylate cyclase interactions (see Birnbaumer, 1973). In a series of papers these workers went on to demonstrate the existence of specific GTP-binding sites in plasma membranes and presented evidence for the occurrence of multi-site transition states involving guanylnucleotides, in the stimulation of adenylate cyclase by hormones (Rodbell et al, 1974; Salomon and Rodbell, 1975). These observations resulted in the demonstration that GTP affects hormone receptor conformation and adenylate cyclase activity through independent guanylnucleotide sites with different properties, and that the hormone receptor is a separate entity,

distinct from adenylate cyclase (Lad _et al_,1977; Welton _et al_, 1977). Binding of GTP to the nucleotide site associated with the receptor (N_1) was shown to alter the conformation of the receptor in such a way as to increase its affinity for glucagon, a process that probably involves dissociation of the receptor-nucleotide binding site complex. The resulting hormone-receptor complex was then proposed to bind uniquely to adenylate cyclase only when the nucleotide site associated with the cyclase (N_2) was occupied by GTP, the resulting 'coupled' adenylate cyclase forming a high affinity receptor state, stabilised by the hormone, and exhibiting a high V_{max} for the catalysis of cAMP production. These interrelationships are illustrated diagramatically in Fig. 1.4.

The possible role of an adenylate cyclase-associated GTPase activity in the regulation of this enzyme, suggested by some of the observations noted above, received direct support from the results of Cassel and Selinger (1976) who described the stimulation of such an activity in turkey erythrocyte membranes by adrenalin. They distinguished two types of GTPase activity in these membranes; one, a non-specific or basal GTPase which operated in the absence of hormone effectors and was apparently unconnected to adenylate cyclase regulation, and another which only became apparent on addition of adrenalin and which exhibited a high specificity and affinity for GTP. Levinson and Blume (1977), in a detailed study of the effects of guanylnucleotides and divalent cations on the activity of adenylate cyclase, also implicated the importance of a Mg^{++}-dependent GTPase activity in the regulatory processes. The studies with cholera toxin described in the following section have helped to clarify the role of guanylnucleotides in the regulation of adenylate cyclase.

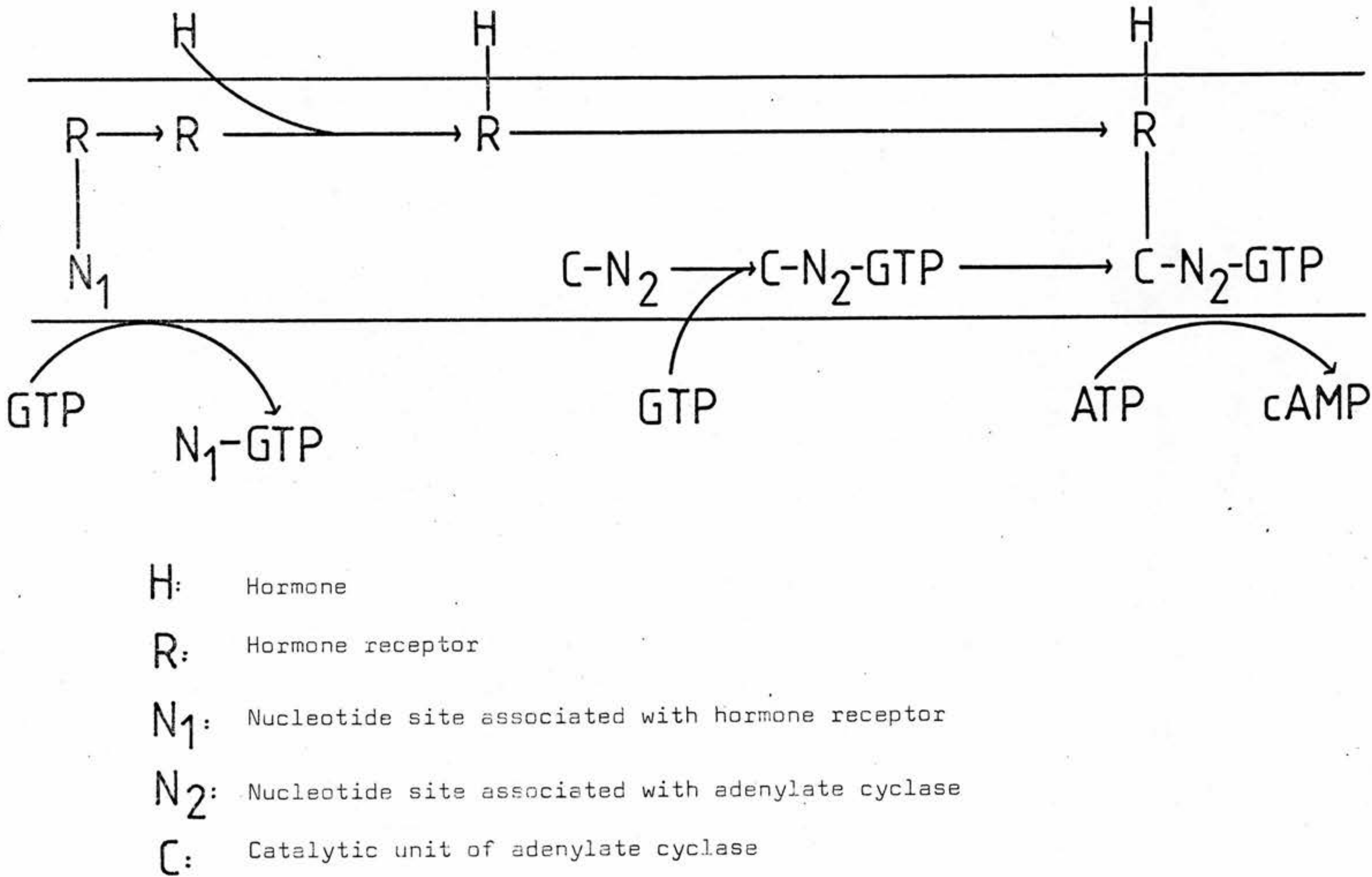


Figure 1.4. Hormone-Receptor-Adenylate Cyclase Interactions

1.12. Effects of cholera toxin on hormone-responsive adenylate cyclase systems and the molecular mechanism of toxin action.

Several studies on the kinetics of adenylate cyclase after activation by cholera toxin have indicated that toxin stimulation occurs without a change in the apparent K_m of the cyclase for its substrate ATP-Mg^{++}, and that activation is best described by an increase in the V_{max} of the enzyme (Franks, 1976; Glossmann and Struck, 1977). However several other properties of the enzyme are altered dramatically after toxin stimulation. Generally, toxin-treated adenylate cyclase displays enhanced responses to a variety of hormones. For example, the toxin caused an apparent increase in the sensitivity of turkey erythrocytes to catecholamines (Rudolph et al, 1977) and increased the apparent affinity of isoprotorenol, ACTH, glucagon, and vasoactive intestinal polypeptide (VIP) for cyclase activation in rat adipocytes (Bennett et al,1975b). Field (1974) has also reported an increase in the maximum adrenalin-stimulatable activity of adenylate cyclase after toxin-treatment of turkey erythrocytes, and adenylate cyclase responses to prostaglandin E_1 in liver were enhanced after prior incubation with cholera toxin (Su et al, 1976). In intestinal mucosa however, the prostaglandin response was unaltered by cholera toxin (Kimberg et al, 1971, 1974.)

The above are only a small selection of the recorded hormonal enhancement effects of cholera toxin and a more comprehensive list of references has been compiled by Gill (1977). It is interesting to note that at least some of these effects may be of direct relevance to

clinical cholera. VIP for example is capable of activating adenylate cyclase in intestinal mucosa (Simon and Kather, 1978) where it may have an important physiological role in the regulation of intestinal fluid secretion. The increased responsiveness of adenylate cyclase to VIP stimulation after toxin-treatment of fat cells was also observed in intestinal mucosal cells (Bennett et al, 1975b), and these workers suggested that enhanced VIP responsiveness may at least contribute to the fluid losses observed in clinical cholera.

Contrary to its effect on hormonal responses, cholera toxin treatment often results in a decreased ability of adenylate cyclase to respond to stimulation by fluoride ions (Field, 1974; Petrella and Zenser, 1976), and Field (1974) has suggested that adenylate cyclase in the turkey erythrocyte membrane may exist in two interconvertible forms; one which is catecholamine-responsive but fluoride-insensitive, and another which is fluoride-sensitive but not coupled to β-adrenergic receptors. In this model cholera toxin would stabilise the cyclase in its catecholamine receptor-coupled form, thereby explaining the toxin-induced enhancement of hormonal responsiveness at the expense of fluoride sensitivity.

In the enhancement of catecholamine responsiveness, cholera toxin apparently does not increase either the number or affinity of β-adrenergic receptors, since the binding characteristics of (-) alprenolol (a β-blocker), and its displacement by isoprotorenol, were unchanged after toxin treatment (Rudolph et al, 1977). The observations of Katz and Greenough (1975) also suggest that cholera toxin does not interact with hormone receptor sites.

Propanolol, a β-blocker, inhibited adrenalin-stimulation of adenylate cyclase in isolated fat cells but had no effect on cholera toxin action. Conversely, choleragenoid, which inhibited the action of toxin, did not alter cyclase activation by adrenergic agents. A complete α and β receptor blockade by combined treatments with propanolol and adrenalin (the latter also interacts with α-adrenergic receptors) did not inhibit the action of cholera toxin, suggesting that no relationship or interactions existed between the toxin receptor and either α or β-adrenergic receptors. In the enhancement of peptide hormone responsiveness on the other hand, cholera toxin has been reported to increase the apparent affinity of several hormones for their receptors, and to decrease the rate of dissociation of ^{125}I-glucagon from rat hepatocytes (Bennett et al, 1975b).

The first clue as to the detailed mechanism of cholera toxin action was provided by Cassel and Selinger (1977). After their previous demonstration that catecholamines induced a specific adenylate cyclase-associated GTPase activity in pigeon erythrocyte membranes (Cassel and Selinger, 1976), the same authors reported that cholera toxin selectively inhibited about 80% of the catecholamine-stimulated GTPase while having no effect on the basal GTPase activity. As the concentration of cholera toxin was increased, catecholamine-stimulated GTPase activity was progressively inhibited, and both basal and isoprotorenol-stimulated adenylate cyclase activities increased in parallel. The authors reasoned therefore that if cholera toxin action was dependent upon the inhibition of a cyclase-associated GTPase activity, then after cholera toxin treatment, GTP should act in the same manner as the non-hydrolysable analogue Gpp (NH)p in causing

high and persistent activation of the enzyme. Experimental data were completely in agreement with this hypothesis and isoprotorenol+ GTP synergistically activated adenylate cyclase in toxin-treated membranes to the same extent as isoprotorenol + Gpp (NH)p in untreated membranes (Cassel and Selinger, 1977).

On the basis of these observations, a model was proposed for adenylate cyclase regulation in which the guanylnucleotide binding site (the N_2 of Rodbell and coworkers) plays a dual role as depicted in Fig. 1.5. In the presence of hormone and GTP, adenylate cyclase is activated through an increased affinity for GTP at the regulatory site. The active adenylate cyclase is then returned to its inactive state by hydrolysis of GTP at the same site - 'the turn-off GTPase.' Thus hormones act by continually generating an active cyclase species through an increased affinity for GTP, and the activation induced by hormones can only be transitory because of the continual regeneration of the inactive cyclase through the internal GTPase reaction. The extent of cyclase activation induced by a particular hormone will presumably be dependent upon the extent of increased affinity for GTP and will be limited by the rate of the GTPase reaction. Since cholera toxin inhibits the catecholamine-induced GTPase activity, it was proposed that the toxin catalysed an irreversible covalent modification of the guanylnucleotide site which prevents GTP hydrolysis and thereby 'traps' the adenylate cyclase in its active state. Gpp(NH)p and similar non-hydrolysable GTP analogues mimic the action of cholera toxin by binding to the guanylnucleotide regulatory site in the same way as GTP, but because of their subsequent inability to participate in the GTPase reaction, the cyclase is again

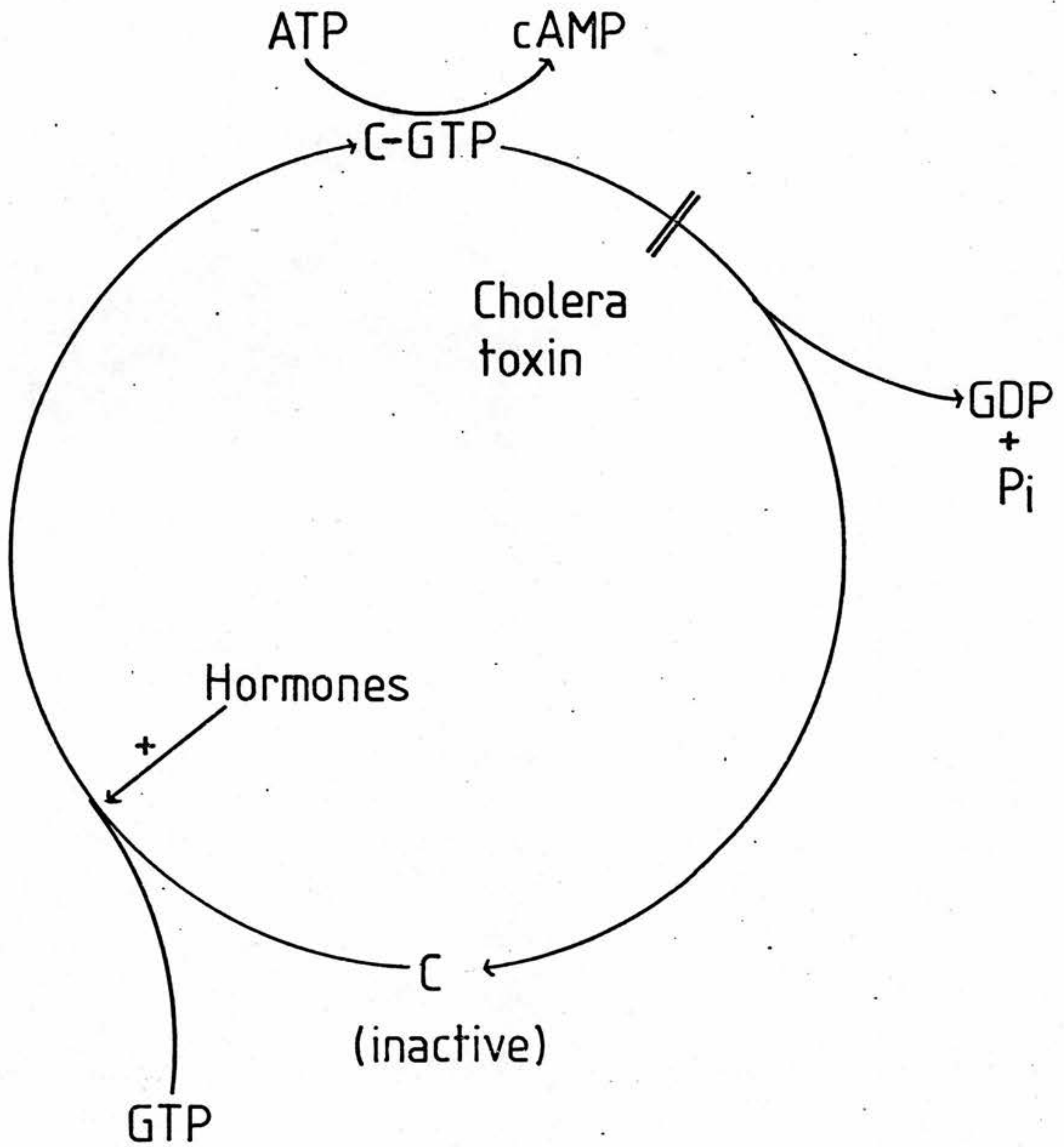


Figure 1.5. The regulatory GTPase cycle of adenylate cyclase after Cassel and Selinger (1977).

trapped in its active state. The model can also explain the enhancement of hormonal effects by cholera toxin. After toxin-treatment the hormone-induced binding of GTP to the regulatory site will generate the active form of the enzyme and because the GTPase is inhibited, this form will accumulate to an extent not normally seen in the presence of hormone alone and the stimulatory effects of the latter will therefore be magnified.

The above model of toxin action implies that GTP itself must be a necessary cofactor for cholera toxin action, while in few previous experiments was GTP knowingly added to cholera toxin incubations with membranes. However the guanylnucleotides in general are effective in extremely low concentrations, and the success of previous studies probably reflects either that sufficient endogenous GTP was present, or that sufficient GTP had in fact been added along with commercial ATP preparations, in which GTP is a common contaminant (Kimura et al, 1976). That GTP is in fact essential for toxin stimulation of adenylate cyclase was demonstrated by Lin et al (1978) who observed that if careful steps were taken to remove all traces of endogenous GTP, and if highly purified ATP preparations were employed during assay, then toxin-stimulation of cyclase activity could only be expressed if GTP was added after the toxin treatment. Since GTP was only required after toxin treatment, and addition of GTP after removal of toxin by extensive washing could still elicit high and persistent activation, the nucleotide is not strictly a cofactor for the modification of adenylate cyclase by cholera toxin, but in the absence of GTP, this modification cannot be expressed as increased adenylate cyclase activity.

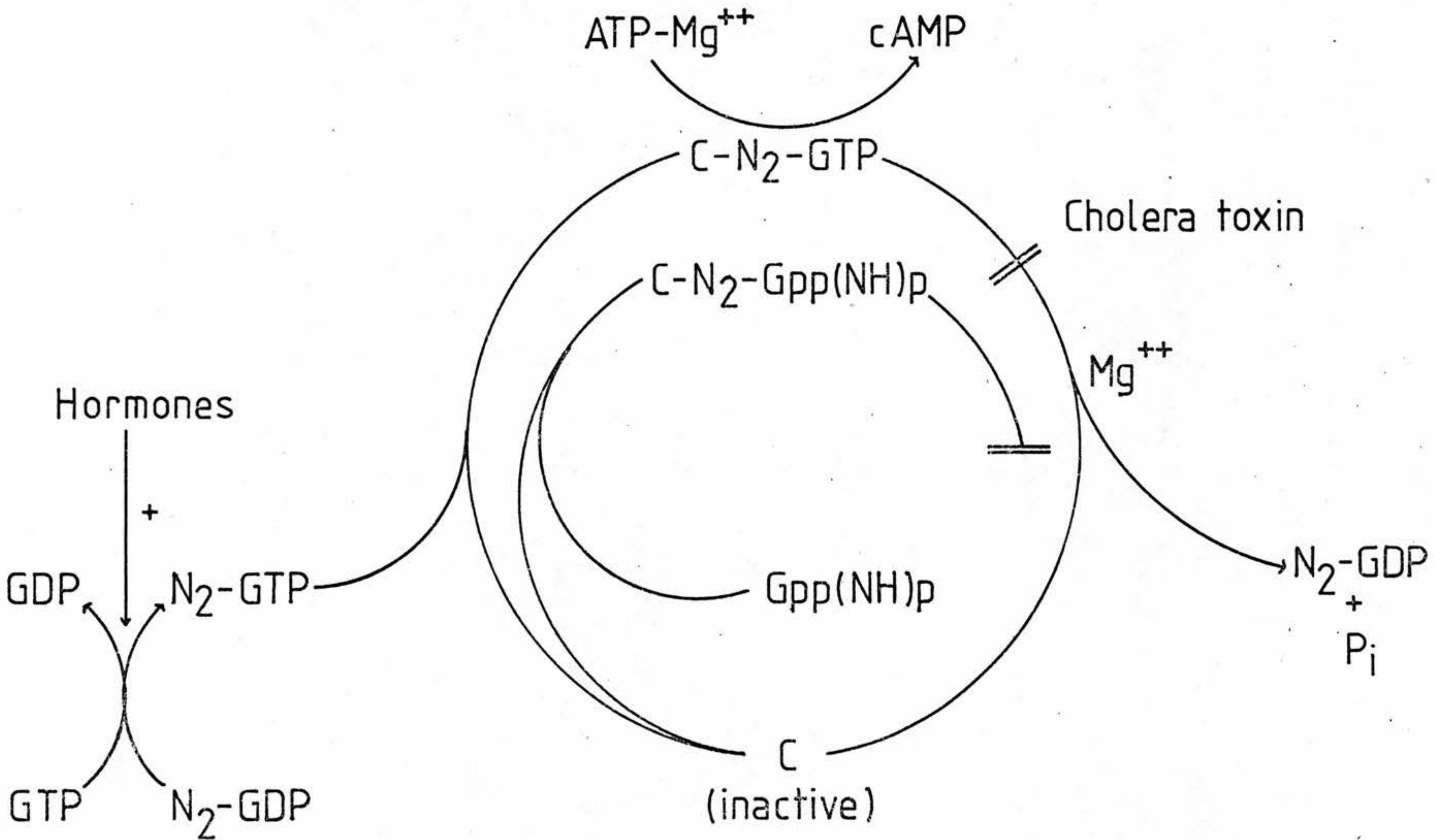
Direct evidence in support of this model of toxin action was provided by the combined observations of Pfeuffer (1977) and of Cassel and Pfeuffer (1978). The former author, in an extension of his previous studies (Pfeuffer and Helmreich, 1975), had described dissociation and purification by affinity chromatography of a 42,000 dalton guanylnucleotide binding protein from solubilised preparations of pigeon erythrocyte membranes. The latter workers then demonstrated the specific incorporation of $[^{32}P]$ from NAD^+ radioactively labelled in the ADP-ribose moiety, into a 42,000 dalton protein from the same membranes, catalysed by cholera toxin. The labelled protein possessed a GTP-binding activity and evidence was presented to show that the covalent modification of this protein by cholera toxin was probably an ADP-ribosylation and that this modification was responsible for the toxin-induced activation of adenylate cyclase.

At about the same time, Gill and Meren (1978), also using pigeon erythrocytes, obtained essentially similar results although in this case, there were substantial toxin-specific ADP-ribosylations of several other proteins. Nevertheless, the 42,000 dalton peptide was the major product and was ADP-ribosylated before all others. To date, similar experiments have been conducted in mouse S49 lymphoma cells, turkey erythrocytes, human erythrocytes (Kaslow et al, 1979) and rat adipocytes (Malbon and Gill, 1979), and in all cases, a 42,000 dalton GTP-binding protein has been the major substrate for ADP-ribosylation by cholera toxin.

From several recent observations (Levinson and Blume, 1977; Cassel and Selinger, 1978), it seems likely that the GTPase activity does not result in the dissociation of GDP from the guanylnucleotide site, since catecholamines were shown to induce the displacement of bound GDP by GTP. The regulatory GTPase cycle of adenylate cyclase is therefore probably more accurately represented as shown in Fig. 1.6. This diagram also takes account of the possibility that the GTPase cycle involves an association-dissociation step in which the guanylnucleotide binding protein with bound GTP only acquires a GTPase activity when in association with the catalytic unit of adenylate cyclase (Ross and Gilman, 1977 , Levinson and Blume, 1977; Johnson et al, 1978a; 1978b). After GTP hydrolysis, the guanylnucleotide binding protein-GDP complex dissociates, and reassociation with adenylate cyclase occurs only after displacement of bound GDP by GTP, a process which is stimulated by hormones.

Since cholera toxin can activate adenylate cyclase in the absence of hormones, it follows that the GTPase cycle must be in continual operation with the enzyme oscillating between active and inactive states, although to date it has not been possible to detect a cyclase-associated GTPase activity in the absence of hormones. This may simply reflect the fact that in the basal activity state, the rate of the internal GTPase may be so low as to be undetectable with conventional techniques. If the cycle is in continual operation then, in an analogous fashion to the 'futile cycle' of fructose diphosphatase where the continual hydrolysis of ATP is the price paid for a sensitive control mechanism, GTP hydrolysis may be the expense incurred in maintaining adenylate cyclase under strict and

Figure 1.6. Regulation of adenylate cyclase activity by hormones and guanylnucleotides and the mechanism of cholera toxin action.


ATP-Mg++
cAMP
C-N2-GTP
Cholera toxin
C-N2-Gpp(NH)p
Mg++
Hormones
+
GDP
N2-GTP
Gpp(NH)p
N2-GDP
+
Pi
GTP
N2-GDP
C
(inactive)

sensitive regulatory control.

The current state of knowledge on the interaction of hormone receptors with adenylate cyclase, on the action of cholera toxin, and on the role of guanyl-nucleotides in these events has been discussed recently by Cassel et al (1977), Pfeuffer (1979) and Levitzki and Helmreich (1979).

1.13. Enzymic activities of cholera toxin

From the previous section, it is clear that there is now good evidence to suggest that cholera toxin catalyses the NAD^+ - dependent ADP-ribosylation of a guanylnucleotide binding protein associated with adenylate cyclase. However these observations have only recently come to light and for several years prior to this, and since the demonstration of an NAD^+ requirement for toxin action by Gill (1975), various attempts were made to establish the enzymic nature of cholera toxin, including the experiments reported in Ch. 5. of this work. Since the cofactor requirement was for NAD^+ and since diphtheria toxin was already known to have certain structural similarities with cholera toxin and to catalyse the ADP-ribosylation of elongation factor 2 (see Ch. 6), it was reasonable to postulate that a similar enzymic activity may be a feature of cholera toxin. Nevertheless, attempts by Gill (1975) to deomonstrate cleavage of NAD^+ in pigeon erythrocyte lysates in the presence of cholera toxin were unsuccessful. In these experiments Gill used concentrations of toxin which, although sufficient to cause full adenylate cyclase activation, in retrospect were probably insufficient to demonstrate detectable cleavage of NAD^+.

The first reported enzymic activity of cholera toxin was the observation by Moss et al (1976b) of a toxin-associated NADase activity. The authors demonstrated hydrolysis of NAD^+ by cholera toxin which was dependent on time and on the concentration of toxin, although activity could only be easily observed with extremely large amounts of toxin (100 to 500 µg/ml), and only in the presence of certain buffers. NADase activity was detected in sodium acetate, pH 6.2 but not in Tris/HCl, pH 8.0, glycine/HCl, pH 8.0 or 8.5, or hydrazine/HCl, pH 9.5. The use of phosphate buffers produced the highest NADase activities and surprisingly, activity was stimulated 10-fold by increasing the potassium phosphate concentration from 50mM to 400mM. All of the phosphate buffers described were used at pH 7.0.

NADase activity was also dependent on the presence of DTT in high concentrations (20mM) and the authors suggested that this dependence may reflect a necessity for dissociation of Subunit A or peptide A_1, before expression of activity. The effects of phosphate and acetate in high concentrations were explained in a similar fashion. Subunit A of the toxin was shown to be the active species and NADase activity comigrated with the toxin on polyacrylamide gel electrophoresis and on gel filtration with Biogel P-60. The calculated K_m of the toxin for NAD^+ was 3.8 mM.

Because diphtheria toxin was known to catalyse the slow hydrolysis of NAD^+ (a reflection of its true ADP-ribosyltransferase activity), the demonstration of a cholera toxin-associated NADase activity was suggested to be an entirely analogous situation and was therefore investigated further by the same workers. In a subsequent

paper (Moss et al, 1977b) ganglioside Gm_1 (and to a lesser extent Gm_3, Gm_2 and Gd_{1a}) were shown to inhibit the NADase activity of both intact cholera toxin and of subunit A, a surprising result since subunit A exhibits no affinity for, or interactions with, gangliosides (CH.1.9.) Various other compounds also inhibited the reaction, of which adenine and adenosine were the most effective. The relative potencies of the inhibitors tested were essentially similar to those found earlier with diphtheria toxin, with the important difference that K_i values for inhibition of the NADase activity of cholera toxin were always 1 to 2 orders of magnitude higher than those reported for diphtheria toxin (values ranged from 3mM for adenine to 160mM for nicotinamide mononucleotide). The authors pointed out that this difference may have been due to the higher salt concentrations employed in their assays which routinely contained 200 to 400 mM-potassium phosphate.

Since cholera toxin had been shown to possess an NADase activity, it was logical to investigate the possibility that it could ADP-ribosylate as well. Moss and Vaughan (1977) reported enhancement of the NADase activity by L-arginine, D-arginine, and the most effective compound, L-arginine methyl ester (ARG-OMe). In the presence of 75mM ARG-OMe NADase activity was elevated about 5-fold, and again maximal activity was observed, both in the presence and absence of ARG-OMe, in phosphate buffers where activity increased dramatically with increasing phosphate concentration. Direct evidence for an ADP-ribosyltransferase activity of cholera toxin was also presented. After incubation of the toxin in the presence of [adenine U - ^{14}C] NAD^+ and [^3H] arginine, a reaction product, containing both [^3H] and [^{14}C]

in the ratio to be expected in an ADP-ribose-arginine adduct, was isolated by thin layer chromatography.

Some of the results reported in Ch. 5. of this work argue against the validity of drawing meaningful conclusions about the action of cholera toxin from experiments of the above type. A full discussion of these arguments is presented in Ch. 6. but it should be noted at this stage that, unlike studies on the NADase activity of diphtheria toxin where results are generally of direct relevance to the action of the toxin as an ADP-ribosyltransferase, there are few observations arising from studies on the NADase activity of cholera toxin which can be related to the action of this toxin on adenylate cyclase. For example the K_m value for NAD^+ (3.8mM) is about four times greater than the concentration of NAD^+ with which maximum cyclase activation is usually observed in broken cell systems, and the dependence of the NADase reaction on unusually high concentrations of particular salts, especially phosphate, has no similarities to the toxin's action on adenylate cyclase which can occur in many different simple buffer systems. Similarly the inhibition of NADase activity by gangliosides is difficult to reconcile with the ineffectiveness of these molecules in inhibiting the action of either intact toxin or of subunit A in broken cell systems. Probably, as suggested by van Heyningen (1977a), this is a non-specific effect as is often seen with gangliosides, particularly when present at the high concentrations (50μM) used in this work: the critical micellar concentration of ganglioside Gm_1 has been reported to be around $1\text{-}2 \times 10^{-10}$M or less (Formisano <u>et al</u> 1978).

Finally, the concentrations of the substances tested for enhancement of NADase activity were extremely high (75mM) making it difficult to derive any meaningful comparisons to the action of cholera toxin on adenylate cyclase, either in vivo or in vitro.

Nevertheless there are some interesting observations related to the above work from which the latter derives some support, and which may have some relevance to the mechanism of toxin action. Trepel et al (1977), using similar incubation conditions to those of the above workers, demonstrated the self-ADP-ribosylation of cholera toxin in which subunit A acted both as catalyst and acceptor protein. About 0.8 pmoles of ADP-ribose were incorporated per pmole of toxin. In a completely unrelated and earlier peice of work, Holmgren and Lonnroth (1976) described several treatments which inhibited the action of cholera toxin in mouse thymocytes, one of which was the chemical modification of the toxin with arginyl-directed agents (1,2-cyclohexanedione and phenanthrenequinone).

Self-ADP-ribosylation is a reaction also catalysed by diphtheria toxin. Again, this occurs at a much slower rate than the physiologically significant reaction and is not considered to be of any importance in the toxin's action on elongation factor 2. Similarly Trepel and coworkers concluded that self-ADP-ribosylation of cholera toxin, although providing more similarities with diphtheria toxin, could not as yet be assigned any definite physiological significance. However it is not inconceivable that once inside a cell, the action of subunit A on adenylate cyclase may be enhanced, or even dependent upon, the initial self-ADP-ribosylation of the subunit using the cell's endogenous

stores of NAD^+. Since arginine was reported to enhance the NADase activity of cholera toxin, presumably by acting as an acceptor of ADP-ribose (Moss and Vaughan, 1977), and since toxin action is inhibited by specific arginyl-directed reagents (Holmgren and Lonnroth, 1976), it remains a possibility that self-ADP-ribosylation of an arginine moiety is of importance in the early stages of toxin action. Experimental investigation of such a hypothesis would be difficult, owing to the very small amounts of ADP-ribose incorporated into subunit A, but the use of $[^{32}P]$ NAD^+, instead of the $[^{3}H]$ NAD^+ employed by Trepel and coworkers, may facilitate such studies.

Moss and Vaughan (1978) have also been able to isolate a protein from turkey erythrocyte cytosol which is capable of ADP-ribosylating several purified proteins and of activating adenylate cyclase, albeit to a very small extent (about 1.5-fold in the presence of NAD^+ and ATP). The authors suggested that ADP-ribosylation of adenylate cyclase may already be a normal regulatory control mechanism within the cell which is exploited to its fullest extent by cholera toxin. If this were true, one would expect to find activities capable of removing the ADP-ribose residues so incorporated. For cholera toxin at least however, no cellular factors capable of reversing the toxin-induced modification of adenylate cyclase have as yet been detected.

1.14. Aims of the project

The aims of the work presented in this report can be summarised briefly as follows:

(i) to develop a simple method for the purification of cholera toxin from crude culture filtrates of V. cholerae.

(ii) to investigate the NADase activity of cholera toxin in relation to its effects on adenylate cyclase.

(iii) to investigate the possible enzymic nature of cholera toxin in activating adenylate cyclase in rat liver membranes, and in particular, to study the putative ADP-ribosylation.

The various assay systems which were set up for the above purposes are described fully in Ch. 3.

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Chapter 2 METHODS

2.1. Purification of cholera toxin

Crude culture filtrates of the bacterium *Vibrio cholerae*, obtained from the Wellcome Research Laboratories (see Appendix I), were the starting material for preparation of purified cholera toxin. The filtrates were centrifuged at 100,000g x 30 min. on a Beckman L2-65B ultracentrifuge and the supernatant, after dialysis against 50mM-Tris/HCl, pH 7.4, was subjected to the purification scheme outlined below. All operations were performed at $4^{o}C$.

The pellet from the 100,000g centrifugation, which probably consisted of dead bacteria and insoluble cell fragments, contained no cholera toxin antigen and was discarded.

2.1.1. Ammonium sulphate fractionation

Samples of culture filtrate supernatant were diluted 10-fold with 50mM-Tris/HCl, pH 7.4, and solid, finely ground ammonium sulphate was added slowly and with stirring to 60% of room temperature saturation. After stirring for 30 min, the mixture was centrifuged at 20,000g x 20min. in an MSE-18 high speed centrifuge, the supernatant was discarded, and the pellet resuspended in, and dialysed against, 50mM-Tris/HCl, pH 7.4.

2.1.2. DEAE-cellulose column chromatography

The dialysed ammonium sulphate fraction was applied to a 2.5 cm x 7 cm column of DEAE-cellulose equilibrated in 50mM-Tris/HCl, pH 7.4 and eluted overnight with 200ml of a linear 0-100mM NaCl gradient

at a flow rate of 15ml/hour. 3ml-fractions were collected and after gradient elution, the column was washed with 20ml of 2M-NaCl before being re-equilibrated in 50mM-Tris/HCl, pH 7.4.

Protein elution profiles were monitored by measuring the absorbance at 280 nm. of each fraction, and alternate fractions were tested for the presence of cholera toxin as described in Ch. 2.2.1. Fractions containing toxin were pooled, dialysed against 0.1M-Tris/HCl, 0.1M-NaCl, pH 7.4, and freeze-dried. The product was resuspended in a small volume of the same buffer.

2.1.3. Gel filtration on Ultrogel ACa-44

The resuspended fractions from the previous step were applied to a 2cm x 92 cm column of Ultrogel ACa-44 gel filtration medium, equilibrated in 0.1M-Tris/HCl, 0.1M-NaCl, pH 7.4, and eluted overnight with the same buffer at a flow rate of 5cm/hour. 2ml-fractions were collected and analysed as described above for the DEAE-cellulose column. Fractions containing cholera toxin were pooled, dialysed against 50mM-Tris/HCl, pH 7.4, freeze-dried and resuspended in a small volume of the same buffer. Purified toxin was stored in small aliquots at -20°C before use. Purification of the toxin by the above method is discussed fully in Ch. 4.

2.2. Immunological techniques

2.2.1. Ouchterlony immunodiffusion.

Qualitative detection of cholera toxin was routinely performed by testing for reaction against antitoxin using the technique of Ouchterlony immunodiffusion.

3.15g of noble agar and 12 mg of sodium azide were added to 60 ml of water. The mixture was heated in a boiling water bath until homogeneous, and 5 ml aliquots were pipetted into each of 12 vials. For use, 5 ml of 0.1M-Na_2PO_4, 1.8% NaCl, pH 7.0 were added to one vial and after heating in a boiling water bath until homogeneous, 2ml of the suspension were pipetted into each of five glass microscope slides (pre-washed in chromic acid). The agar was allowed to set and slides were stored in a sealed, humid container before use.

Using an Ouchterlony cutter, two rings of six wells each with a central well (all of 6 µl capacity) were cut on each slide Antiserum raised against cholera toxin in rabbits (see below) or purified antitoxin therefrom, was placed in the central wells and samples for analysis, in the surrounding wells. Diffusion was allowed to occur in a humid atmosphere overnight and toxin-antitoxin precipitates were viewed under a desk lamp on the following day. For strongly antigenic solutions, immunoprecipitates were visible after 3 to 4 hours diffusion time.

2.2.2. Preparation of cholera antitoxin

Antiserum against purified cholera toxin, prepared in rabbits as described earlier (van Heyningen, 1976a), was provided. For some experiments it was necessary to purify antitoxin IgG in order to remove contaminating enzyme activities in the crude antiserum.

The serum was dialysed overnight at room temperature against 18% Na_2SO_4, pH 7.0. Precipitated protein was collected by centrifugation at 1,200g x 10 min, the supernatant discarded, and the pellet resuspended in a small volume of water. After a further dialysis against 15% Na_2SO_4 the precipitate, containing antitoxin IgG, was collected by centrifugation as above and resuspended in a small volume of 50mM-Na_2PO_4, 0.9% NaCl, pH 7.0, Antitoxin was stored at -20^oC when not in use.

2.3. Electrophoretic techniques

2.3.1. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis.

5% and 10% polyacrylamide-SDS gels were prepared according to Weber et al (1972). Protein samples (20 - 100µl) were mixed with 5 µl of 50% 2-mercaptoethanol in water, 5µl of 1.0mM-phenylmethylsulphonylfluoride (PMSF) in propan-2-ol, and 5µl of 10% SDS. After incubation in a boiling water bath for 1 min. and cooling to room temperature, 5µl of 0.1% bromophenol blue marker dye were added, followed by one drop of glycerol. Samples were

applied to gels and electrophoresed at 8mA/gel for about 5 hours, when the marker dye was about 1 cm. from the bottom of each gel. Gels were stained either for 1 to 2 hours at 37°C or overnight at room temperature in 0.25% (w/v) Coomassie Brilliant Blue R-250, 45.4% methanol, 9.2% glacial acetic acid, and destained electrophoretically (50V) in 5% methanol, 7.5% glacial acetic acid for 30 to 60 min. Destained gels were stored in 7% acetic acid.

For determination of molecular weights, gels were calibrated with the following standard proteins: cytochrome C (11,700), myoglobin (17,200), γ-globulin L-chain (23,500), aldolase (40,000), ovalbumin (43,000), γ-globulin H-chain (50,000), and bovine serum alubumin (68,000). Using these proteins the standard curve shown in Fig. 2.1. of electrophoretic mobility on 10% gels against log of molecular weight, was obtained. This curve was subsequently usedfor molecular weight determinations in the range 10,000 to 70,000.

2.3.2. Native polyacrylamide gel electrophoresis.

Native 6.3% polyacrylamide gels were prepared according to Davis (1964) without a stacking or sample gel. Protein samples (20-100µl) were mixed with 5µl of 0.1% bromophenol blue marker dye and one drop of glycerol, applied to gels, and electrophoresed at 1 - 2 mA/gel for 1-2 hours when the marker dye was about 1 cm from the bottom of each gel. Gels were stained and destained as described above for SDS-polyacrylamide gels.

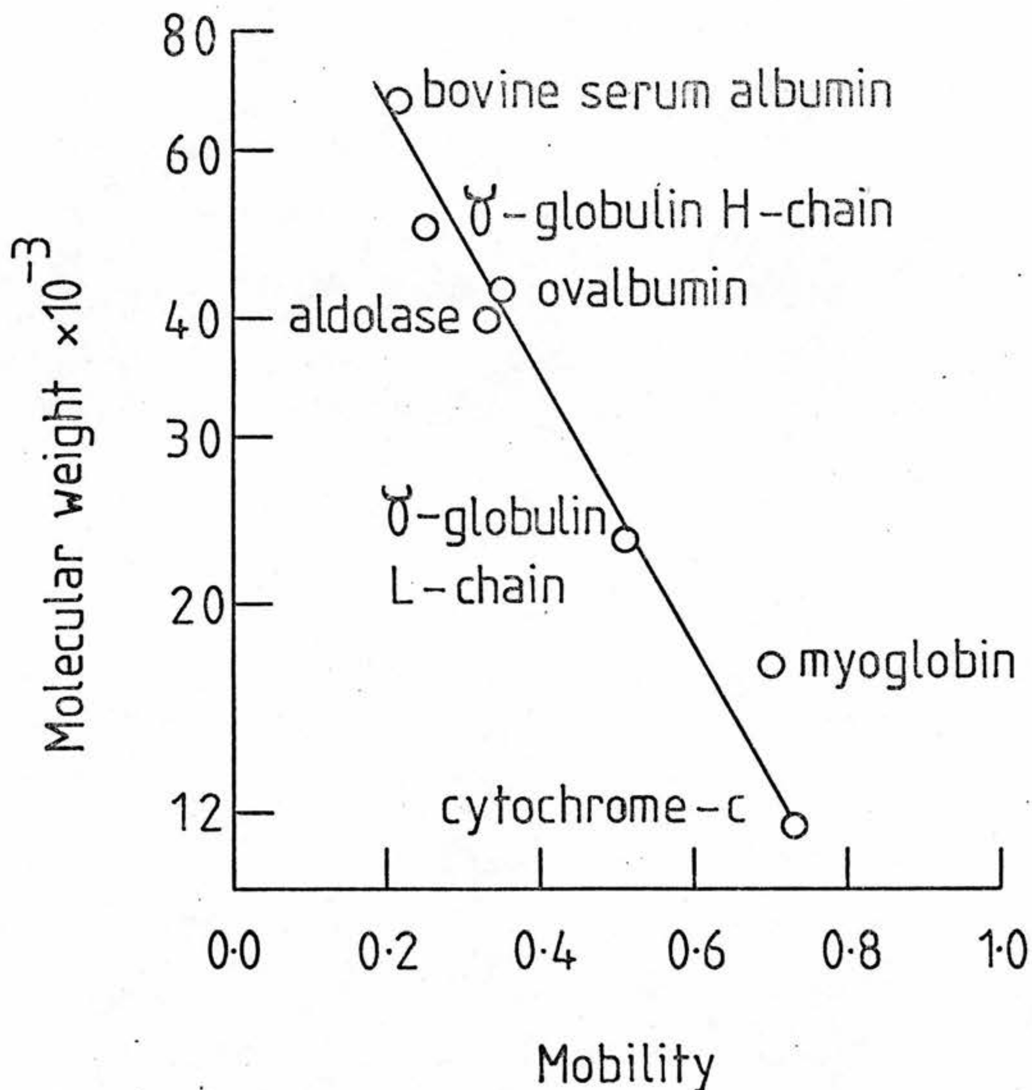


Fig.2.1. Calibration of 10%-polyacrylamide SDS gels for molecular weight determinations.

25μl of 1mg/ml solutions of the standard proteins were electrophoresed on 10%-polyacrylamide SDS gels as described in Ch.2.3.1. After staining and destaining, the mobilities relative to the dye front were recorded.

2.3.3. Elution of active proteins from native polyacrylamide gels

In some experiments native polyacrylamide gels were not stained for protein but were used for elution and subsequent assay of active protein species from slices of the gels.

Samples were electrophoresed as described above and the distance travelled by the marker dye noted. Gels were sliced into 2 mm-segments which were eluted for 24 hours in a shaking water bath at 25°C into about 0.4 ml of water. Gel slices were removed, the eluates freeze-dried, and the residues resuspended in 30 µl of water for assay.

2.3.4. Determination of radioactive proteins in polyacrylamide gel slices.

Samples containing radioactively labelled proteins were electrophoresed in either native or SDS polyacrylamide gels as described above. Gels were sliced into 2 mm-segments and each slice placed in a scintillation vial immersed in 0.2 ml of NCS tissue solubiliser (see Appendix I). The vials were capped and heated in an oven at 50°C for 2 hours. After cooling to room temperature, 5ml of toluene-based scintillator (Ch.2.6.1.) were added, and samples counted for radioactivity in a Searle Mk III liquid scintillation spectrometer.

2.4. Column chromatographic techniques

2.4.1. Preparation and storage of columns

All ion exchange and gel filtration media were suspended in the desired buffer, mixed, and the resin allowed to settle. The supernatant was decanted and the procedure repeated several times. This served both to remove 'fines' and to allow rapid equilibration after pouring the columns. Resins were finally suspended in an equal volume of buffer, the slurry poured into glass wool-plugged columns and packing was allowed to proceed under gravity. Several column volumes were passed through the packed columns until the pH of the effluent was identical to that of the applied buffer, the criterion taken to indicate that equilibration was complete. When not in use, columns were stored at 4°C in the presence of 0.02% sodium azide.

2.4.2. Use of columns

All column chromatographic procedures were conducted at 4°C and the pH of the buffers quoted was the pH at this temperature. Elution of columns was usually achieved with the aid of a peristaltic pump situated after the column and buffer reservoir, and connected to a Gilson automatic fraction collector. Pump speeds were selected to ensure that the pressures exerted on columns did not exceed the maxima stated in the manufacturer's handbook.

2.5. Preparation of tissues for assay.

The tissue chosen as the starting material for preparation of adenylate cyclase fractions was rat liver. Several different buffer systems were employed for the preparation of rat liver homogenates.

2.5.1. Buffer systems

A. Rat livers were perfused, washed, and homogenised in 75mM-Tris/HCl, 12.5 mM-$MgCl_2$, pH 7.4.

B. Rat livers were perfused with 130 mM-KCl, 20mM-potassium acetate, pH 7.4, washed in 10mM-Tris/HCl, pH 7.4, and homogenised in 10mM-Tris/HCl, 20mM-$MgCl_2$, 5mM-EDTA, 0.25% sucrose, pH 7.4.

C. Rat livers were perfused and washed as in Buffer System B, and homogenised in 10 mM-Tris/HCl, 20mM-$MgCl_2$, 5mM-EDTA, 25% sucrose, pH 7.4.

D. Rat livers were perfused, washed, and homogenised in 10mM-Hepes, 1mM-$MgCl_2$, 0.25M-sucrose, pH 7.4.

The procedures described below were common to all of the above buffer systems and in any one preparation, one buffer system was adhered to throughout. All operations were performed at 0-4°C.

2.5.2. Preparation of rat liver homogenates

A male rat was killed by a blow to the head, the liver was exposed and perfused *in situ* through the portal vein. The liver was excised, placed in about 50 ml of washing medium, and after blotting on filter paper, weighing, and cutting into small pieces, the tissue was homogenised in 2-3 volumes of the appropriate buffer. Homogenisation was carried out by hand using 10 strokes of a teflon-on-glass homogeniser. After filtration through one layer of muslin, the filtrate was taken as crude rat liver homogenate.

2.5.3. Preparation of crude rat liver membranes

A homogenate prepared as described in Ch. 2.5.2. was centrifuged at 2,000g x 10 min. on an MSE-18 centrifuge. The supernatant was discarded, the pellet resuspended, washed, and centrifuged a further two times, and finally resuspended in a small volume of the appropriate homogenisation buffer.

2.5.4. Preparation of rat liver cytosol.

The supernatant from the first low-speed centrifugation described in Ch. 2.5.3. was centrifuged at 100,000g x 30 min. on a Beckman L2-65B ultracentrifuge and the supernatant taken as rat liver cytosol.

2.5.5. Preparation of purified rat liver plasma membranes

Plasma membranes were purified from rat liver as described by Pilkis et al (1974).

2.5.6. Preparation of solubilised adenylate cyclase fractions

Solubilisation of adenylate cyclase from the particulate preparations described above was achieved by adding a one-tenth volume of Lubrol-PX in the appropriate buffer to give the desired final concentration (%, v/v) of detergent. After mixing for 30 s. and centrifugation at 100,000g x 30 min. on a Beckman L2-65B ultra-centrifuge, the clear supernatant was taken as the solubilised preparation.

2.5.7. Preparation of pigeon erythrocyte cytosol.

Pigeon erythrocyte lysates were obtained as described earlier (van Heyningen, 1977b) and cytosol was prepared by centrifugation at 100,000g x 30 min. as described above for rat liver cytosol (Ch. 2.5.4.).

2.6. Protein estimation

Protein was determined by the method of Bradford (1976). Protein reagent was prepared by dissolving 100mg of Coomassie Brilliant Blue G in 50 ml of 95% ethanol, adding 100 ml of 88% orthophosphoric acid, and diluting to 1 litre with distilled water.

For protein estimation, 0.1 ml of sample (containing 10 to 100μg of protein) was added to 5 ml of protein reagent and the resulting blue colour was measured after 2 min, and before 1 hour by recording the absorbance at 595 nm. Blanks consisted of 0.1 ml of the appropriate buffer in which the protein samples were dissolved. For each batch of protein reagent made up, a standard curve of protein concentration (μg per 0.1 ml of sample) against A_{595} nm was constructed using bovine serum albumin as standard. An example of such a curve is illustrated in Fig. 2.2. All experimental determinations were made on the linear portions of standard curves.

2.7. Liquid scintillation counting techniques

2.7.1. Scintillators

All radioactive samples, except those from adenylate cyclase assays, were counted in 5 ml of a toluene-based scintillator containing 7g PPO, 0.6g dimethyl POPOP, 150g naphthalene, 300 ml ethyleneglycol monoethylether, and toluene to 1 litre. Adenylate cyclase assay samples were counted in 10 ml of a scintillator containing 3g PPO, 257 ml Triton X-100, 37 ml ethyleneglycol monoethylether, 106 ml ethanol, and xylene to 1 litre.

2.7.2. Background and standard vials

During preparation of samples for counting, care was taken to ensure that all samples were identical with respect to volume of scintillator, amount of quencher, etc. and for each experiment, background and standard vials were also counted.

The background vial was identical to all other experimental vials except that it contained no radioactive material. Counts arising

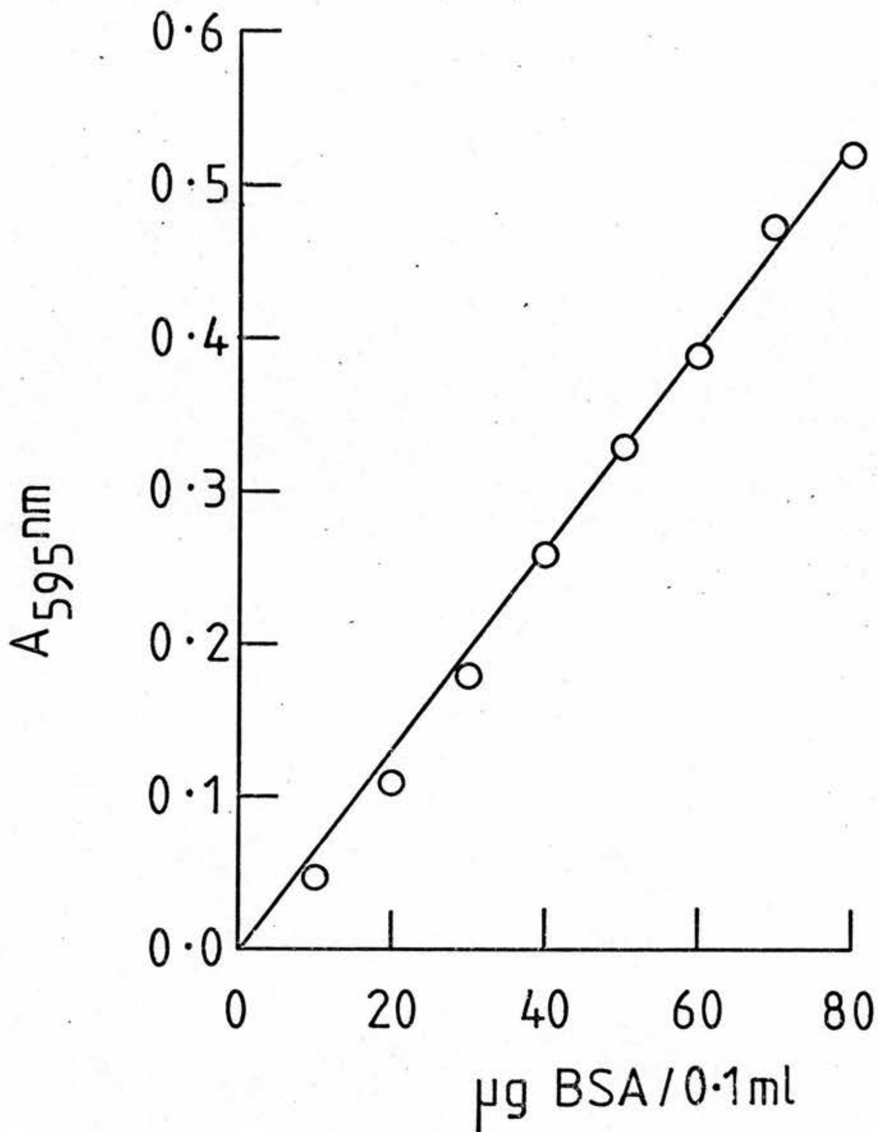


Fig.2.2. Standard curve of protein concentration against absorbance at 595nm. in the Bradford method of protein estimation.

A 1mg/ml solution of BSA in water was made up and increasing volumes up to 80µl were placed in a series of tubes. The volume in each tube was made up to 0.1ml with water, 5ml of protein reagent were added and the absorbance at 595nm was determined as described in Ch.2.6.

in this vial were subtracted from the values obtained in the experimental vials.

The standard vial for any particular experiment consisted of a known amount of the assay medium (containing radioactive substrate) used in the experiment, under identical conditions to assay samples in the experimental vials. Counts arising in this vial served as an estimate of the c.p.m. or d.p.m. of radioactive substrate added to each assay.

2.7.3. Use of the Searle Mk III liquid scintillation spectrometer

Radioactive samples were usually counted in the Searle Mk III liquid scintillation spectrometer. This was a computerised machine which was programmed before use with sets of quenched $[^{3}H]$ and $[^{14}C]$ standards supplied by Amersham/Searle. Each standard in the $[^{3}H]$ or $[^{14}C]$ series contained the same number of d.p.m. but owing to the variation in quench, each was counted with a different efficiency. During programming, the counter was instructed to store each standard counted, along with its counting efficiency, in the memory banks which therefore retained quench correction curves for both $[^{3}H]$ and $[^{14}C]$ isotopes. For any sample counted subsequently, the machine was therefore able to calculate the counting efficiency and print out absolute d.p.m. values directly. The counter was usually used in the variable quench mode in which optimum discriminator settings were established automatically for each sample before commencing counting. For most samples, individual counting efficiencies did not vary significantly, but for the complex adenylate cyclase assay samples (Ch. 3.3.) efficiences tended to vary to a greater extent.

2.7.4. Use of the Packard Tricarb liquid scintillation spectrometer

Counting samples in the Packard Tricarb liquid scintillation spectrometer was exactly as described above except that, in this case, the machine was unable to compute d.p.m. values directly. However since all vials in any particular experiment (including the standard vial) contained the same volume of sample and scintillator etc. it was assumed that all vials would be counted with the same efficiency (counting the same samples in the Searle Mk III on the variable quench mode confirmed this assumption). Because of this, and since the standard vial provided an estimate of the c.p.m. of labelled substrate added to each assay, computation of absolute d.p.m. values was unnecessary and specific enzyme activities could be calculated directly from c.p.m. values.

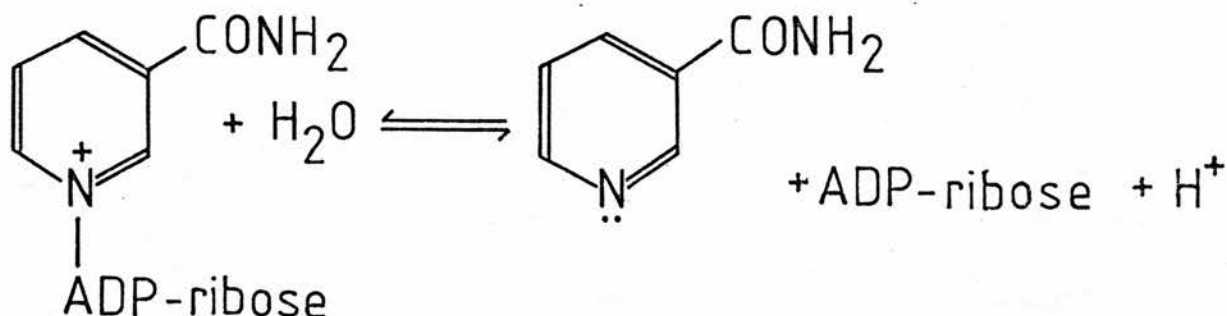
The packard Tricarb counter was however of limited use when applied to the complex adenylate cyclase assay samples and for this reason, these samples were always counted in the Searle Mk III counter.

Chapter 3. Assay Methods

3.1. NAD^+ GLYCOHYDROLASE ASSAY.

3.1.1. Introduction

NAD^+ glycohydrolases (NADases) are a class of enzymes (EC 3.2.2.5.) which catalyse hydrolysis of the nicotinamide-ribose bond of NAD^+ yielding nicotinamide, adenosine-diphosphate ribose (ADP-ribose) and a proton:

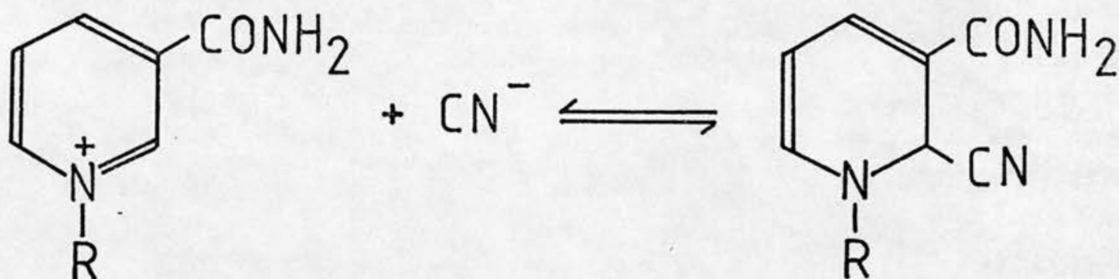


Most early assays of NADase activities depended on the measurement of residual levels of NAD^+ after incubation with enzyme. NAD^+ was originally assayed as 'V-factor' in cultures of _Haemophilus parainfluenzae_. Lwoff and Lwoff (1937) noted that the growth of this bacterium was dependent on the presence of a vitamin (V)-factor (now known to be NAD^+) in the culture medium, and they developed an assay for NAD^+ based on the extent to which an unknown sample containing NAD^+ could support the growth of _H. parainfluenzae_ cultures. The theory and specificity of this assay have been discussed by Kohn (1938).

A more convenient assay for NAD^+ was introduced by Negelein and Wulff (1937) who used ethanol and alcohol dehydrogenase to convert NAD^+ to NADH when the concomitant increase in absorbance at 340 nm was a measure of the original amount of NAD^+ in a sample.

The assay was further characterised and simplified by Racker (1950).

NAD^+ determinations were again simplified by Colowick <u>et al</u> (1951) who exploited the observation that NAD^+ reacted with cyanide or bisulphite to form complexes having absorption spectra resembling those obtained by enzymatic reduction of NAD^+. These authors developed a method for the determination of NAD^+ by measuring the amount of cyanide complex which formed on addition of an assay sample to a standard 1M-KCN solution. NADase activities determined by this method were essentially identical to those obtained by the alcohol dehydrogenase method. Cyanide addition occurs on the nicotinamide ring of N-substituted nicotinamides:



The fact that NAD^+ hydrolysis liberates a proton has also been exploited in the development of manometric (McIlwain and Rodnight, 1949) and potentiometric (Zatman <u>et al</u>, 1954) assay methods.

Most of the above assays, requiring large volumes for analysis, were unsuitable for the present work where the limited availability of cholera toxin necessitated the use of assays on a miniature scale, and a radioactive assay was therefore employed. NAD^+ radioactively labelled with $[^{14}C]$ in the carbonyl moiety was used as substrate, and the product, $[carbonyl\text{-}^{14}C]$nicotinamide, was

separated from unreacted [carbonyl-^{14}C] NAD^+ before counting. Separation of nicotinamide and NAD^+ on small columns of Dowex 1-formate (X2: 200-400 mesh) has been described by Ueda et al (1975) and a similar method was also employed by Moss et al (1976b)in their original demonstration of the NADase activity of cholera toxin. In the work reported here, a simple and efficient separation of nicotinamide and NAD^+ was effected by chromatography on strips of DEAE-cellulose paper, in a method similar to that already described for separation of NAD^+ and $NADP^+$ (Apps and Nairn, 1975).

3.1.2. Method.

3.1.2.1. Incubations and Assay Media.

NADase activities of cholera toxin fractions were determined initially in the assay medium described by Moss et al (1976b). Assays, in a total volume of 15 to 50µl contained 100 to 400 mM potassium phosphate ($KH_2PO_4 \cdot 12H_2O$), 20 mM-DTT, 0.5 to 5mM-NAD^+, and 4 to 20 nCi of [carbonyl-^{14}C] NAD^+, pH 7.0. In later experiments, low concentrations of potassium phosphate (10mM), 200mM-sodium acetate, pH 6.2, or 50mM-Tris/HCl, pH 7.4 were substituted for the high concentration phosphate buffer.

NADase activities of rat liver fractions were conducted in the appropriate homogenisation buffer (Ch.2.5.1.) containing NAD^+ and [carbonyl-^{14}C] NAD^+ as above.

Assays were initiated by the addition of enzyme sample and after an incubation at 25 or 37°C, reactions were terminated by the addition of 25% TCA. Precipitated protein was removed by centrifugation at 1,200g x 5 min. and aliquots of each supernatant (usually 10 µl) taken for estimation of [^{14}C]-nicotinamide.

3.1.2.2. Separation of [^{14}C]Nicotinamide.

[^{14}C] nicotinamide produced during assay was separated from unreacted [carbonyl-^{14}C] NAD^+ by chromatography on DEAE-cellulose paper. Sheets of Whatman DE-81 paper (46 cm x 57 cm) were cut into 2 cm x 20 cm strips and an assay sample was applied 2 cm from one end of each strip. 2µl of a 100 mg/ml solution of nicotinamide were also applied along with each sample to enable visualisation of nicotinamide spots under short-wave ultraviolet light. The strips were suspended in small chromatography tanks (up to 20 per tank) and ascending development in 0.12M-formic acid / NH_3, pH 3.1 was allowed to proceed for about 1 hour, when the solvent front was about 1 cm from the top of each strip. The strips were dried in an oven at 50°C, viewed under short-wave ultraviolet light, and the nicotinamide spots marked in pencil.

3.1.2.3. Liquid Scintillation Counting Conditions

The nicotinamide spots were cut out directly into scintillation vials containing 5 ml of toluene-based scintillator (Ch.2.7.1.) and counted for radioactivity either in a Packard Tricarb or a Searle Mk. III liquid scintillation spectrometer. Background vials contained

an equivalent blank piece of DE-81 paper and standard vials contained known amounts of the assay media used in each experiment, (containing [carbonyl-^{14}C] NAD^+ substrate) dried onto an equivalent piece of DE-81 paper.

3.1.3. Characterisation of Assay Method

3.1.3.1. Separation of NAD^+ and its' hydrolysis products on DE-81 paper

The separation of NAD^+ and nicotinamide on DE-81 was assessed visually. 100mg/ml solutions of the two compounds in water were made up and applied to 3 strips as follows:

Strip 1 3 µl of nicotinamide

Strip 2 3 µl of NAD^+

Strip 3 3 µl of nicotinamide + 3 µl of NAD^+

After development and visualisation under U.V. light, nicotinamide on strip 1 had migrated with an R_f value of 0.9 and NAD^+ on Strip 2 with an R_f of 0.5. (R_f values are quoted as distance from origin to front of spot / distance from origin to solvent front). On strip 3 the two compounds had migrated with the same R_f values as in strips 1 and 2 and were clearly separated from each other. A similar experiment in which 10 µl of 1M-$KH_2PO_4 \cdot 12H_2O$ were applied along with each sample gave identical results showing that samples of high ionic strength could also be separated efficiently in this system.

Separation was assessed quantitatively by applying a sample of partially hydrolysed NAD^+ (from an incubation with cholera toxin as described in the previous section) to a DE-81 strip. The toxin had been incubated in the simultaneous presence of 2nCi of [carbonyl-^{14}C] NAD^+ and 2 nCi of [adenine U - ^{14}C] NAD^+ and therefore the sample should have contained a mixture of unreacted NAD^+ and equal radioactive counts of [carbonyl-^{14}C] nicotinamide and [adenine U-^{14}C] ADP-ribose. After development, the chromatogram was dried and cut into 1 cm-long segments each of which was counted for radioactivity as described in Ch. 3.1.2.3. As shown in Fig. 3.1. three peaks of radioactivity were observed. Since the positions of NAD^+ and nicotinamide had already been established visually. the third slow-moving peak was presumably ADP-ribose. Nicotinamide was detected between R_f values 0.69 - 0.94, NAD^+ between 0.38 - 0.63 and ADP-ribose between 0.06-0.31. Nicotinamide and ADP-ribose were present in approximately equivalent amounts, about 94% of all applied radioactivity was recovered, and all three compounds were clearly separated from each other on the DE-81 strip.

3.1.3.2. Recovery of radioactivity from DE-81 paper

[Carbonyl-^{14}C] NAD^+ was diluted with water to give an approximately 1µCi/ml solution, and increasing volumes up to 50µl were placed in a series of vials containing 5 ml of scintillator. An identical series of aliquots were adsorbed onto pieces of DE-81 paper and after drying in an oven at $50^{o}C$, were also placed in vials as above. The total volume of aqueous solution in each vial was made up to 50 µl with water, and vials were counted for radioactivity.

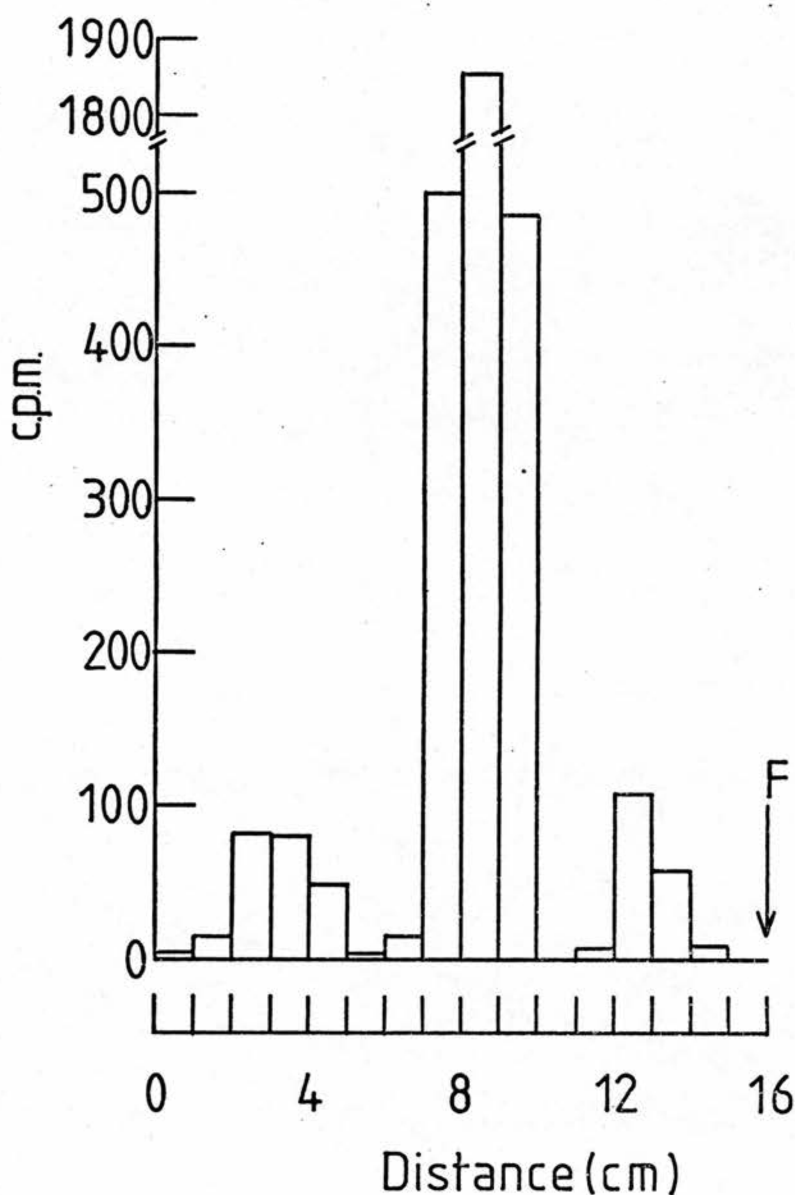


Fig.3.1. Migration of NAD^+ and its hydrolysis products on DE-81 strips.

A mixture was prepared containing 200mM-potassium phosphate, 20mM-DTT, 1mM-NAD^+, 2nCi of [carbonyl-^{14}C]NAD^+, 2nCi of [adenine U-^{14}C]NAD^+, and 118μg/ml cholera toxin, pH 7.0. After 3hr at 37°C, 10μl were applied to a DE-81 strip which was developed and analysed for radioactivity as described in the text (Ch.3.1.3.1.)

Reported c.p.m. values are corrected for the total 20μl assay volume. 'F' indicates the position of the solvent front.

Fig. 3.2. illustrates the quantitative recovery of radioactivity from [carbonyl-^{14}C] NAD^+ adsorbed onto DE-81 paper and the method was therefore judged suitable for quantitative assay determinations without the need for corrections for self-absorption of counts. The experiment also indicates that the presence of DE-81 paper in a vial does not per se affect the observed count rate and therefore no special precautions to ensure the presence of an exact amount of paper in each vial were necessary.

3.1.3.3. Breakdown of NAD^+ on DE-81 paper.

NAD^+ was found to be unstable on DE-81 paper at room temperature, and Fig. 3.3. shows a slow time-dependent release of [^{14}C] nicotinamide after application of [carbonyl-^{14}C] NAD^+ to DE-81 strips which were left for various times before development. After 6 hours about 5% of the applied NAD^+ had been broken down.

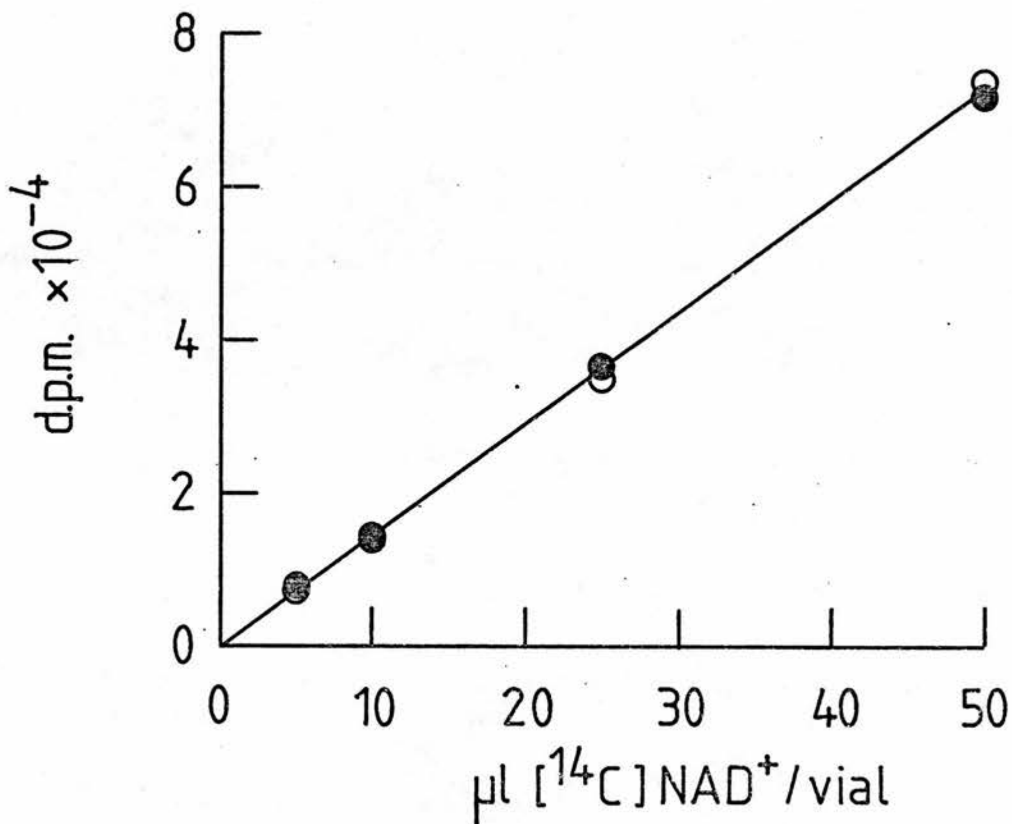


Fig.3.2. Recovery of radioactivity from Whatman DE-81 Paper.

Aliquots of an approximately 1µCi/ml solution of [carbonyl-^{14}C]NAD$^+$ in water were either added directly into scintillation vials (o—o) or adsorbed onto pieces of DE-81 paper and dried (●—●). The total volume of water in each vial was made up to 50µl with water, 5ml of toluene-based scintillator was added, and vials were counted for 10 min. All values were single determinations only.

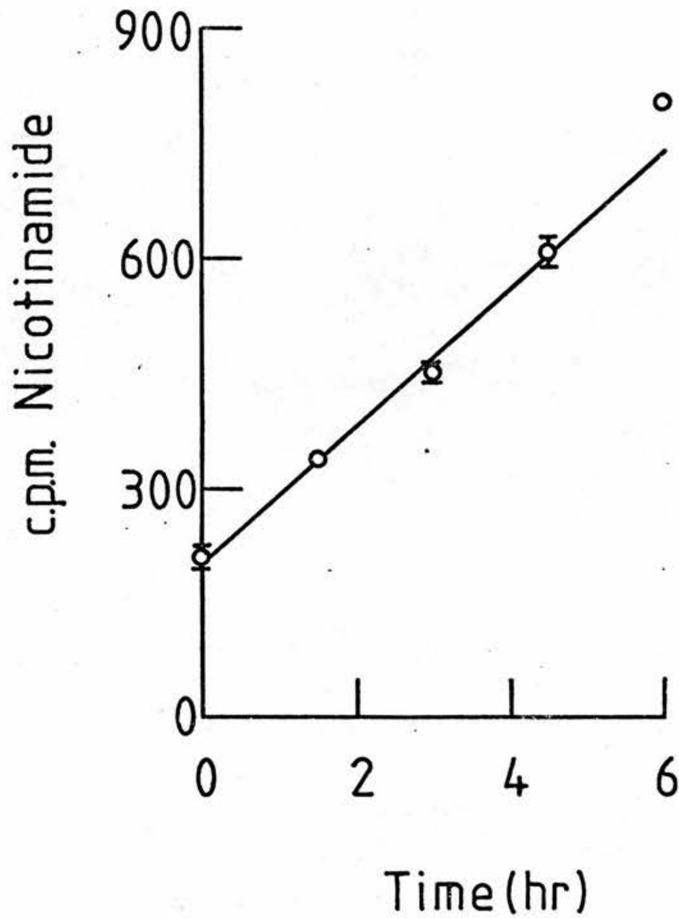


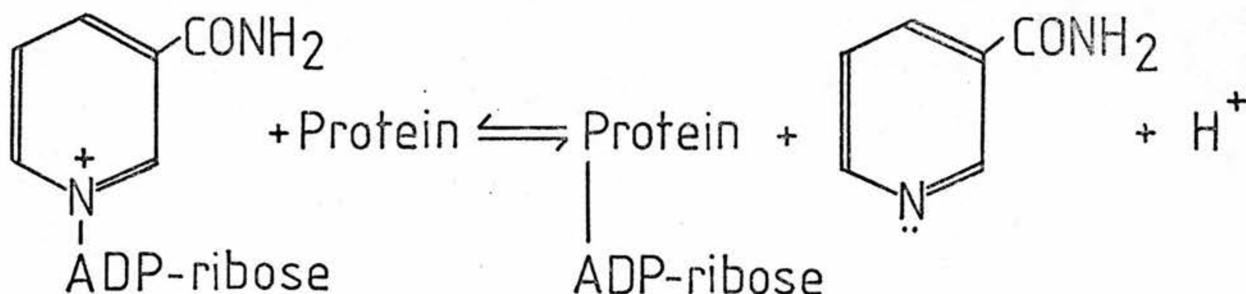
Fig.3.3. Breakdown of NAD^+ on DE-81 paper.

10nCi of [carbonyl-^{14}C]NAD^+ were applied to a series of DE-81 paper strips and after the indicated times at room temperature, the strips were chromatographed and nicotinamide was determined as described in Ch.3.1.2. All values except the 6hr sample, were the means of duplicates.

3.2. ADP-RIBOSYLTRANSFERASE ASSAY

3.2.1. Introduction

ADP-ribosyltransferases are responsible for catalysing the transfer of the ADP-ribose moiety of NAD^+ to an acceptor protein, in the process liberating free nicotinamide and a proton:



The NADase activity discussed in Ch. 3.1. may be thought of as an ADP-ribosylation reaction in which the protein acceptor is replaced by water and in theory therefore, the assay method described in Ch. 3.1.2. for NADase activity should also be suitable for the measurement of ADP-ribosyltransferase activity (assuming that the transferase is pure and devoid of all NADase activity . However, the diversity of NAD^+-utilising enzymes in the crude rat liver fractions used in this work, of which only a small proportion are ADP-ribosyltransferases, invalidated the use of any assay method based on the measurement of nicotinamide production which can serve only as a measure of the total activities of all enzymes in a solution which release nicotinamide from NAD^+ during catalysis.

For this reason, NAD^+ labelled universally with $[^{14}C]$ in the adenine ring was chosen as a substrate and after an incubation with enzyme sample, the amount of $[^{14}C]$associated with TCA-precipitable material was determined. In common with all published methods for the

estimation of ADP-ribose incorporation, the method described here involved the use of a filtration step during which TCA precipitates were applied to filters (in this case Whatman GF/C filters) and all non-covalently associated [^{14}C] was washed free. Several similar methods to the one described here have been reported previously. (Fujimura and Sugimura, 1971; Nishizuka et al, 1971; Haines et al, 1969; Gill, 1972).

3.2.2. Assay Method

3.2.2.1. Incubations and assay media

ADP-ribosyltransferase activities in rat liver fractions were determined in the appropriate homogenisation buffer (Ch.2.5.1.) containing various concentrations of NAD^+, ATP, DTT, GTP, 50-100 nCi of [adenine U-^{14}C] NAD^+, cholera toxin and other additions as indicated in the Results section (Ch. 5.). Assays were usually conducted in a total volume of 100µl and were initiated by the addition of rat liver fraction. After incubation at 25°C, 25% TCA was added to terminate the reactions and aliquots from each assay were taken for estimation of ^{14}C ADP-ribose incorporation.

3.2.2.2. Estimation of ADP-ribose incorporation

100 µl of assay mixtures were applied to 2.5 cm diameter Whatman GF/C glass fiber filter discs, pre-wetted in 5% TCA and mounted on a Millipore filtration device. After attaching the top of the Millipore apparatus the filters were washed with 2 x 20 ml aliquots of 5% TCA from a 20 ml syringe. Excess moisture was removed by forcing

air through the apparatus twice from the syringe and filters were removed and dried in an oven at 50°C. The filters were finally placed in scintillation vials containing 5 ml of toluene-based scintillator (Ch. 2.7.1.) and counted for $[^{14}C]$radioactivity in a Searle Mk. III liquid scintillation spectrometer.

3.2.3. Characterisation of assay method

3.2.3.1. Recovery of radioactivity from Whatman GF/C filters

An approximately 1 µCi/ml solution of $[$adenine U-$^{14}C]$ NAD^+ was made up in water and increasing volumes up to 50 µl were placed directly into scintillation vials containing 5 ml of toluene-based scintillator. An identical series of aliquots were placed on Whatman GF/C filters, dried at room temperature, and added to scintillation vials as above. The total volume of aqueous medium in each vial was made up to 50µl with water, and vials were counted for 10 min. in the Searle Mk. III liquid scintillation spectrometer.

In both cases, a linear relationship was obtained between amount of $[^{14}C]$ NAD^+ added, and observed d.p.m. (Fig. 3.4.) although d.p.m. values were always slightly greater when the isotope was adsorbed onto GF/C filters. A possible reason for this phenomenon is discussed later (Ch. 3.4.)

3.2.3.2. Recovery of radioactivity in the presence of precipitated protein

In routine assays, radioactivity on GF/C filters was determined in varying amounts of TCA-precipitated protein and it was

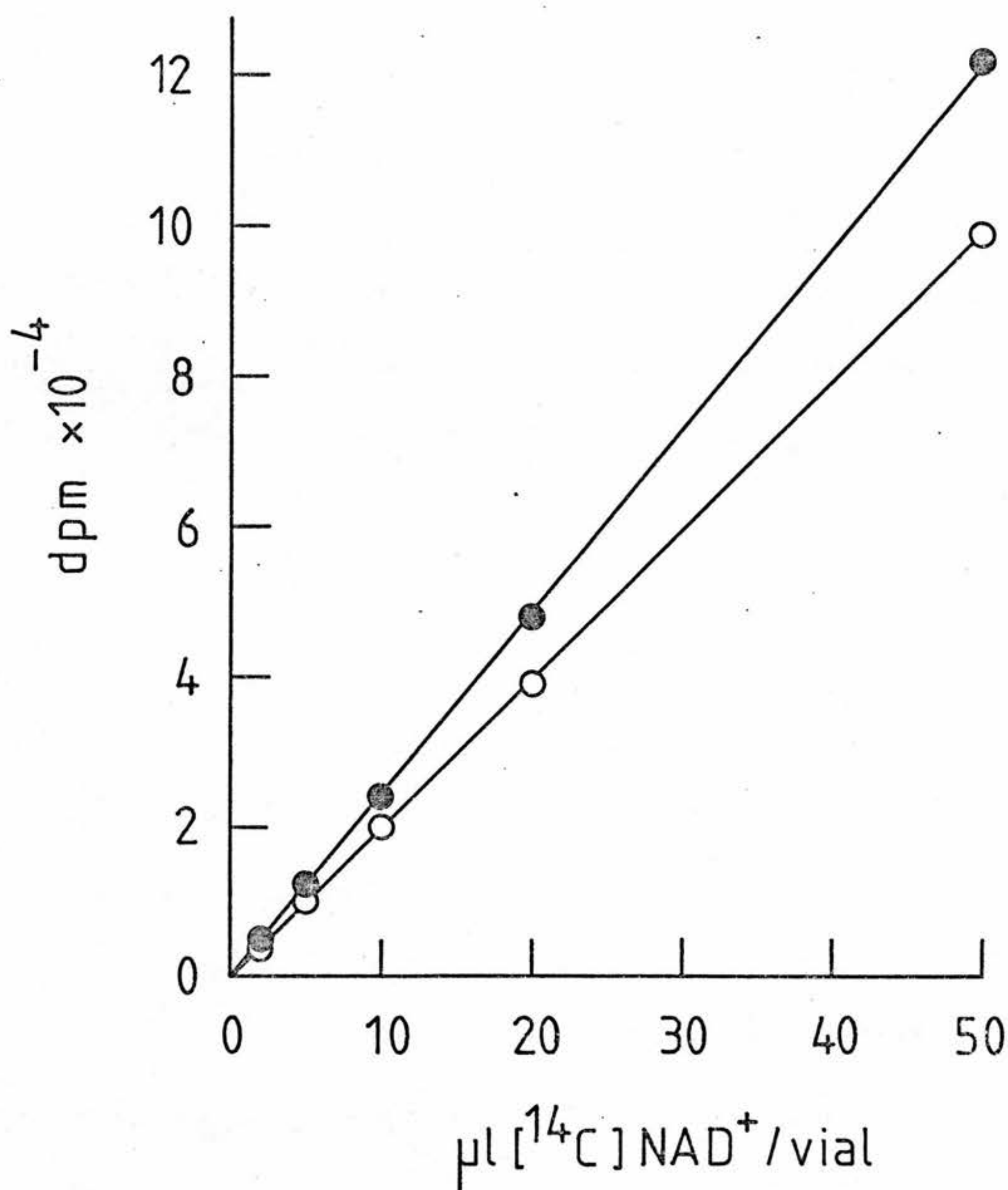


Fig.3.4. Recovery of radioactivity from Whatman GF/C filters.

An approximately 1µCi/ml solution of [adenine U-^{14}C]NAD^{+} was made up in water and increasing volumes were either added directly to scintillation vials (o—o) or applied to Whatman GF/C filters and dried (●—●). The total volume of water in each vial was made up to 50µl, 5ml of scintillator were added, and vials were counted for 10min.

therefore essential to establish that precipitated protein did not produce a 'masking' effect on the observed count rate.

80μl aliquots of increasing concentrations (up to 20 mg/ml) of BSA in water were added to tubes containing 20 μl of an approximately 1μCi/ml solution of [adenine U-^{14}C] NAD^{+}. 10 μl of 25% TCA were added and after mixing, 100 μl from each tube were applied to GF/C filters, dried, and counted in the Searle Mk. III as above. As Fig. 3.5. shows, the presence of precipitated protein over a wide range had no effect on the observed d.p.m. values.

3.2.3.3. Efficiency of washing procedure

The sensitivity of the assay method depends on the efficiency with which non-covalently attached [^{14}C] is removed from the protein precipitates in the washing step.

80μl of 10 mg/ml BSA were added to 20μl of an approximately 1μCi/ml solution of [adenine U-^{14}C]NAD^{+} followed by 10μl of 25% TCA. After mixing, 100 μl from each of 5 similar tubes were applied to pre-wetted GF/C filters and washed with increasing numbers of 20 ml aliquots of 5% TCA as described in Ch. 3.2.2.2. The filters were dried, placed in scintillation vials and counted in the Searle Mk. III as above.

As the results in Table 3.1. indicate, the majority of radioactivity was removed from the filter after the first wash, and after two washes almost all of the applied counts were removed. Subsequent washes then had little effect. In routine assays, 2 x 20 ml washes were always employed.

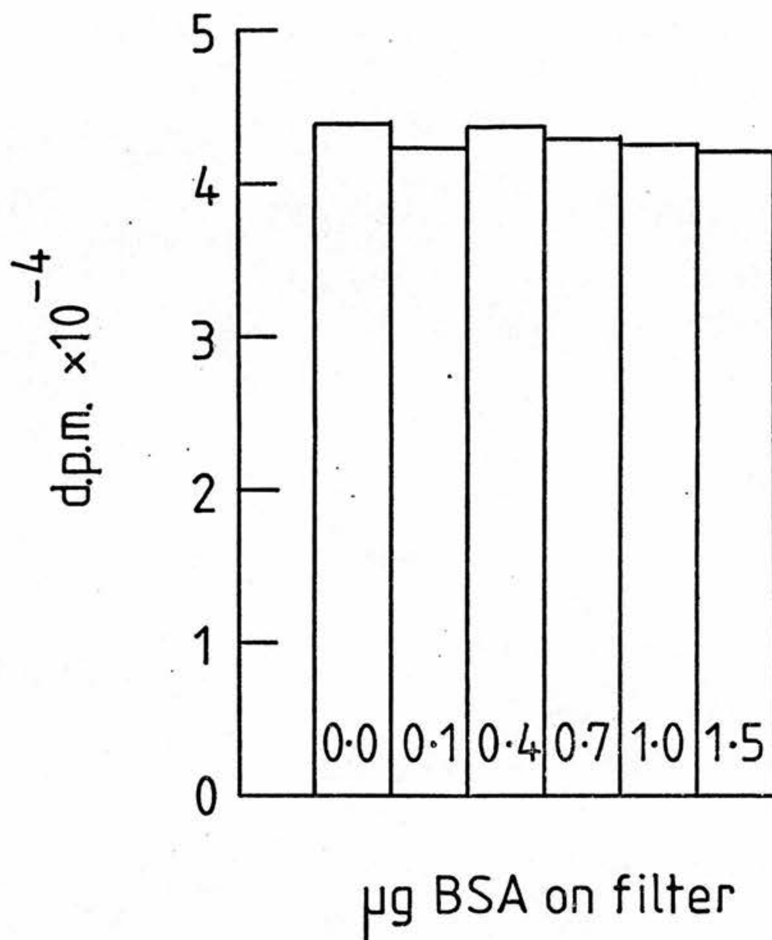


Fig.3.5. Effect of precipitated protein on the recovery of radioactivity from Whatman GF/C filters.

80μl of increasing concentrations of BSA in water were added to 20μl of a 1μCi/ml solution of [adenine U-^{14}C]NAD^{+}. 10μl of 25%-TCA were added and 100μl aliquots were applied to GF/C filters which were dried, placed in 5ml of scintillator, and counted for 10min.

Table 3.1. Efficiency of washing procedure in ADP-ribosyltransferase assay.

80μl of 10 mg/ml BSA were added to 20μl of an approximately 1μCi/ml solution of [adenine U-^{14}C] NAD^+ followed by 10μl of 25%-TCA. 100μl aliquots were applied to Whatman GF/C filters and washed with increasing numbers of 20ml-aliquots of 5%-TCA as described in Ch. 3.2.2. Filters were dried, placed in 5 ml of scintillator and counted for 10 min. All values were single determinations only.

No. of 20ml-washes	d.p.m. [^{14}C]
0	38248
1	209
2	95
3	78
4	76

3.3. ADENYLATE CYCLASE ASSAY

3.3.1. Introduction

Adenylate cyclase (ATP pyrophosphate-lyase (cyclising) EC 4.6.1.1.) is an integral component of cellular plasma membranes and is responsible for the formation of cyclic adenosine 3',5'-monophosphate (cAMP) from ATP.

Since the discovery of this activity (Rall and Sutherland, 1957) and the subsequent isolation and characterisation of cAMP (Rall and Sutherland, 1958a), research has continually been hampered by the lack of simple and efficient methods of assay. Most of the earlier methods for estimating adenylate cyclase activity (and some more recent ones) have employed non-radioactive ATP as a substrate, and a variety of techniques for measuring the small amount of unlabelled cAMP produced have been reported. For example, cAMP was originally assayed by its ability to activate liver glycogen phosphorylase and thus to stimulate glycogenolysis. Levels of glycogen, by reaction with iodine (Rall and Sutherland, 1958b) or production of glucose-1-phosphate, by coupling to $NADP^+$ reduction through phosphoglucomutase and glucose-6-phosphate dehydrogenase (Brown et al 1963; Scott and Falconer, 1965), were used as estimates of the amount of cAMP in a sample. A number of methods then appeared all of which were dependent on the conversion of cAMP to, and subsequent determination of, some other nucleotide (Breckenridge, 1964; Goldberg et al, 1969; Hardman et al, 1966; Ebashi et al 1971). All of these methods were extremely laborious requiring the preparation of several different purified enzyme systems. They also

lacked sensitivity, many of the enzymes used being responsive to variations in the levels of compounds other than cAMP itself (Krebs et al 1959).

More recent methods for cAMP determination have concentrated on measuring the nucleotide directly and have thus achieved a markedly greater sensitivity. The isolation of cAMP-binding proteins (cAMP-dependent protein kinases) from skeletal muscle and beef adrenal cortex enabled the development of saturation binding assays for cAMP (Gilman, 1970; Walton and Garren, 1970; Brown et al 1970; 1971), and a specific cGMP-binding protein from lobster muscle (Kuo and Greengard, 1970a) enabled Murad and Gilman (1971) to assay cAMP and cGMP simultaneously. The ability of cAMP to activate the cAMP-dependent protein kinases has also been exploited. Thus Kuo and Greengard (1970b) and Wastila et al (1971) incubated assay samples in the presence of protein kinase and [γ-^{32}P] ATP and measured [^{32}P] incorporation into histones and casein respectively.

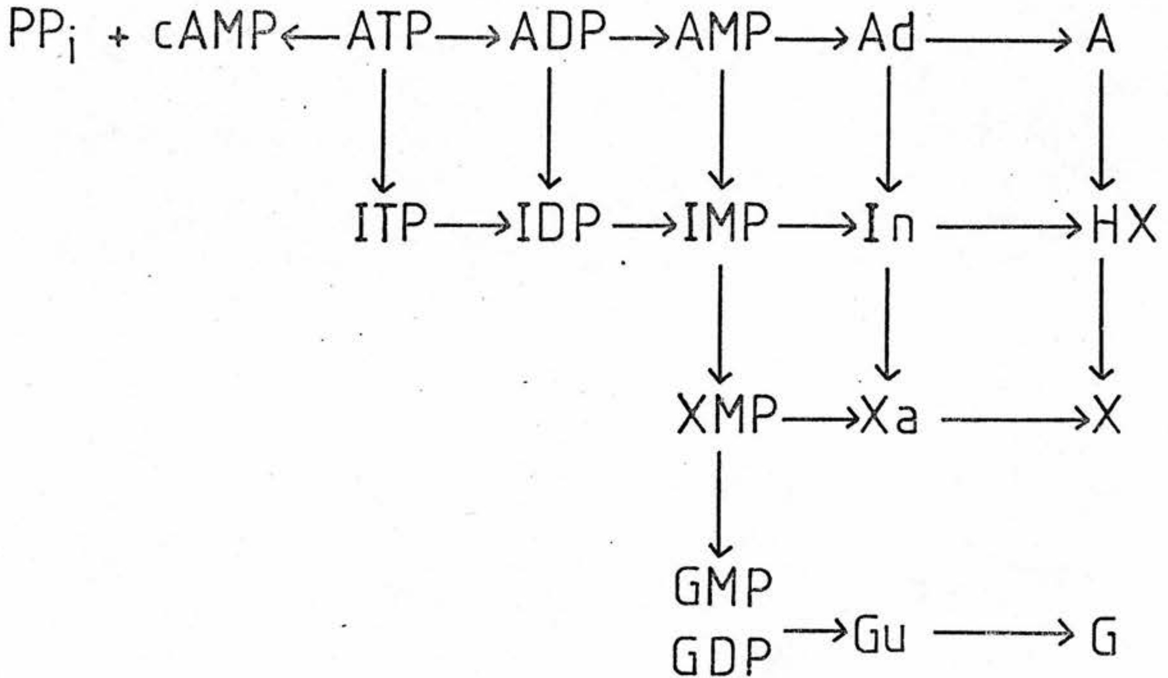
The specificity of cAMP-phosphodiesterase, the enzyme responsible for degradation of the nucleotide, formed the basis of an enzymatic radioisotopic displacement assay for cAMP (Brooker et al 1968), and radioimmunoassays for cAMP and cGMP have also been reported (Steiner et al 1969; 1972; Wehmann et al, 1972).

Some of the above methods, notably the saturation binding techniques, have provided fairly simple and sensitive adenylate cyclase assays but again, to a greater or lesser extent, all require the preparation of other protein/enzyme systems, each fresh preparation

having to be extensively characterised before use. A high-pressure liquid chromatographic method for cAMP determination has been described (Brooker, 1971) but, while eliminating the need for other protein systems, it required extensive prior purification of samples before chromatography. Furthermore, all adenylate cyclase assay methods using unlabelled ATP as substrate suffer from the criticism that tbe rate of the adenylate cyclase reaction itself is not being measured. Only the levels of total cAMP after an incubation are determined and although the two measurements are unlikely to differ markedly, it is possible that endogenous pools of cAMP in a tissue could lead to erroneous results, particularly when attempting to measure low basal activity states of adenylate cyclase. In order to measure the rate of cAMP production by adenylate cyclase, it is necessary to use radioactively-labelled ATP as substrate and determine the amount of radioactive cAMP produced.

There are two major difficulties in assays of this sort. Firstly, the basal activity of the enzyme is low and only a small fraction of substrate is converted to product. Secondly, since purification of adenylate cyclase without the loss of regulatory properties is not yet possible, the enzyme must be assayed in relatively crude tissue fractions, which contain numerous contaminating ATP-utilising activities. This necessitates the use of a high concentration of substrate (0.5-2mM) in order to ensure saturation of all activities, and since the fractional conversion to cAMP is low, ATP of high specific radioactivity must be used in a minimal assay volume. Radioactive cAMP produced must then be separated from a preponderance of radioactive contaminants and the sensitivity of any assay is therefore dependent upon the efficiency of cAMP purification. Most of the major possible

radioactive contaminants resulting from an incubation of a crude tissue fraction with radioactive ATP are illustrated below; (see list of abbreviations).



The popular use of [α - ^{32}P] ATP as a substrate, stems from the fact that only phosphorylated contaminants then have to be separated from cAMP, thereby simplifying the subsequent purification steps.

A major improvement in adenylate cyclase assay techniques was provided by Krishna et al (1968), who noted that treatment of assay samples with $ZnSO_4$ /$Ba(OH)_2$ quantitatively precipitated the vast majority of adenine-derived compounds leaving cAMP in solution. cGMP was also precipitated. Adenosine, which was not precipitated, could be removed by Dowex-50H^+ ion-exchange chromatography. Barium ions however, are known to catalyse the non-specific formation of cAMP from ATP (Cook et al, 1957), a problem which can be overcome by adding

a slight excess of $ZnSO_4$ to minimise the free Ba^{++} concentration, and processing samples rapidly at low temperature (Ebashi et al,1971) or by prior acid-heat destruction of ATP before the Zn-Ba treatment (Nakai and Brooker, 1975).

Separation of cAMP by paper chromatographic procedures (Dousa and Rychlik , 1970; Streeto and Reddy, 1967; Pauk and Reddy, 1967) and paper chromatography combined with high-voltage paper electrophoresis (Straub, 1969) has not generally been very successful and usually involves tedious procedures taking several days to complete.

The most successful adenylate cyclase assays have resulted from the initial observations of White and Zenser (1971) and Ramachandran (1971) who independently isolated cAMP in a one-step procedure using columns of neutral alumina. At pH 7.6, the alumina was found to discriminate between the cyclic nucleotides and most other derivatives of ATP which are more highly charged at this pH and were retained on the column. The alumina also discriminated between cAMP and cGMP, the latter eluting behind cAMP. Thompson et al (1973) reported several minor modifications to this procedure resulting in greater reproducibility, lower blanks and enhanced stability of the alumina columns. However, even with this method, the complete elimination of radioactivity from reaction blanks was not achieved. Salomon et al (1974) and later, Wincek and Sweat (1975) were able to achieve virtually complete elimination of all radioactive contaminants from cAMP using sequential Dowex-alumina column chromatography, and the sensitivity of this assay procedure is reflected in its almost routine use in adenylate cyclase work at the present time. However, the method does not separate cAMP from neutral contaminants and

therefore depends heavily on the use as substrate of [α - ^{32}P]ATP which is both expensive and hazardous to use routinely at the levels common in adenylate cyclase assay mixtures (up to 10 million cpm.)

For these reasons, in the work reported here, an assay of as high a sensitivity as possible, and using either [^{3}H] or [^{14}C]ATP as substrate, was desired. The method of Keirns et al (1975) involving the use of [^{3}H] ATP as substrate and separation of [^{3}H]cAMP by thin-layer chromatography on polyethyleneimine-impregnated cellulose (PEI-cellulose), appeared to separate cAMP from all possible mono- di- and tri-phosphates as well as neutral contaminants, and was therefore set up for routine use. Several minor modifications were made to the method during the course of this work and the procedure is therefore described in detail in the following sections.

3.3.2. Assay method

3.3.2.1. Pre-activation of cholera toxin

In some experiments, cholera toxin was 'preactivated' essentially as described by Gill (1976b). The toxin solution was added to a one-tenth volume of activation solution to give final concentrations of 0.01% - SDS, 10mM-DTT in 50mM -Tris/HCl, pH 7.4. After incubation at 25°C or 37°C for 30 mins., one tenth volume of this solution was added to adenylate cyclase preincubation mixtures to give the desired final concentration of cholera toxin and where the final SDS concentration (0.001%) was sufficiently low to have no effect on adenylate cyclase.

3.3.2.2. Preincubation of adenylate cyclase preparations with cholera toxin

Adenylate cyclase preparations were preincubated with cholera toxin before assay in the presence of varying concentrations of NAD^+, ATP, DTT, GTP and other additions as indicated in the Results Section (Ch. 5.). Preincubations were carried out at 25°C or 37°C in a total volume of 30 - 200µl and were terminated in one of two ways.

(i) an aliquot of the mixture was removed and transferred directly into adenylate cyclase assay medium.

(ii) an excess of the appropriate buffer (Ch. 2.5.1.) at 0°C was added, membranes were precipitated at 1,200g x 5 min. resuspended in a small volume of buffer, and aliquots added to adenylate cyclase assay medium.

3.3.2.3. Adenylate cyclase incubations and assay media.

Adenylate cyclase assays were initiated by the addition of 15µl aliquots of preincubation mixtures (Ch. 3.3.2.2.) to 10µl of assay medium. After incubation at 25°C or 37°C, reactions were terminated by the addition of 5µl of 25% TCA, precipitated protein was removed by centrifugation at 1,200g x 10 min, and 10µl of each supernatant taken for estimation of [^{3}H] cAMP.

Two different adenylate cyclase assay media were used during the course of this work.

Medium I:

The final concentrations of components in the 25µl assay volume were, 25mM-Tris/HCl, 5mM-$MgCl_2$, 5mM-KCl, 5mM-PEP, 0.5mM-cAMP, 0.5mM-BMX, 1mM-DTT, 0.5 - 2mM-ATP, 0.5-1µCi of [8-^{3}H]ATP, 80µg/ml pyruvate kinase, and 80µg/ml adenylate kinase, pH 7.4.

Assay medium was made up by mixing, for each assay, 5µl of $MgCl_2$-KCl-PEP-cAMP-BMX solution (assay buffer), 1µl of DTT solution, 1µl of ATP, 1µl of [8-^{3}H]ATP, 1µl of pyruvate kinase and 1µl of adenylate kinase. 10µl of this mixture were placed in assay tubes and on addition of 15µl of adenylate cyclase preparation, the final concentrations indicated above were obtained.

Medium II:

The final concentrations of components in the 25µl assay volume were, 9mM-Hepes, 0.22M-sucrose, 5mM-$MgCl_2$, 5mM-PEP, 1mM-DTT, 0.1mM-cAMP, 0.1mM-BMX, 0.5-1mM-ATP, 80µM-GTP, 1µCi of $[8-^3H]$ ATP, 80µg/ml pyruvate kinase and 80µg/ml adenylate kinase, pH 7.4.

Assay medium was made up by mixing, for each assay, 3µl of PEP, 1µl of cAMP-BMX, 1µl of DTT, 1µl of ATP, 1µl of GTP, 1µl of $[8-^3H]$- ATP, 1µl of pyruvate kinase and 1µl of adenylate kinase. 10µl of this mixture were placed in assay tubes and on addition of 15µl of adenylate cyclase preparation, the final concentrations indicated above were obtained.

All solutions in assay medium I were made up in the appropriate homogenisation buffer (Ch. 2.5.1.) and all solutions in assay medium II were prepared in Buffer System D (Ch.2.5.1.). The pH was adjusted to 7.4 with either 1M-NaOH or 1M-HCl using 10µl of 0.2mg/ml phenol red per ml of solution as an internal indicator. ATP and DTT solutions were made up fresh prior to each assay and all other solutions were made up fresh at regular intervals of 2-3 weeks and stored at -20°C when not in use.

3.3.2.4. Purification of $[^3H]$cAMP

$[^3H]$cAMP produced during assay was separated from other radioactively labelled contaminants by two-solvent descending thin-layer chromatography (TLC) on polyethyleneimine-impregnated cellulose (PEI-cellulose) essentially as described by Keirns et al (1974).

Preparation of TLC plates

A stock solution of 1%-PEI was made up by dissolving 20g of commercial 50%-PEI in about 700 ml of water, adjusting to pH 6.0 with concentrated HCl, and diluting to 1 litre with distilled water. 20 cm x 50 cm glass plates, used as the support for the thin-layers, were cleaned before use with 95% ethanol, rinsed in distilled water, and dried with a clean towel.

For each plate to be coated, 10g of cellulose microcrystalline (Merck) were suspended to a volume of 60 ml in 1% PEI-solution, and after stirring the suspension for 1 hour the plates were coated (50 ml of PEI-cellulose/plate) to a layer thickness of 0.3 mm using an automated TLC spreader leaving the top 10 cm of each plate uncoated. The plates were allowed to dry at room temperature on a level surface overnight.

After drying, the edges of each layer were trimmed 1 cm inwards, twelve 1.5 cm-wide channels were cut lengthwise using the edge of a spatula, and the bottom of each layer was cut in a saw-tooth design to ensure even flow of solvent off the ends of the layers. Plates were wrapped in aluminium foil and stored at toom temperature for no more than 1 month before use.

Development of thin layers

10 µl aliquots from adenylate cyclase assay supernatants were applied, with drying, 3 cm from the top of each thin layer (12 samples/plate). 5µl of a solution containing 10mM-cAMP and 2-5 nCi of [adenine U-^{14}C]cAMP were applied along with each sample, the unlabelled cAMP allowing visualisation of cAMP spots under short-wave ultraviolet light, and the [^{14}C]- cAMP enabling calculation of per cent recovery of each sample from the thin layer.

A piece of filter paper (20 cm x 15 cm) was wetted with Solvent I (Methanol: 1M-ammonium acetate, 5:2) and placed on top of each plate overlapping the layer by about 5mm. Polyethylene solvent troughs were attached to the plates with perspex screw-clips and the paper wick folded back inside the trough as illustrated in Fig. 3.6. The plates were placed upright in large chromatography tanks, the solvent troughs (Capacity: 30 ml) were filled with Solvent I, and descending development was allowed to proceed in a sealed atmosphere for 6 - 8 hours by which time the solvent front had travelled about 20 - 25 cm down the layers.

The plates were removed, solvent troughs detached, and the layers dried with a hot air blower. cAMP spots were visualised under short-wave ultraviolet light, a line was scratched about 1 cm behind the cAMP spots with the edge of a spatula, and the entire area of the layer behind this line was shaved off and discarded. The plates were re-wicked as above (the wick overlapping the layers by about 2mm), and descending development was continued for 12 - 16 hours in Solvent II

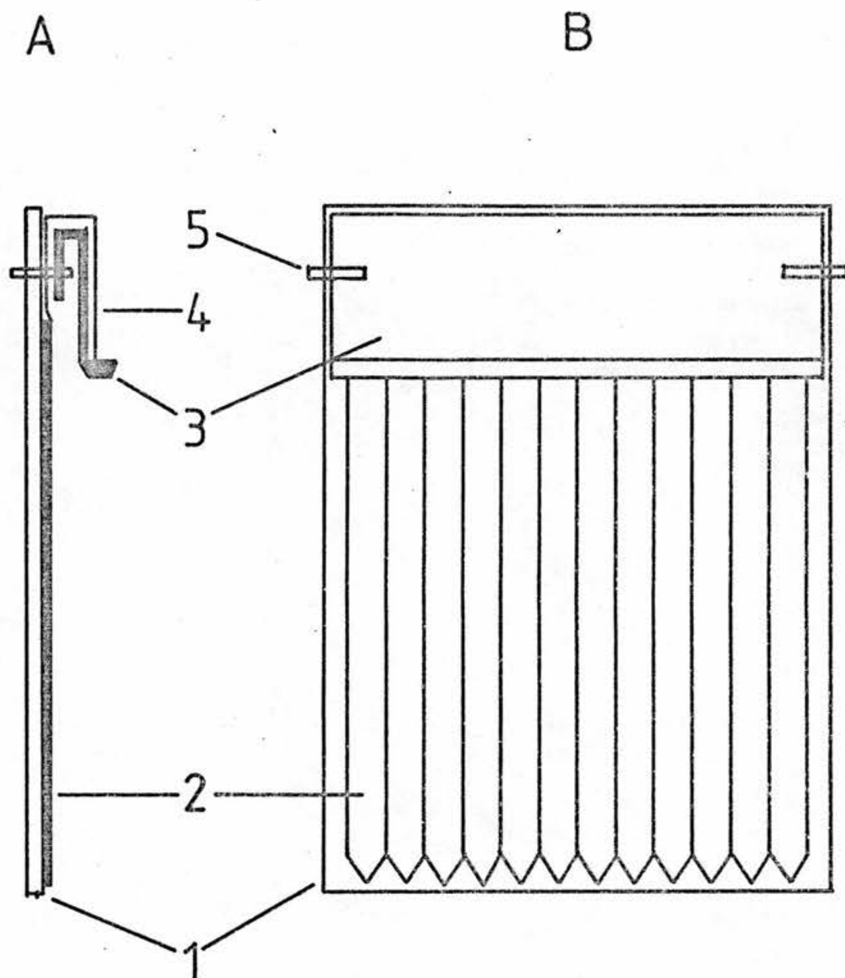


Fig. 3.6. Apparatus for descending PEI-cellulose thin layer chromatography.

A: Side view.

B: Front view.

1: Glass plate support.

2: Thin layer.

3: Perspex solvent troughs.

4: Filter paper wick.

5: Perspex screw clips.

(n-Butanol: Acetic acid : H_2O, 2:1:1). During this stage the solvent front was allowed to flow off the ends of the layers.

Plates were finally removed, solvent troughs detached, the layers dried in an oven at 50^oC and the positions of the cAMP spots, seen under short-wave ultraviolet light, were marked with the edge of a spatula.

3.3.2.5. Recovery of cAMP from thin layers and counting conditions

The area of the cAMP spot in each channel was shaved off with the edge of a spatula and PEI-cellulose scrapings were sucked directly into scintillation vials using an apparatus connected to a water pump similar to that originally described by Bjork and Svensson (1967) and illustrated in Fig. 3.7. Each vial contained 2 ml of 25mM-$MgSO_4$ acting both as a trap for the cellulose particles (preventing losses through the outlet tube) and as a solubiliser, eluting cAMP from the PEI-cellulose particles. The presence of 2 ml of aqueous medium in each vial, necessitated the use of an unusual scintillation fluid which was capable of accepting large volumes of water, and the xylene-triton X-100- based mixture of Fricke (1975), described in Ch.2.7.1., was found to be suitable. After addition of 10 ml of scintillator, vials were counted on the double isotope programme of the Searle Mk. III liquid scintillation spectrometer, in the variable quench mode.

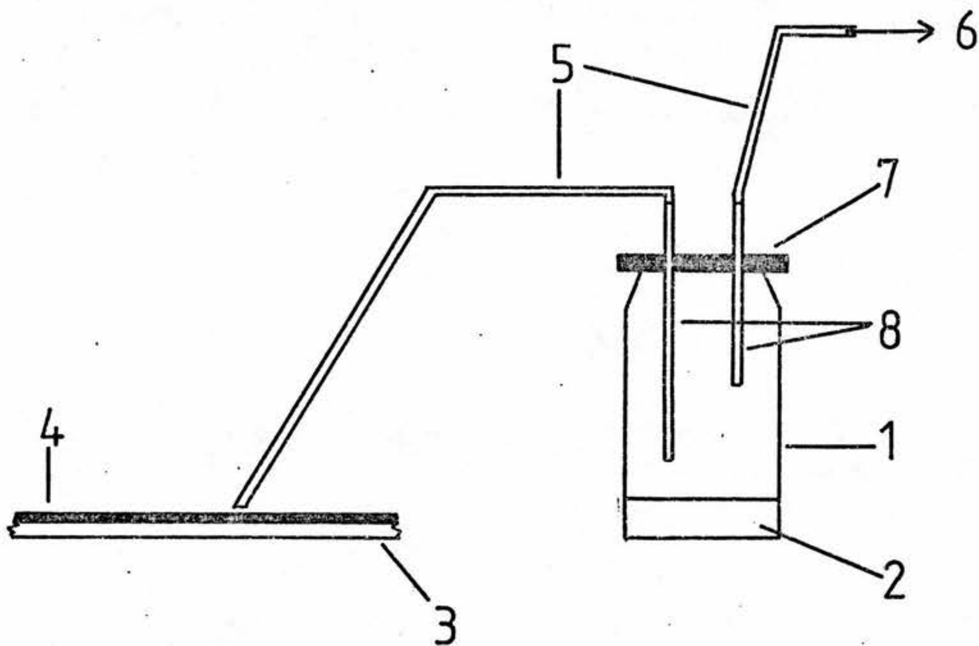


Fig.3.7. Apparatus for removing PEI-cellulose particles into scintillation vials.

1: Scintillation vial.

2: 2ml of 25mM-$MgSO_4$.

3: Glass plate support.

4: Thin layer.

5: Plastic tubing.

6: To water pump.

7: Rubber seal.

8: Glass tubing.

3.3.3. Characterisation of Assay Method

3.3.3.1. Use of Searle Mk. III liquid scintillation spectrometer

All adenylate cyclase assay samples were counted in the Searle Mk. III liquid scintillation spectrometer using the double isotope programme in the variable quench mode, in which optimum discriminator settings for each sample are established automatically prior to commencing counting. Before use the counter was programmed with sets of quenched [^{3}H] and [^{14}C] standards as described in Ch. 2.7.3. [^{3}H]cAMP and [^{14}C]cAMP standards made up under adenylate cyclase assay conditions (ie the isotopes adsorbed onto PEI-cellulose particles in vials before addition of 2 ml of 25mM-$MgSO_4$ and 10ml of scintillator) were readily recognised by the counter as being [^{3}H] and [^{14}C] standards. Thus despite the complex nature of the assay samples the quenbh correction data stored in the counter's memory were sufficient to enable recognition of the isotopes under cyclase assay conditions and the inclusion of a new programme specifically for these samples was unnecessary.

3.3.3.2. Solubilisation of cAMP from PEI-cellulose particles

In order to ensure consistent detection of all radioactivity in assay samples, it was necessary to acheive complete solubilisation of cAMP from PEI-cellulose particles thereby enabling the nucleotide to be counted free in solution after allowing cellulose particles to settle. 25mM-$MgSO_4$, as suggested by Keirns et al (1974) was chosen as the solubilising agent and it's ability to elute cAMP bound to PEI- was investigated as described below.

Two series of vials were prepared, all containing 50mg. of PEI-cellulose

particles. [8 - ^{3}H]cAMP ($\sim$ 100nCi) or [adenine U-^{14}C] cAMP ($\sim$20 nCi) were placed directly on the cellulose particles from a 50µl microsyringe and allowed to dry at room temperature. Increasing volumes (up to 5 ml) of 25 mM-$MgSO_4$ were added and mixed, followed by 10 ml of scintillator and all vials were counted for 10 min. in the double-isotope programme of the Searle Mk. III. Identical series of vials, containing the same amounts of radioactive cAMP free in solution in the absence of PEI-cellulose particles, were also prepared for each isotope and counted as above.

As shown in Fig. 3.8. both [^{3}H] and [^{14}C] counting efficiencies decreased linearly with increasing volumes of $MgSO_4$ solution, either in the presence or absence of PEI-cellulose particles. Efficiencies in the presence of cellulose were always about 2% lower than those in the absence of cellulose for both isotopes. The corresponding d.p.m. values obtained for these samples are illustrated in Fig. 3.9. When cAMP was free in solution, addition of a small volume of $MgSO_4$ eluant (0.5-1ml) caused a marked decrease in observed d.p.m. for both isotopes, the count rate being regained upon further addition of eluant. When 2 - 5 ml of $MgSO_4$ were present in each vial, d.p.m. values did not vary significantly and were equal to the observed d.p.m. in the absence of eluant. When either [^{3}H]or [^{14}C]cAMP was adsorbed to PEI-cellulose particles, very few counts were detected in the absence of $MgSO_4$. Addition of as little as 0.5 ml of $MgSO_4$ caused the majority of counts to be solubilised and for both isotopes, solubilisation was maximal after addition of 1 ml of $MgSO_4$. Further addition of eluant had no further effect. For [^{3}H]cAMP solubilised from PEI-cellulose particles, d.p.m. values were the same as those obtained for the

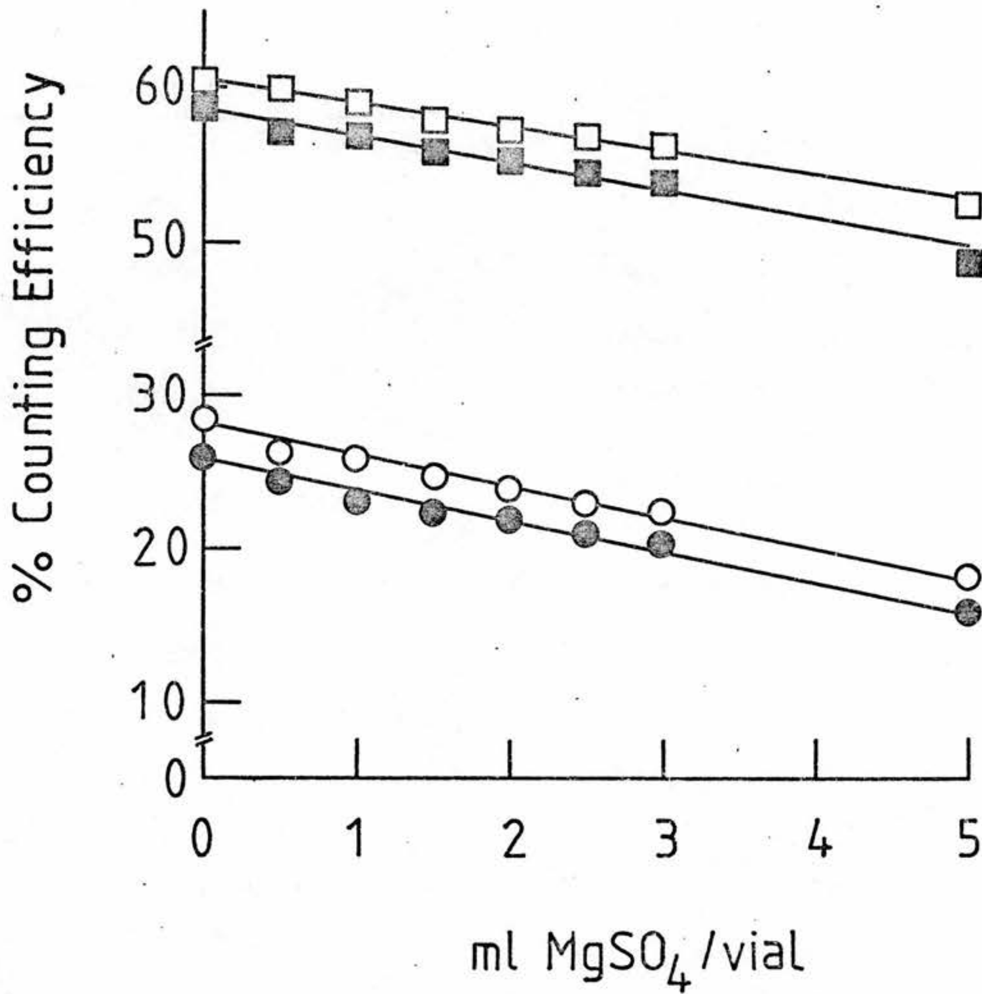


Fig.3.8. Decrease in [^{3}H] and [^{14}C]cAMP counting efficiency with increasing volumes of 25mM-$MgSO_4$ solution.

Scintillation vials contained 100nCi of [8-^{3}H]cAMP (○,●) or 20nCi of [adenine U-^{14}C]cAMP (□,■), either adsorbed to 50mg of PEI-cellulose particles (●,■) or free in solution (○,□). The indicated volumes of 25mM-$MgSO_4$ were added followed by 10ml of scintillator and vials were counted for 10 min. All values were single determinations only.

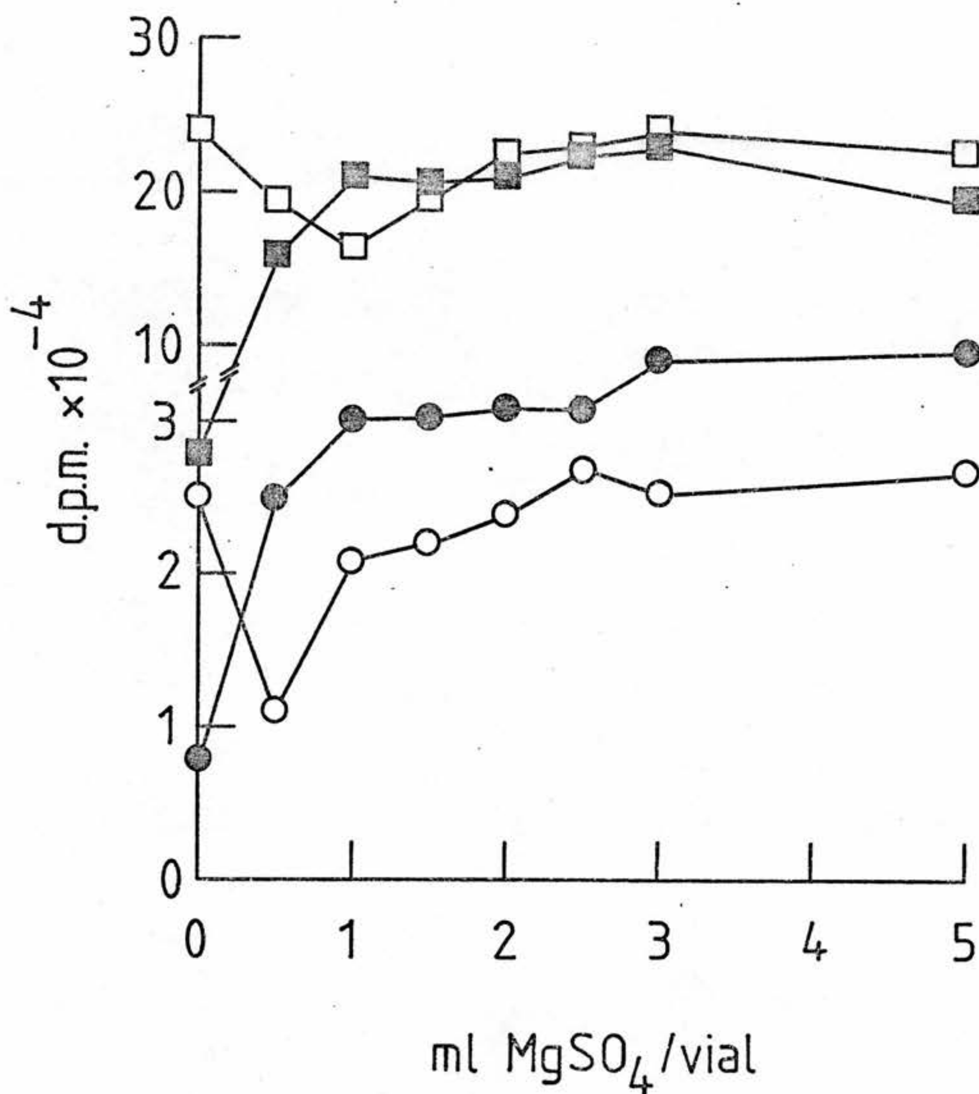


Fig.3.9. Effect of 25mM-$MgSO_4$ on the count rate of [^{3}H] and [^{14}C]cAMP in the presence and absence of PEI-cellulose.

Scintillation vials contained 100nCi of [8-^{3}H]cAMP (□,■) or 20nCi of [adenine U-^{14}C]cAMP (○,●), either adsorbed to 50mg of PEI-cellulose particles (●,■) or free in solution (○,□). The indicated volumes of 25mM-$MgSO_4$ were added followed by 10ml of scintillator and vials were counted for 10min. All values were single determinations only.

nucleotide free in solution, but for $[^{14}C]$ cAMP, d.p.m. values after maximal solubilisation from PEI-cellulose were always significantly greater than those observed for the nucleotide free in solution. This observation (similar to that seen with [adenine U-^{14}C] NAD^+ on Whatman GF/C filters: Ch. 3.2.3.1.) is more clearly illustrated in the following sections and is discussed in Ch. 3.4.

3.3.3.3. Recovery of $[^3H]$ and $[^{14}C]$cAMP from PEI-cellulose Particles

The results presented above indicated that 2 ml of 25mM-$MgSO_4$ per 10 ml of scintillator was able to elute cAMP quantitatively from PEI-cellulose particles while at the same time giving acceptable counting efficiencies of 22% and 55% for $[^3H]$ and $[^{14}C]$ respectively. The validity of this conclusion was tested by adsorbing increasing amounts of either $[8-{}^3H]$ cAMP or [adenine U-^{14}C] cAMP to 50 mg of PEI-cellulose particles in scintillation vials and adding 2 ml of 25mM-$MgSO_4$ and 10 ml of scintillator as above. An identical series of vials for each isotope containing the radioactive cAMP free in solution was also prepared, and all vials were counted on the double isotope programme of the Searle Mk. III for 10 min.

Fig. 3.10. shows that for $[^3H]$ cAMP, identical d.p.m. values were recorded whether the nucleotide was free in solution, or whether initially adsorbed on PEI-cellulose particles and subsequently eluted with 2 ml of 25 mM-$MgSO_4$. Although $[^{14}C]$cAMP, as noted above, produced higher count rates after adsorption and elution from PEI-cellulose (fig. 3.11.), under both conditions there was a linear increase in d.p.m. values with increasing amounts of isotope added.

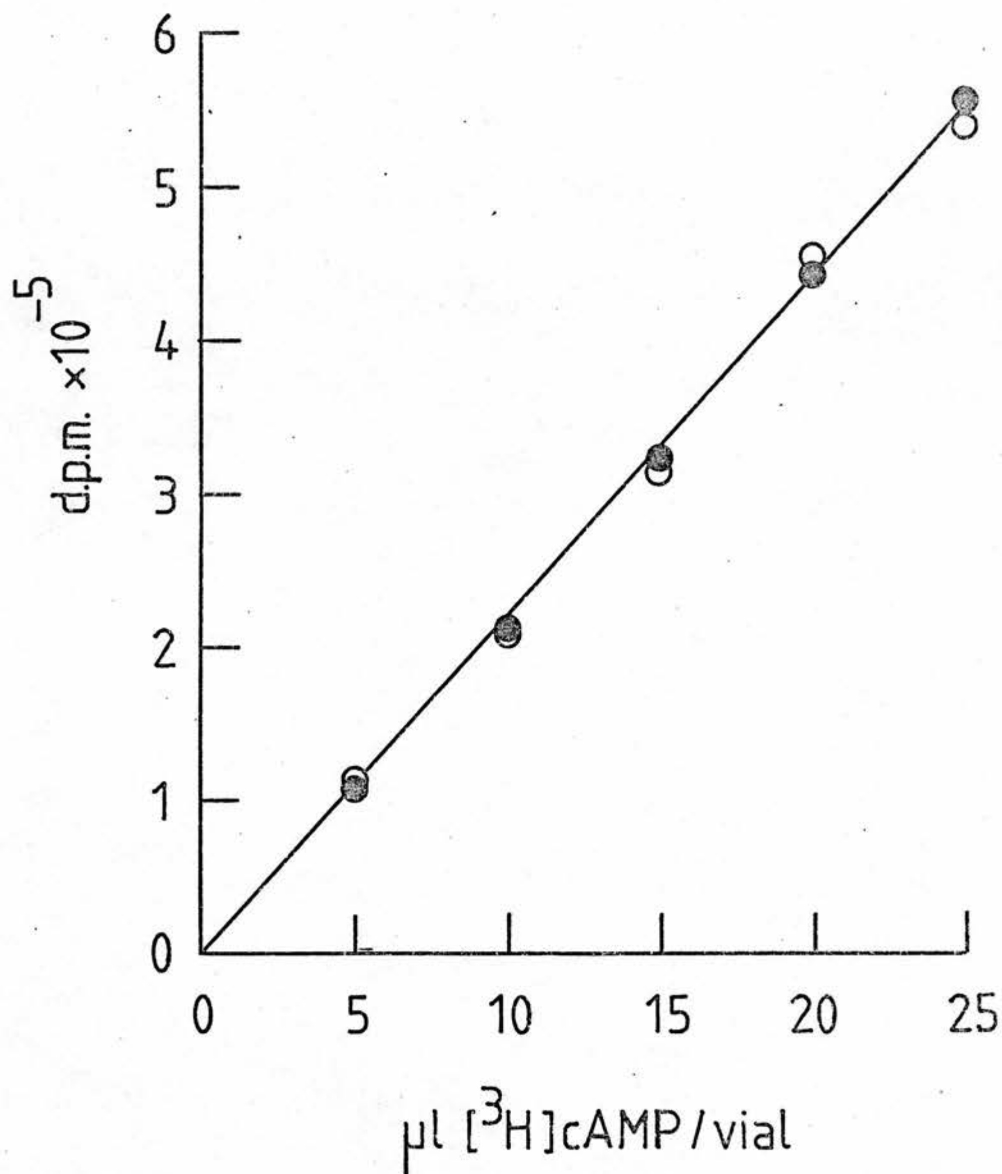


Fig.3.10. Recovery of [^{3}H]cAMP from PEI-cellulose particles.

Scintillation vials contained increasing volumes of 10µCi/ml [8-^{3}H]cAMP either free in solution (○—○) or adsorbed to 50mg of PEI-cellulose particles (●—●). 2ml of 25mM-$MgSO_4$ were added followed by 10ml of scintillator and vials were counted for 10min.

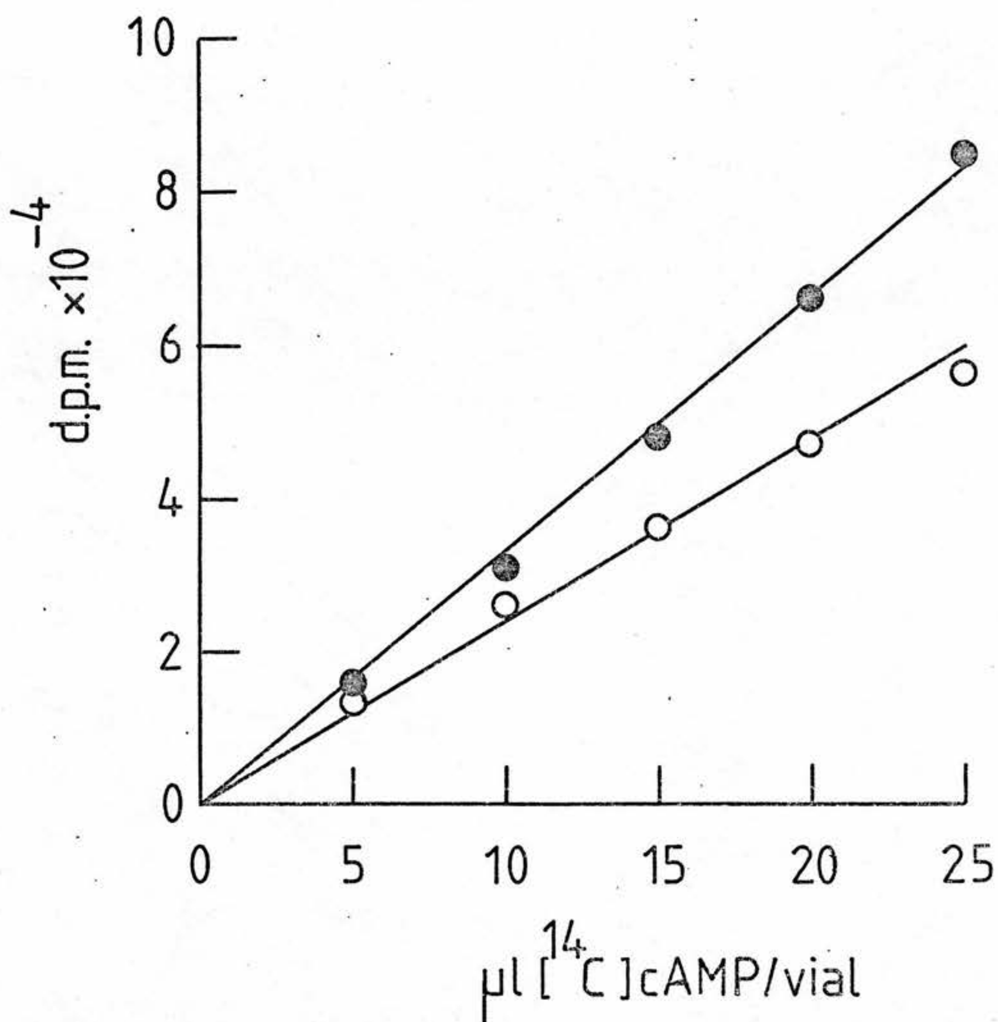


Fig.3.11. Recovery of [^{14}C]cAMP from PEI-cellulose particles.

Scintillation vials contained increasing volumes of [adenine U-^{14}C]cAMP either free in solution (o—o) or adsorbed to 50mg of PEI-cellulose particles (●—●). 2ml of 25mM-$MgSO_4$ were added followed by 10ml of scintillator and vials were counted for 10min.

3.3.3.4. Effect of PEI-cellulose particles on $[^3H]$ and $[^{14}C]$ counting characteristics.

The experiments described above were conducted with a constant amount (50 mg) of PEI-cellulose particles in each vial. Although possible, it would be extremely laborious to ensure that, in routine assays, all vials contained exactly the same amount of cellulose particles. The effect of varying the content of PEI-cellulose over a wide range was therefore studied.

Series of vials were prepared containing either $[8\text{-}^3H]$ cAMP (~100 nCi) or $[\text{adenine U-}^{14}C]$cAMP (~ 20 nCi) adsorbed to increasing amounts (0-100 mg) of PEI-cellulose particles in scintillation vials. 2 ml of 25 mM-$MgSO_4$ were added followed by 10 ml of scintillator and vials were counted for 10 min. in the double isotope programme of the SearleMk. III.

Counting efficiencies for $[^3H]$ and $[^{14}C]$cAMP were found to vary in a similar fashion (Fig. 3.12.) with each displaying a curious peak at 20 mg of cellulose per vial. The corresponding d.p.m. values (Fig. 3.13) indicated that the amount of PEI-cellulose present had little effect on $[^3H]$ cAMP counting and the recorded d.p.m. were similar at all amounts of cellulose tested. The counting characteristics of $[^{14}C]$ cAMP however varied in a complex fashion with PEI-cellulose quantities. As little as 10 mg of cellulose per vial was sufficient to cause the apparent increase in count-rate noted earlier and from 20-100 mg of cellulose per vial,d.p.m. values decreased gradually at a rate of about 40 d.p.m. mg of cellulose added. These observations are discussed fully in Ch. 3.4.

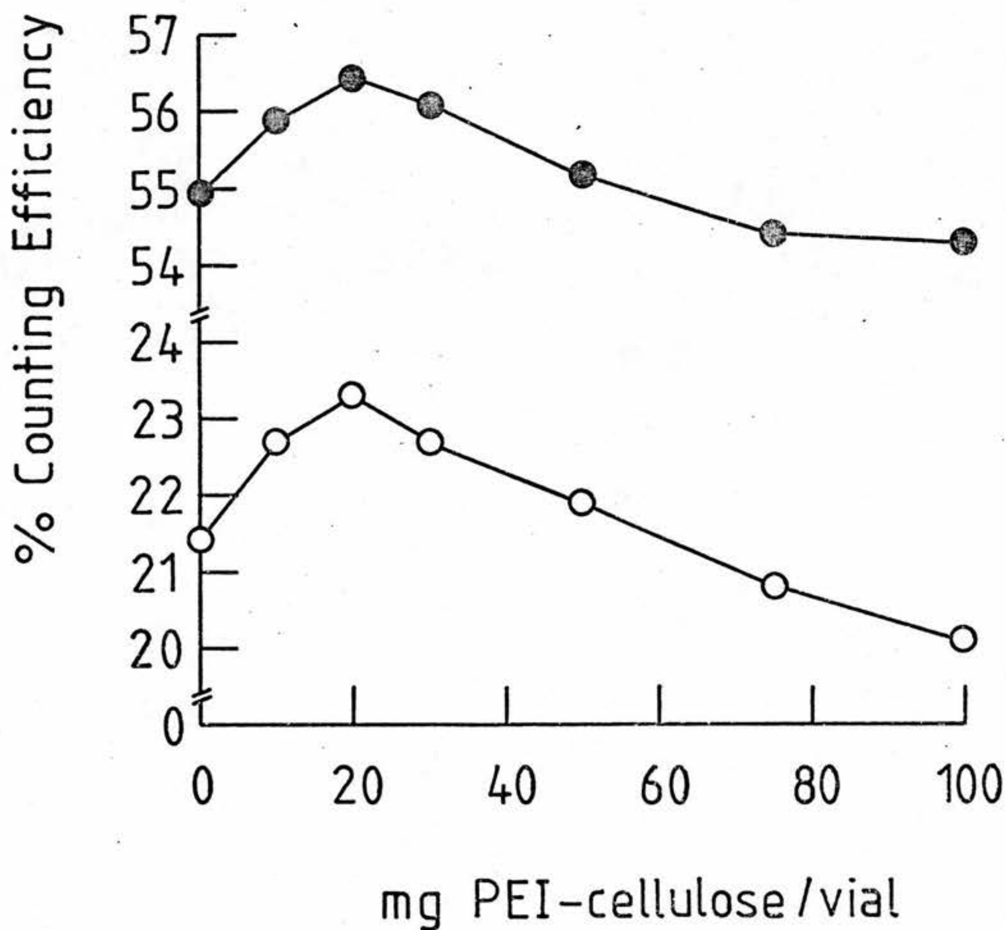


Fig.3.12. Effect of PEI-cellulose particles on the counting efficiencies of [^{3}H] and [^{14}C]cAMP.

Scintillation vials contained 100nCi of [8-^{3}H]cAMP (o—o) or 10nCi of [adenine U-^{14}C]cAMP (●—●) adsorbed to the indicated amounts of PEI-cellulose particles. 2ml of 25mM-$MgSO_4$ were added followed by 10ml of scintillator and vials were counted for 10min.

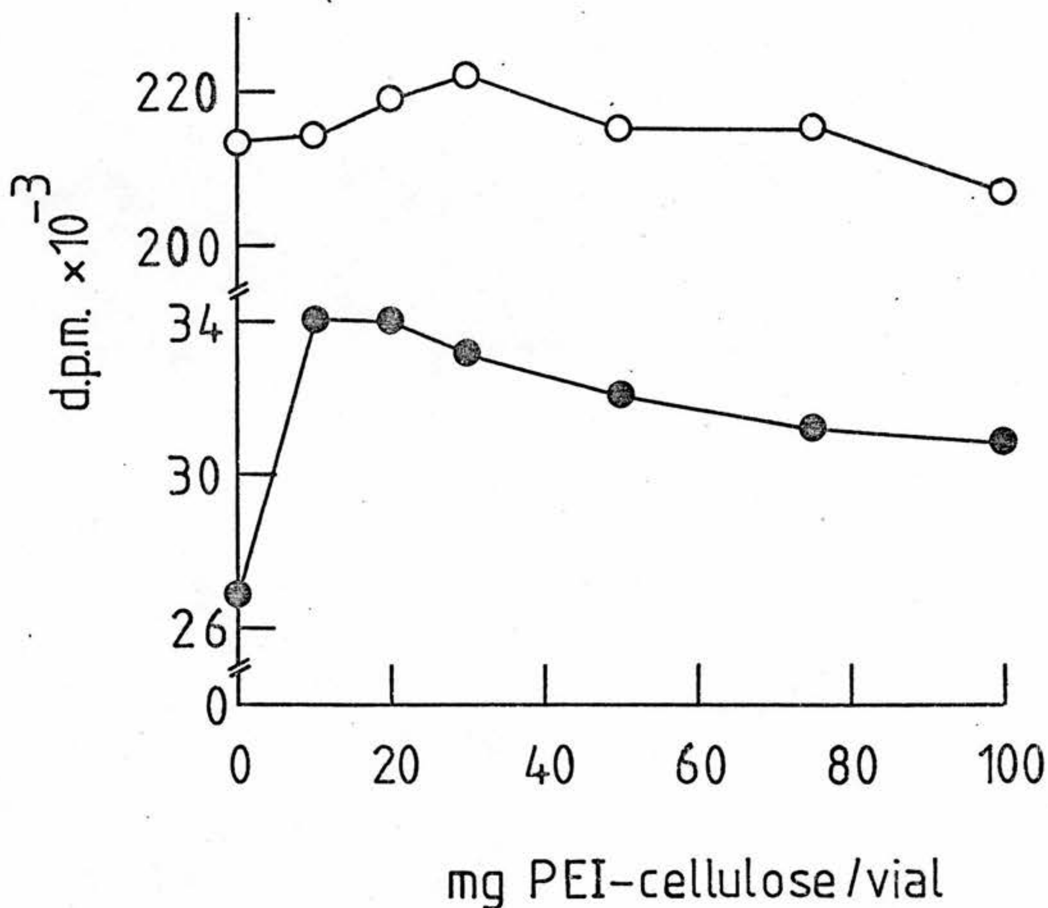


Fig.3.13. Effect of PEI-cellulose particles on the count rate of [^{3}H] and [^{14}C]cAMP.

Scintillation vials contained 100nCi of [8-^{3}H]cAMP (o—o) or 10nCi of [adenine U-^{14}C]cAMP (●—●) adsorbed to the indicated amounts of PEI-cellulose particles. 2ml of 25mM-$MgSO_4$ were added followed by 10ml of scintillator and vials were counted for 10min.

3.3.3.5. Simultaneous determination of $[^{3}H]$ and $[^{14}C]$cAMP.

The counting conditions determined above for $[^{3}H]$ and $[^{14}C]$ counting in separate vials were tested for their suitability in measuring both isotopes simultaneously in the same vial. Solutions of the two isotopes were mixed in equal amounts to give a solution which was approximately 5nCi/10µl in $[^{3}H]$ cAMP and 1 nCi/10µl in $[^{14}C]$ cAMP. Increasing volumes of this mixture (0-100µl) were adsorbed onto 50 mg of PEI-cellulose particles in scintillation vials, 2ml. of 25mM-$MgSO_4$ were added followed by 10ml. of scintillator, and vials were counted for 10min.

Fig. 3.14 shows that, for both isotopes, a linear increase in d.p.m. values was obtained with increasing volume of double-isotope mixture added and the conditions were therefore considered to be suitable for the simultaneous determination of $[^{3}H]$ and $[^{14}C]$cAMP in adenylate cyclase assay samples.

3.3.3.6. Migration of cAMP on PEI-cellulose TLC plates

In routine work, adenylate cyclase assay samples were applied to TLC plates along with [adenine U-^{14}C] cAMP and unlabelled carrier cAMP, and the position of cAMP after development of the chromatograms was determined by visualisation of the carrier under short-wave ultraviolet light. To ensure that this was a valid procedure a mixture of $[^{3}H]$ cAMP (~ 250 nCi) and $[^{14}C]$ cAMP (~ 50 nCi) were applied to a TLC plate along with 5µl of 10 mM-cAMP and after development in Solvent I as described in Ch. 3.2.2.4., 1 cm- long segments up to about 1 cm behind the cAMP spot were cut out separately

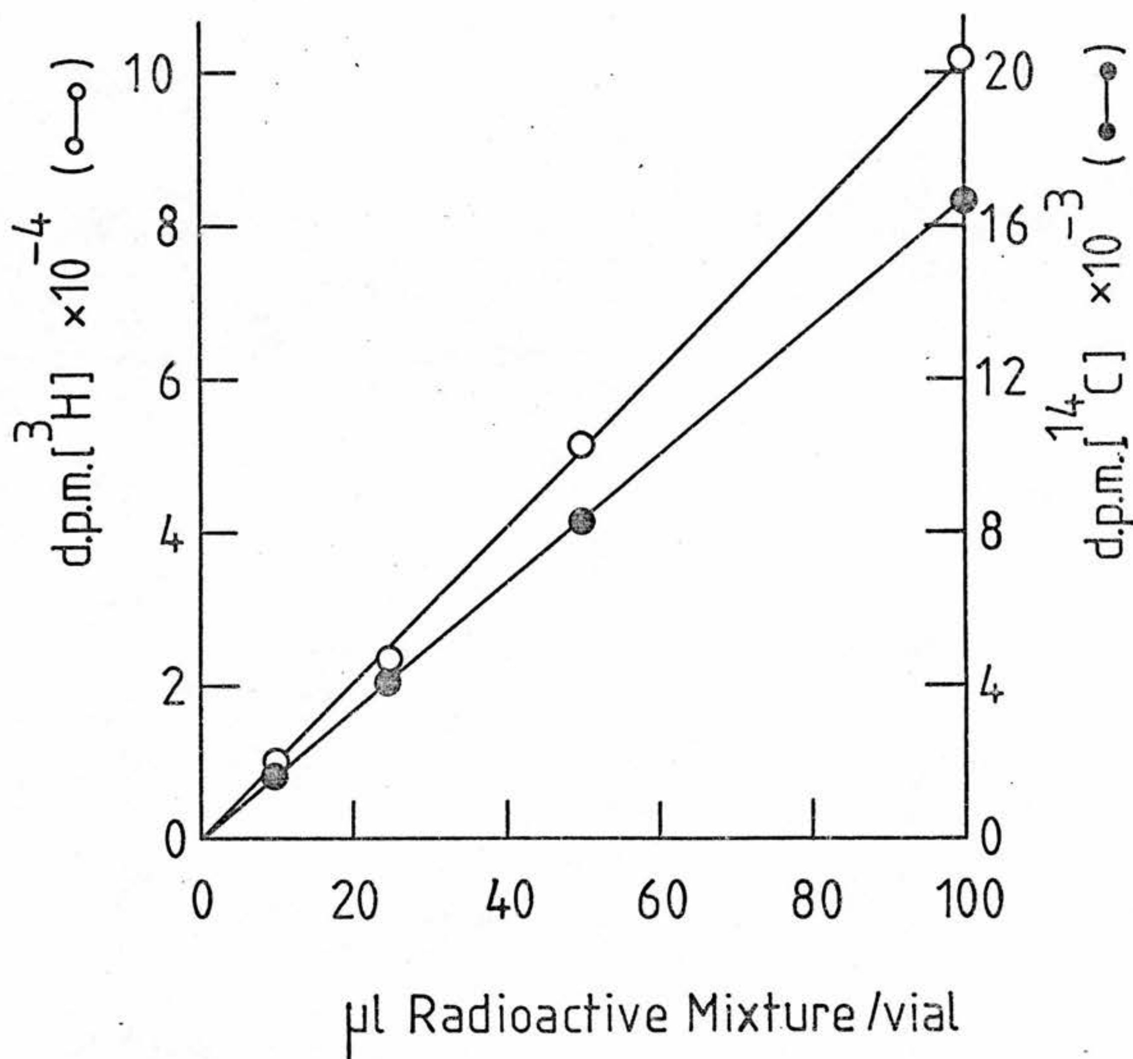


Fig.3.14. Simultaneous counting of [^{3}H] and [^{14}C]cAMP under adenylate cyclase assay conditions.

Scintillation vials contained increasing amounts of both [8-^{3}H]cAMP and [adenine U-^{14}C]cAMP adsorbed to 50mg of PEI-cellulose particles. 2ml of 25mM-$MgSO_4$ were added followed by 10ml of scintillator and vials were counted for 10min.

and counted as described in Ch. 3.2.2.5. After development in Solvent II, the position of cAMP under ultraviolet light was noted and the remainder of the chromatogram was cut out and counted as above. (Fig. 3.15.)

Both [^{3}H] and [^{14}C] cAMP comigrated with unlabelled carrier cAMP in the complete two-solvent system. In solvent I, cAMP was visualised between R_f values 0.49-0.58. The R_f value of cAMP in Solvent II could not be calculated since in this system, the solvent front is allowed to flow off the end of the layer overnight. The total d.p.m. recovered from the plate was 788,031 for [^{3}H] and 83,949 for [^{14}C] and since a duplicate aliquot of the mixture applied to the plate adsorbed directly onto PEI-cellulose in a scintillation vial and counted as described in Ch. 3.2.2.5. gave 804,739 and 87,746 d.p.m. for [^{3}H] and [^{14}C] respectively, the total recovery of counts from the plate was 98% for [^{3}H] and 96% for [^{14}C] cAMP.

A similar experiment in which an aliquot of [8-^{3}H] ATP was applied to a TLC plate and developed in Solvent I only, indicated that ATP remained at the origin during Solvent I development. (Fig. 3.16.)

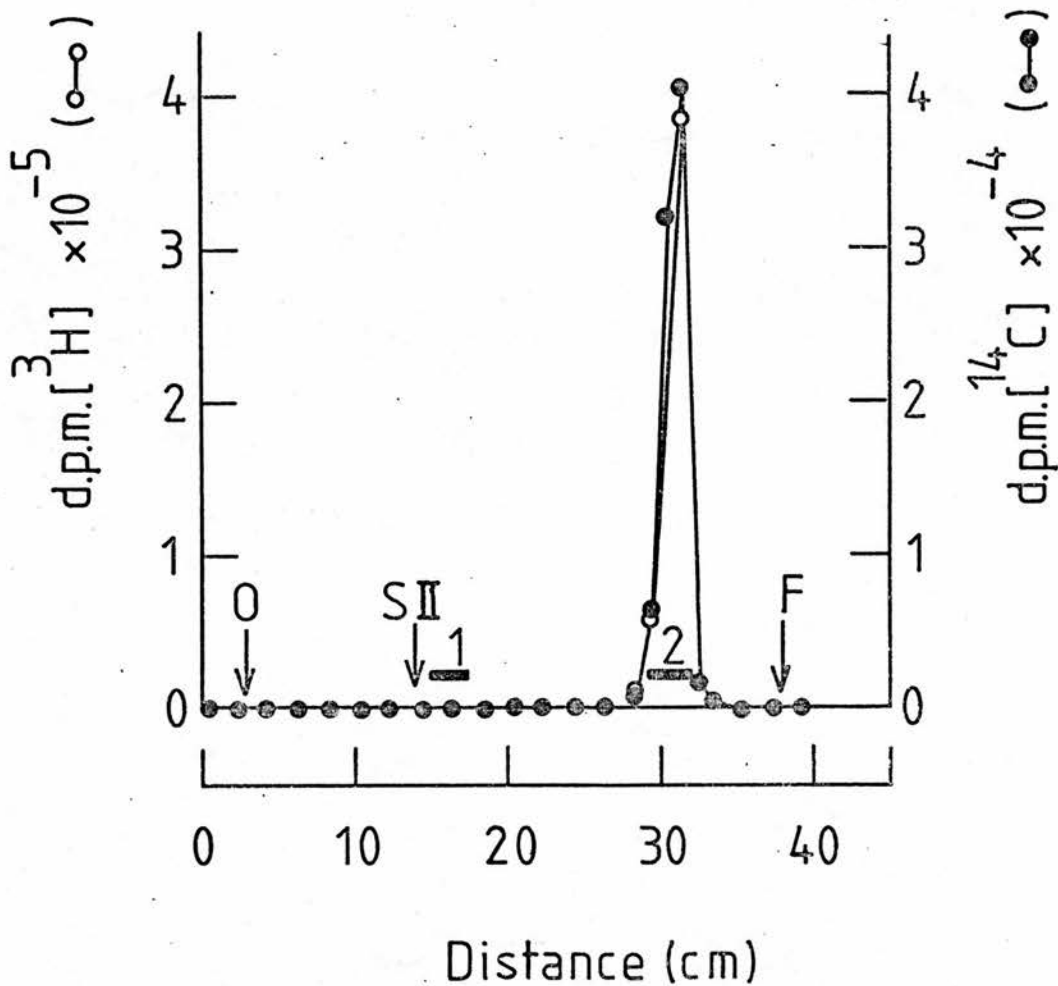


Fig.3.15. Migration of [^{3}H] and [^{14}C]cAMP with unlabelled cAMP on PEI-cellulose thin layers.

[8-^{3}H]cAMP (250nCi) and [adenine U-^{14}C]cAMP (50nCi) were applied to a TLC plate along with 5µl of 10mM-cAMP as described in Ch.3.3.2.4. After development in both solvents, 1cm-long segments of the layer were cut out and counted for [^{3}H] and [^{14}C].

O: origin.

E: end of layer.

SII: start of solvent II.

1 and 2: positions of unlabelled cAMP after development in solvents I and II respectively.

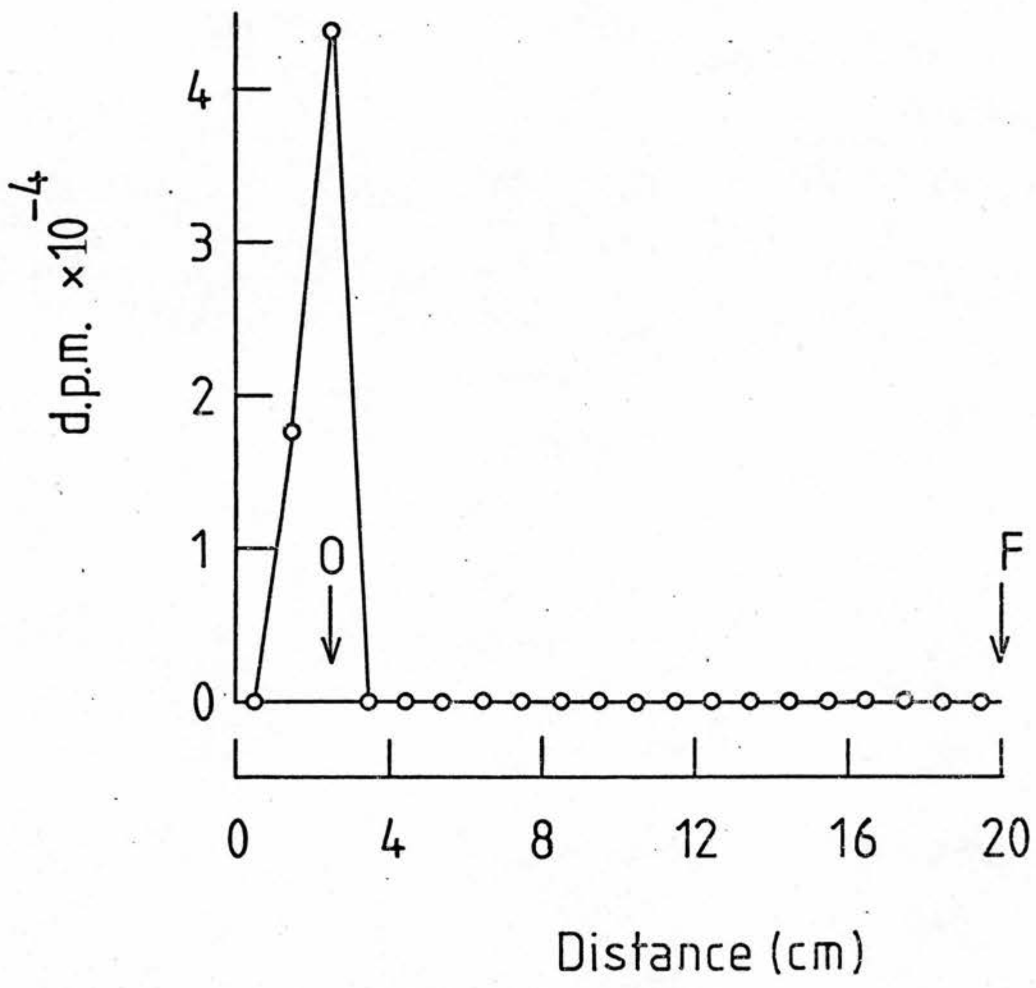


Fig.3.16. Migration of ATP in solvent I on PEI-cellulose thin layers.

50nCi of [8-^{3}H]ATP in a volume of 10µl containing 5mM-unlabelled ATP was applied to a PEI-cellulose thin layer and developed in solvent I as described in Ch. 3.3.2.4. After drying, 1cm-long segments of the layer were cut out and counted.

O: origin.

F: solvent front.

3.4. Discussion

The NADase assay method described in Ch. 3.1. was both rapid and easily performed and DE-81 paper chromatography provided a clear separation of both hydrolysis products of NAD^+ (nicotinamide and ADP-ribose) from each other and from unreacted NAD^+. The data in Fig. 3.1. indicate that NADase activities could theoretically be measured with either [carbonyl-^{14}C] NAD^+ or [adenine U - ^{14}C] NAD^+ by determining production of [^{14}C] nicotinamide or [^{14}C] ADP-ribose respectively. This was in fact confirmed experimentally (results not shown) and estimation of ADP-ribose production could be facilitated by increasing the concentration of formic acid in the developing solvent from 0.12M to 0.3M which resulted in NAD^+ and nicotinamide both migrating near the solvent front leaving ADP-ribose with an R_f of about 0.3 - 0.4. For routine work however, the less expensive [carbonyl - ^{14}C] isotope of NAD^+ was employed.

[^{14}C] counting characteristics were not altered when NAD^+ was adsorbed to DE-81 paper (Fig. 3.2.) and the only potential drawback of the method was the instability of NAD^+ on the paper (Fig. 3.3.) In practise, however, this was not a serious disadvantage since, in most experiments, all samples were chromatographed simultaneously immediately after application to the strips. In assays with large numbers of samples however, when strips were chromatographed in batches of 20-40, corrections for non-specific breakdown on DE-81 paper were made by including a duplicate blank assay for each batch. This procedure was justified by the linearity of NAD^+ breakdown with time in Fig. 3.3. and NADase activities of enzyme samples were identical

after appropriate blank subtraction, between samples chromatographed immediately and samples chromatographed several hours after application to the strips.

The ADP-ribosyltransferase assay method described in Ch. 3.2. was also easily performed and efficient removal of non-covalently attached [^{14}C] material from protein -[^{14}C] ADP-ribose complexes was achieved with 2 x 20 ml washes of the sample on GF/C filters. (fig. 3.6.) As the results in Fig. 3.6. indicate, assay sensitivity (blank approaching zero) was related to the number of washing steps performed, but since little increase in sensitivity was obtained with more than 2 washes, and since physical damage to the filters sometimes occurred after repeated washing, filters were routinely washed with 2 x 20 ml aliquots of 5% TCA in the work reported here.

Unlike the counting characteristics of [^{14}C] NAD^+ on DE-81 chromatography paper, adsorption of [^{14}C] NAD^+ on GF/C filters results in an apparently more than quantitative recovery of d.p.m. from the filters (Fig. 3.4.) This was most likely an artifact of the counting method employed. During computation of d.p.m. values by the Searle Mk. III, several quantities are taken into account, including the number of counts appearing in the tritium channel and the resulting calculated channels ratio. In the presence of GF/C filters, counts in the tritium channel were always higher, leading to a decrease in the channels ratio. Apparently therefore, the emission spectrum of [^{14}C] adsorbed to GF/C filters has been distorted slightly causing a greater proportion of counts to appear in the tritium channel. Because of this, the counter will automatically set new discriminator and attenuation levels and since all of these

factors are accounted for in calculating the d.p.m. values, this may result in the apparent observed increase in count-rate. Since d.p.m. increased linearly with amount of $[^{14}C]$ added to the filters (Fig. 3.4.) this is not a serious complication in itself but it does emphasise the importance of preparing standard vials under identical conditions to those prevalent in the experimental vials. Only then can a standard vial be used for direct calculation of number of d.p.m. added to each assay in an experiment.

The presence of precipitated protein, even in large amounts, had no effect on the observed count rate of $[^{14}C]$ NAD^+ on GF/C filters (Fig. 3.5.) and the method was therefore suitable for estimating $[^{14}C]$ ADP-ribose incorporation into proteins under conditions where the amount of protein applied to filters is not necessarily constant.

The adenylate cyclase assay method described in Ch. 3.3. involved the use of $[8-{}^{3}H]$ ATP as substrate with subsequent purification of $[8-{}^{3}H]$cAMP on PEI-cellulose thin layer chromatograms. Previous attempts to purify cAMP by TLC methods were not always successful and the systems used were often not well characterised. Bar and Hechter (1969) described purification of cAMP on PEI-cellulose thin layers but because only a one-dimensional run with one solvent was used, the authors were forced to employ $[\alpha-{}^{32}P]$- ATP as substrate in order to minimise radioactive contamination. Dinghe et al (1969) using silica gel TLC reported separation of cAMP from only ATP, ADP, 5'-AMP and adenosine. Similarly Upton (1970) who used the tendency of borate ions to form complexes with the 2', 3'-cis diol grouping on simple sugars, developed a method for cAMP purification on

tetraborate-impregnated silica gel thin layers, but again separation of cAMP from only a small number of contaminants was reported. Shimizu et al (1969) also used silica gel TLC and described separation of cAMP from a larger number of compounds but separation from guanine and hypoxanthine was not achieved. The methód was suitable for work on brain tissue which lacks the enzyme adenine deaminase, but would be of limited use with other tissues containing this activity such as rat liver. Woods and Waitzman (1970) have also described purification of cAMP by TLC on a silicic acid-glass microfibre matrix.

A major reason for adopting the PEI-cellulose TLC method of Keirns et al (1974) in this work, was the extensive characterisation of the system carried out by these workers, who demonstrated separation of cAMP from virtually all possible contaminants arising in an adenylate cyclase assay. Development of the layers with Solvent I (Methanol : 1M-ammonium acetate, 5 : 2), left the highly charged polyanionic species ATP, ADP, GTP, GDP (and although not described, probably also ITP and IDP) at or near the origin owing to their high affinity for the polycationic PEI matrix. 2'-AMP, 2'-deoxy-AMP, 3'-AMP, 2'-GMP, 2'deoxy 5'-GMP, 3'-GMP, 5'-GMP and IMP were also significantly retarded and migrated with a lower R_f than cAMP. All of these major contaminants were therefore discarded by removing this part of the layer after Solvent I development.

The neutral contaminants adenosine, 2'- deoxyadenosine, hypoxanthine, adenine, inosine, guanine, guanosine, 2'-deoxyguanosine, and xanthosine, which all migrated with similar or slightly greater

R_f values to that of cAMP in Solvent I were carried well ahead of cAMP in Solvent II (nButanol : Acetic acid: H_2O, 2:1:1) which also served to separate cAMP from such closely related compounds as 2'-deoxy cAMP, 2',3'-cAMP, cGMP, and 2,'3' - cGMP. The only other possible major contaminant not described, xanthine, probably migrates in a similar fashion to adenine and guanine as a result of its structural similarity to these compounds.

Since cAMP was therefore shown to be separated efficiently from all major contaminants, characterisation of the separation system was not repeated here and it was only considered necessary to check the migration of $[^3H]$ATP in Solvent I and of cAMP in Solvents I and II. As shown in Fig. 3.16 $[^3H]$ ATP remained at the origin during Solvent I development and cAMP, $[^3H]$ cAMP and $[^{14}C]$ cAMP all co-migrated in the complete two-solvent system (Fig. 3.15).

However, it was essential to characterise fully the counting conditions for $[^3H]$ and $[^{14}C]$cAMP after removal of samples from the TLC plates. Elution of both isotopes of cAMP from PEI-cellulose particles was complete when 1 - 2 ml of 25mM-$MgSO_4$ were added to the particles in a vial and gently shaken for a few seconds before addition of scintillator (Fig. 3.9.). The low affinity of cAMP for PEI-cellulose was therefore of great advantage and the use of more complicated and tedious elution methods (Randerath, 1967) was unnecessary. A small volume of $MgSO_4$, although probably sufficient to solubilise cAMP produced a cloudy precipitate on addition of 10 ml of scintillator and the loss of counts noted with radioactive cAMP free in solution under this condition (Fig. 3.9.), was probably due

to a proportion of the cAMP being out of solution in the heterogeneous mixture. Addition of more $MgSO_4$ caused the mixture to become clear again and on addition of 3 - 5 ml, a semi-solid gel state was obtained. There was therefore an optimum aqueous volume of 1 - 3 ml of $MgSO_4$ solution per 10 ml of scintillator and in routine assays, 2ml was employed.

With 2 ml of 25mM-$MgSO_4$ per 10 ml of scintillator, recovery of $[^3H]$cAMP from PEI-cellulose was quantitative but recovery of $[^{14}C]$cAMP was apparently more than quantitative (figs. 3.10 and 3.11 respectively). This is a completely analogous phenomenon to that noted above with $[^{14}C]NAD^+$ on GF/C filters and can be explained in the same way as discussed previously. Since the only purpose of $[^{14}C]$-cAMP in this system was to monitor recoveries from the TLC plates, the absolute d.p.m. values were not of great importance. It was sufficient merely that d.p.m. increased linearly with increasing amounts of $[^{14}C]$cAMP initially adsorbed to PEI-cellulose and as Fig. 3.11. shows, this relationship was obtained. However, it was essential that for each experiment, an aliquot of the $[^{14}C]$cAMP solution applied to the TLC plates along with each assay sample, was adsorbed to PEI-cellulose particles in a vial and then eluted and counted as for all other samples. This standard vial could then be used as a true estimate of 100% recovery to which all other experimental samples could be related.

$[^{14}C]$cAMP counting characteristics varied also with the relative amount of PEI-cellulose particles present (Fig. 3.13.) The 'increase' in count-rate observed initially by adding cellulose was progressively reduced on addition of more cellulose between 20 - 100mg per vial. This corresponds to a decrease in count-rate, relative to

the peak rate at 20 mg/vial, of about 0.15% per mg of cellulose added, and was a potentially more serious complication since it would be extremely difficult and tedious to ensure that all vials in an experiment contained exactly the same amount of PEI-cellulose particles. However by cutting out an equal area of TLC plate for each sample in an experiment, the amount of cellulose in each vial did not vary by more than a few milligrams and was usually about 15-20 mg per vial. In this region count rate is independent of the amount of PEI-cellulose and should any particular vial contain significantly more cellulose, a reduction in count-rate of 0.15% per mg of cellulose would only cause a decrease of 7-14 d.p.m for each additional mg of cellulose, (5,000 - 10,000 d.p.m. of ^{14}C cAMP were routinely applied to TLC plates). Errors due to the complex counting characteristics of $[^{14}C]$ cAMP were therfore considered to be insignificant and with appropriate standards and adequate precautions, its use as a monitor of TLC recoveries was judged to be valid. $[^{3}H]$ cAMP counting characteristics were not altered significantly in the presence of varying amounts of PEI-cellulose particles (Fig. 3.13.)

The method therefore appeared to be suitable for quantitative adenylate cyclase assay determinations and had several distinct advantages over other existing methods. The PEI-cellulose layers were themselves inexpensive and easily prepared, and the ability to analyse 12 samples per TLC plate enabled many samples to be handled without great technical difficulty. Pre-washing of the thin layers as described by Randerath (1967), a long and tedious procedure for the large plates used in this assay, was found to be unnecessary. Presumably, washing would be of greater importance when samples were to be eluted and estimated by U.V. absorption

when impurities on the layers may cause interference.

A major advantage of the method is that [8-^{3}H]- ATP can be used as substrate since, although the number of possible contaminants is maximal with this isotope, the purification system is sufficiently sophisticated to eliminate all of the major charged and neutral contaminants. The use of [^{3}H] ATP, rather than [α - ^{32}P] ATP makes the method both considerably less expensive and much less hazardous to use routinely. The long half-life of [^{3}H] over that of [^{32}P] is also advantageous since samples can be left for long periods if necessary before counting, and corrections for decay of any particular batch of ATP during use are unnecessary.

As noted earlier the sensitivity of a radioactive adenylate cyclase assay is increased by incubating with high specific radioactivity ATP in as small a volume as possible. Since a maximum of 10 µl is required for application to TLC plates, assays can easily be carried out in very small volumes (25µl or less) and aliquots taken directly for analysis in a concentrated form, without the need for dilution and the errors involved therein, before application to columns etc.

Assay sensitivity is proportional to the levels of d.p.m. in the blanks and sensitivity is therefore maximal as the blank approaches zero. Using [α - ^{32}P] ATP as substrate with purification of cAMP by sequential Dowex-alumina chromatography, assay blanks equivalent to only 0.0002% of the total [^{32}P] substrate added were reported (Salomon et al, 1974). In the method described here blank values varied from 0.0025 to 0.01% of the total [^{3}H] substrate added

and although the technique was therefore at least 10 times less sensitive than the method of Salomon and coworkers, it was considered to be adequate for this work, where low basal activities of adenylate cyclase were compared with the usually much greater activities after cholera toxin treatment.

Chapter 4. Purification of cholera toxin

4.1. Introduction

Finkelstein and LoSpalluto (1969) were the first workers to report the production of purified cholera toxin. After growing the highly toxigenic 569B Inaba strain of V.cholerae in multi-litre fermenter cultures on a synthetic medium supplemented with casamino acids (syncase medium), cholera toxin was isolated from the culture filtrate by ammonium sulphate fractionation, DEAE-cellulose column chromatography, and gel filtration on columns of Agarose A-5m and Sephadex G-75. The final gel filtration step consisted of two columns linked in series and served to separate whole cholera toxin from choleragenoid. The same workers then reported several modifications to the method which enabled an improvement in yield (Finkelstein and LoSpalluto, 1970) and which included the substitution of several ultrafiltration steps in place of the ammonium sulphate and DEAE-cellulose chromatographic steps in the original method. The toxin isolated by this method was homogeneous as judged by gel filtration, immunoelectrophoresis, ultracentrifugation, ion-exchange chromatography, and immunologic criteria (Finkelstein and LoSpalluto, 1969).

It is of interest to note that the purification of cholera toxin was facilitated by the anomalous behaviour of the toxin on gel filtration media. On Agarose A-5m for example, the toxin behaved as if it had a molecular weight of less than 20,000 daltons and this property enabled an easy separation of toxin from all other proteins of similar molecular weight. The same property was also of great

use in the purification scheme described in this report (see Ch. 4.2.3.).

The quality and yield of the cholera toxin obtained by the purification method of Finkelstein and LoSpalluto is reflected in the almost universal use of toxin prepared in this way in cholera research. However the method involves the use of several large scale column chromatographic steps including a double gel filtration step on Sephadex G-75, during which excessive dilution of toxin fractions will occur. For this reason the method is best suited to the purification of cholera toxin from large amounts of starting material. Since the amount of material available for this work was limited, a simpler purification scheme with fewer stages was desirable. As described below, it proved possible to isolate cholera toxin which was homogeneous as judged by SDS-polyacrylamide gel electrophoresis, with a three-step purification method involving ammonium sulphate fractionation, ion-exchange chromatography on DEAE-cellulose, and gel filtration on Ultrogel AcA-44.

4.2. Results

The results presented below are taken from a representative example of a toxin purification. Results obtained in all other purifications were similar although there were some variations between different batches of crude starting material (see Ch. 4.3.).

4.2.1. Ammonium sulphate fractionation.

A preliminary experiment was conducted on a small scale to determine the optimum conditions for precipitation of cholera toxin by ammonium sulphate from culture filtrates of V. cholerae. 1 ml of the crude toxin supernatant obtained as described in Ch.2.1. was made 30% of room temperature saturation in ammonium sulphate and after 20 min. of stirring, precipitated protein was recovered by centrifugation and resuspended in 0.5 ml of 50mM-Tris/HCl, pH 7.4. Ammonium sulphate was then added to the supernatant to a final concentration of 40% of room temperature saturation and the procedure was repeated. In this way, resuspended precipitates from 0-30%, 30-40%, 40-50%, 50-60%. 60-70%, 70-90%, and supernatant from the final fractionation step were obtained and all were tested for antigenicity against cholera anti-serum as described in Ch. 2.2.1. Cholera toxin was detected in the precipitates from the30-40%, 40-50% and 50-60% fractionations and a 30-60% ammonium sulphate cut was therefore indicated for the precipitation of cholera toxin in large-scale purifications. However since the amount of protein precipitated in the 0-30% fractionation was almost negligible this step could be omitted and a direct 0-60% fractionation did not decrease the puritiy of the final cholera toxin product.

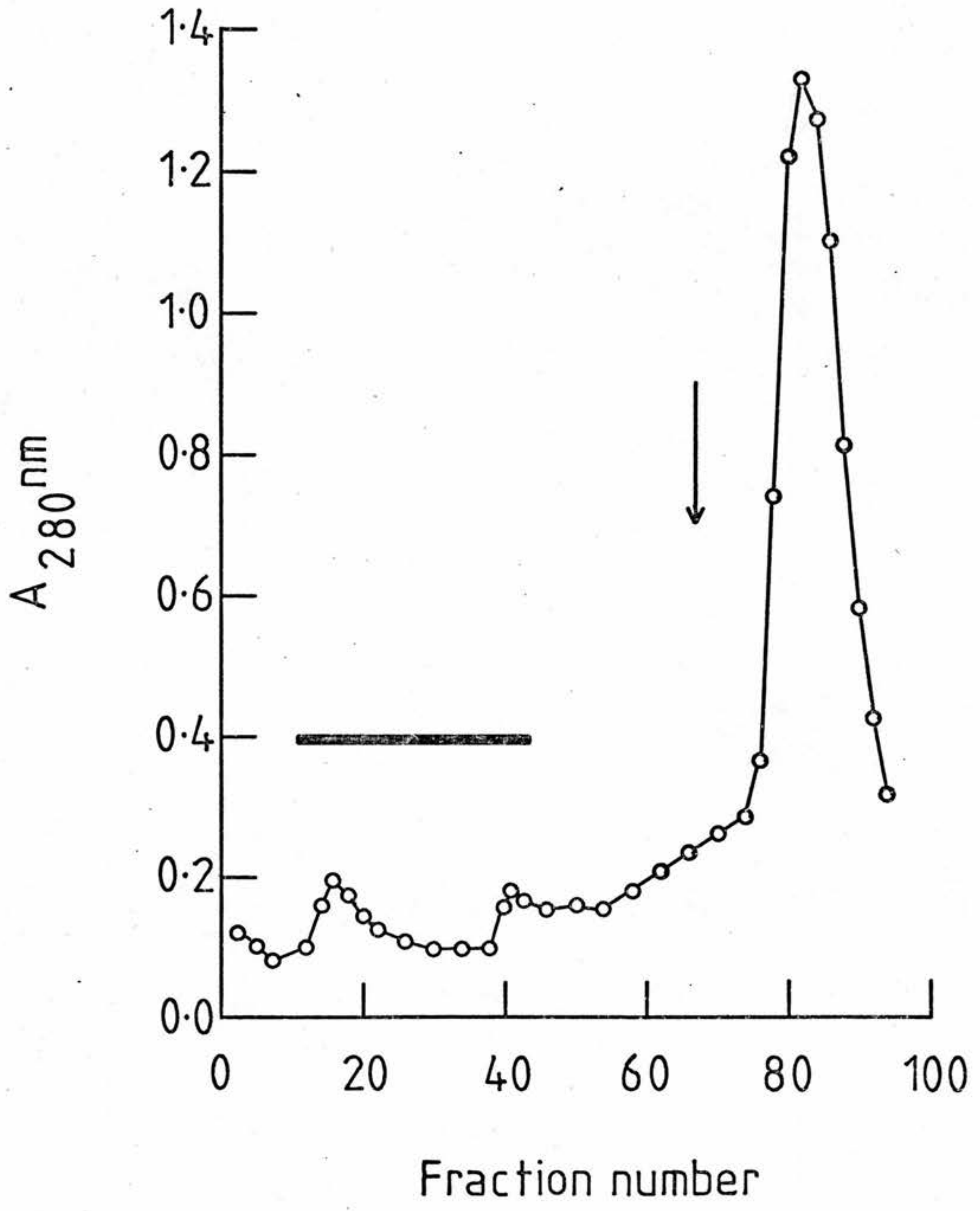
4.2.2. DEAE-cellulose chromatography

Conditions for the elution of cholera toxin from DEAE-cellulose were established initially on a small scale by the application of 1 ml of crude toxin supernatant to a small column of DEAE-cellulose in a Pasteur pipette equilibrated in 50mM-Tris/HCl, pH 7.4. The column was eluted with 3 x 1 ml portions of the same buffer containing 5,10,50,100,250, and 500 mM-NaCl and 1 ml fractions were collected, all of which were tested for antigenicity against cholera anti-serum as described in Ch. 2.2.1. Cholera toxin appeared in three of the column fractions corresponding to elution with 50mM (two fractions) and 100 mM (one fraction) NaCl, and for large scale purifications, a linear 0-100mM-NaCl gradient was therefore employed.

The results of a large-scale elution from DEAE-cellulose of the toxin-containing fraction from ammonium sulphate treatment, are shown in Fig. 4.1. which illustrates the fortuitous phenomenon that the majority of the contaminating proteins in the preparation tend to adhere tightly to DEAE-cellulose at pH 7.4 and are only eluted with a 200mM-NaCl wash after elution with the gradient. In contrast, cholera toxin eluted in a low, broad peak near the beginning of the gradient and these fractions were pooled and concentrated as described in Ch. 2.1.2.

Figure 4.1. Elution of cholera toxin from DEAE-cellulose

Cholera toxin-containing fractions after ammonium sulphate fractionation were dialysed against 50mM-Tris/HCl, pH 7.4 and applied to a column (2.5 cm x 7 cm) of DEAE - cellulose equilibrated in the same buffer. Elution was performed at a flow rate of 20 ml/hr with a linear 0 - 100 mM-NaCl gradient and 3 ml-fractions were collected. The arrow indicates the end of the linear NaCl gradient and the commencement of a 200mM-NaCl wash. Cholera toxin (shaded bar) was detected in the column fractions as described in Ch. 2.2.1.



1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
A_{280} nm
0
20
40
60
80
100
Fraction number

4.2.3. Gel filtration on Ultrogel AcA-44

Prior to use, a column (2cm x 92 cm) of Ultrogel AcA-44 (molecular weight range: 12,000-130,000) was set up, equilibrated in 0.1M-Tris/HCl, 0.1M-NaCl, pH 7.4, and characterised for molecular weight determinations as summarised in Figs. 4.2. to 4.4.

For determination of the included and excluded volumes a mixture of blue dextran and DNP-lysine was passed through the column and as Fig. 4.2. shows, the void volume (V_o) was calculated to be 87.4 ml while DNP-lysine eluted in a total excluded volume of 273.6ml. The latter value agrees well with the total column volume (V_c) calculated from the dimensions of the column, which was 289 ml.

The column was then calibrated with a mixture of three standard proteins; bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), and soya bean trypsin inhibitor (21,000 daltons). The results in Fig. 4.3. illustrate that the three proteins were eluted as three distinct peaks with elution volumes (V_e) of 120, 142.5, and 172.5 ml respectively. The small peak of A_{280}-absorbing material eluted in the void volume was presumably due to contaminants in the standard protein preparations. Using these data, a linear relationship was obtained between log of molecular weight and elution volume (as a percentage of the total column volume) and the standard curve shown in Fig. 4.4. was useful for the determination of molecular weights in the range 20,000 to 70,000 daltons.

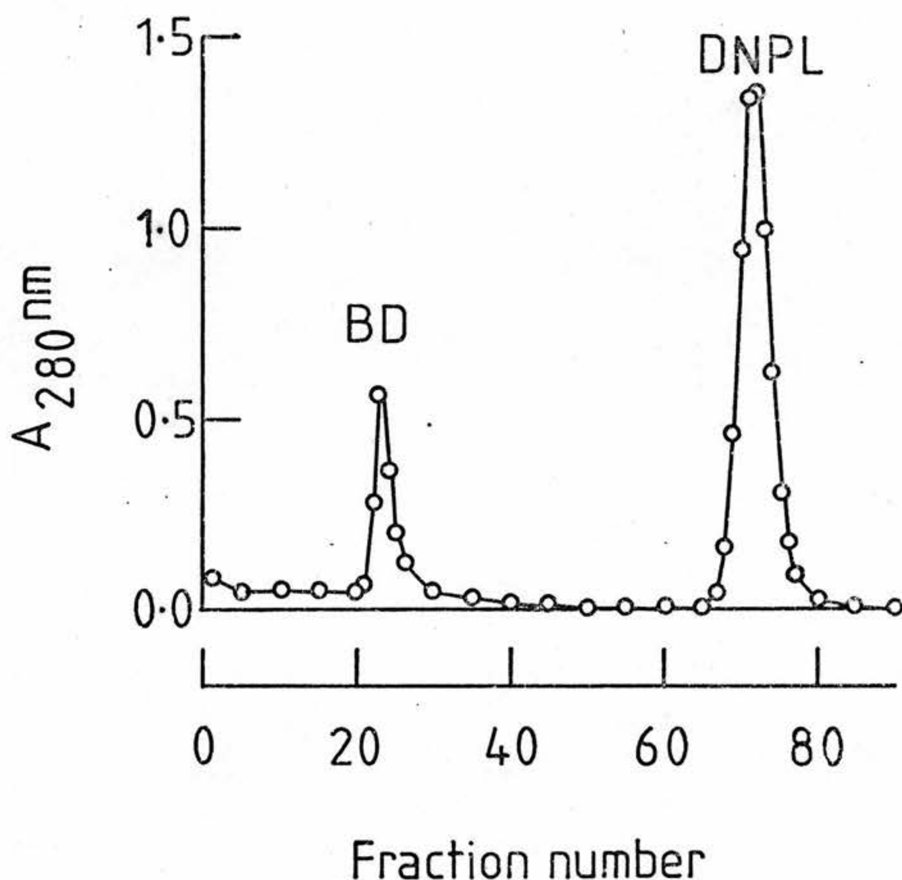


Figure 4.2. Elution of a mixture of Blue Dextran and DNP-Lysine from Ultrogel AcA - 44.

A few milligrams of Blue Dextran were dissolved in 0.1M-Tris/HCl, 0.1M-NaCl, pH 7.4 and filtered. DNP-lysine was then added to the solution until a bright green colour was obtained and 2 ml of the mixture were applied to a 2 cm x 92 cm column of Ultrogel AcA-44 equilibrated in the same buffer. Elution was carried out overnight at a flow rate of 5.8 cm/hr and 3.8 ml - fractions were collected. The absorbance of each fraction at 280nm was recorded.

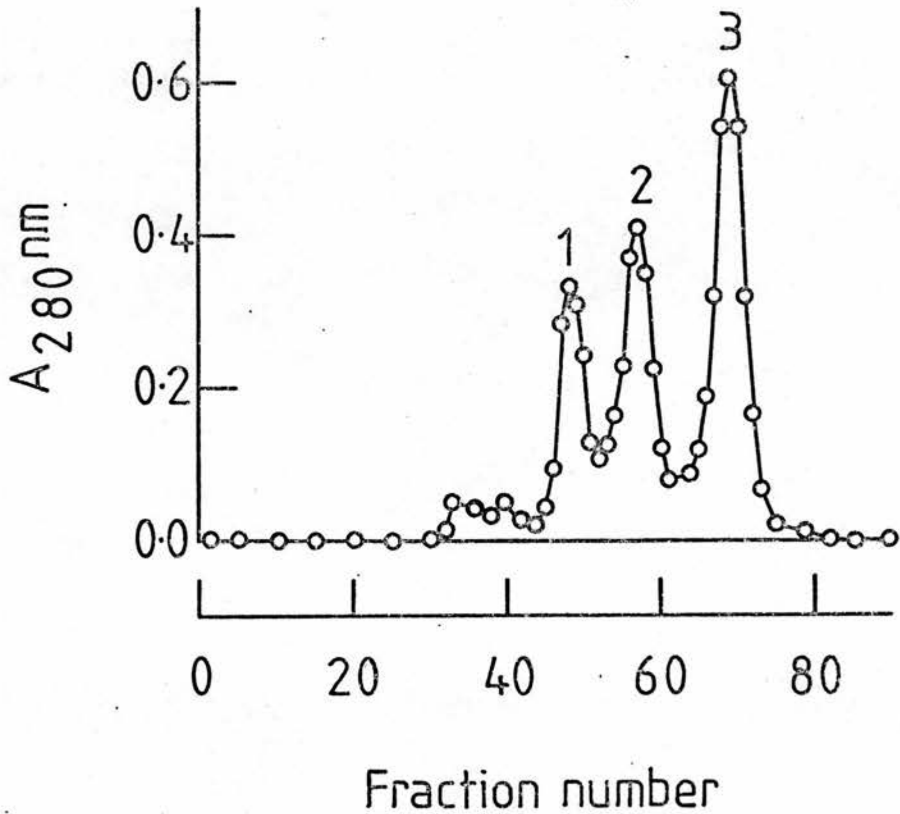


Figure 4.3. Calibration of Ultrogel AcA-44 column with molecular weight standards.

5 ml of a solution in 0.1M-Tris/HCl, 0.1M-NaCl, pH 7.4 containing 10 mg each of bovine serum albumin (1), ovalbumin (2), and soya bean trypsin inhibitor (3) were applied to a 2 cm x 92 cm column of Ultrogel AcA-44 equilibrated in the same buffer. Elution was carried out overnight at a flow rate of 5.8 cm/hr and 2.5 ml fractions were collected. The absorbance of each fraction at 280nm was recorded.

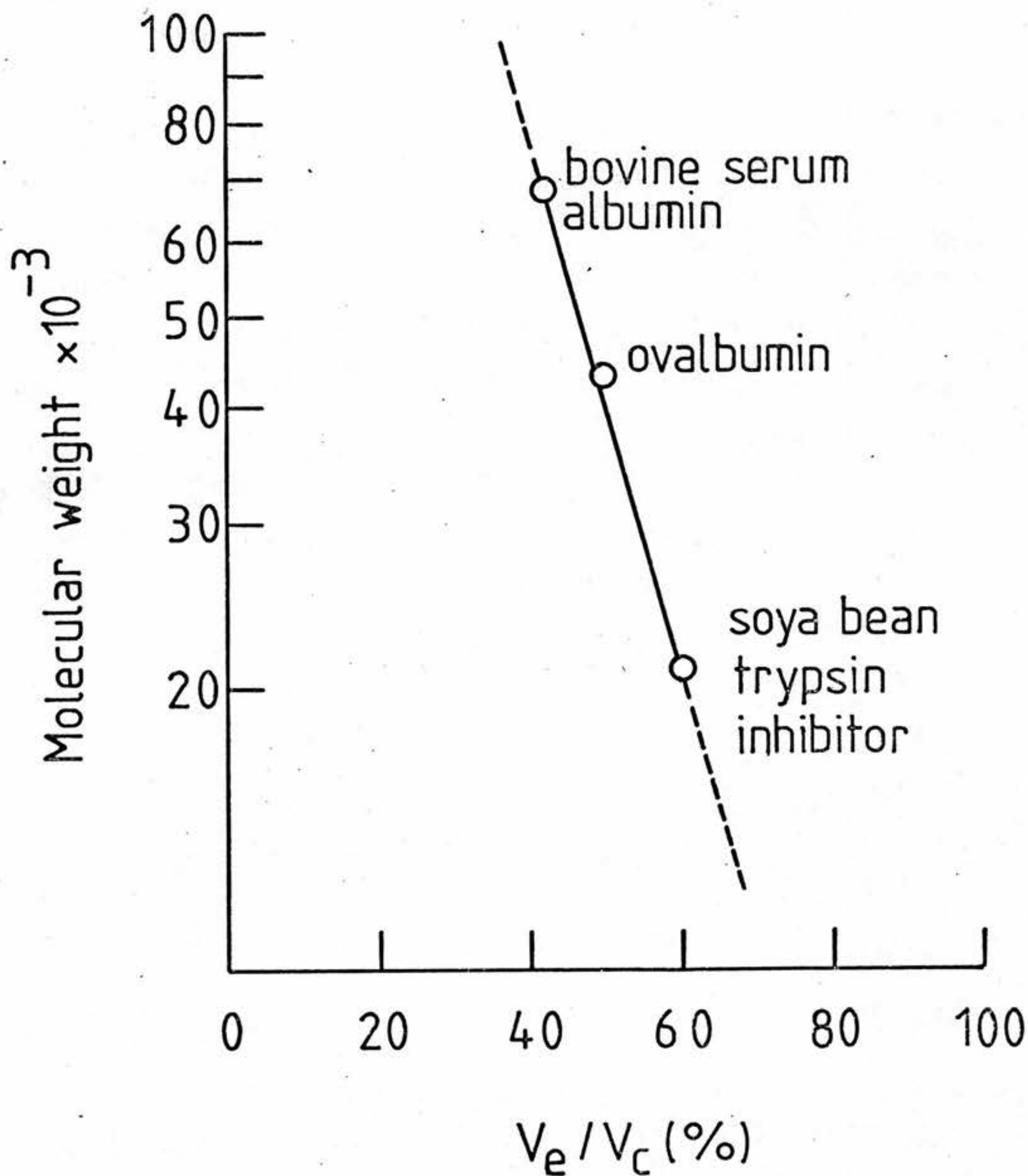


Figure 4.4. Molecular weight standardisation of Ultrogel AcA-44 column

Experimental details are as described in the legend to Fig. 4.3. The molecular weights of the standard proteins are plotted as a function of their elution volumes from Ultrogel AcA-44 (Ve) expressed as a percentage of the total column volume (Vc).

When the pooled cholera toxin-containing fractions from DEAE-cellulose chromatography (Fig. 4.1.) were applied to and eluted from the gel filtration column, the results shown in Fig. 4.5. were obtained. Three major peaks of protein were evident:

(i) a large, sharp peak eluting in the void volume which presumably represents proteins with molecular weights greater than about 80,000 daltons.

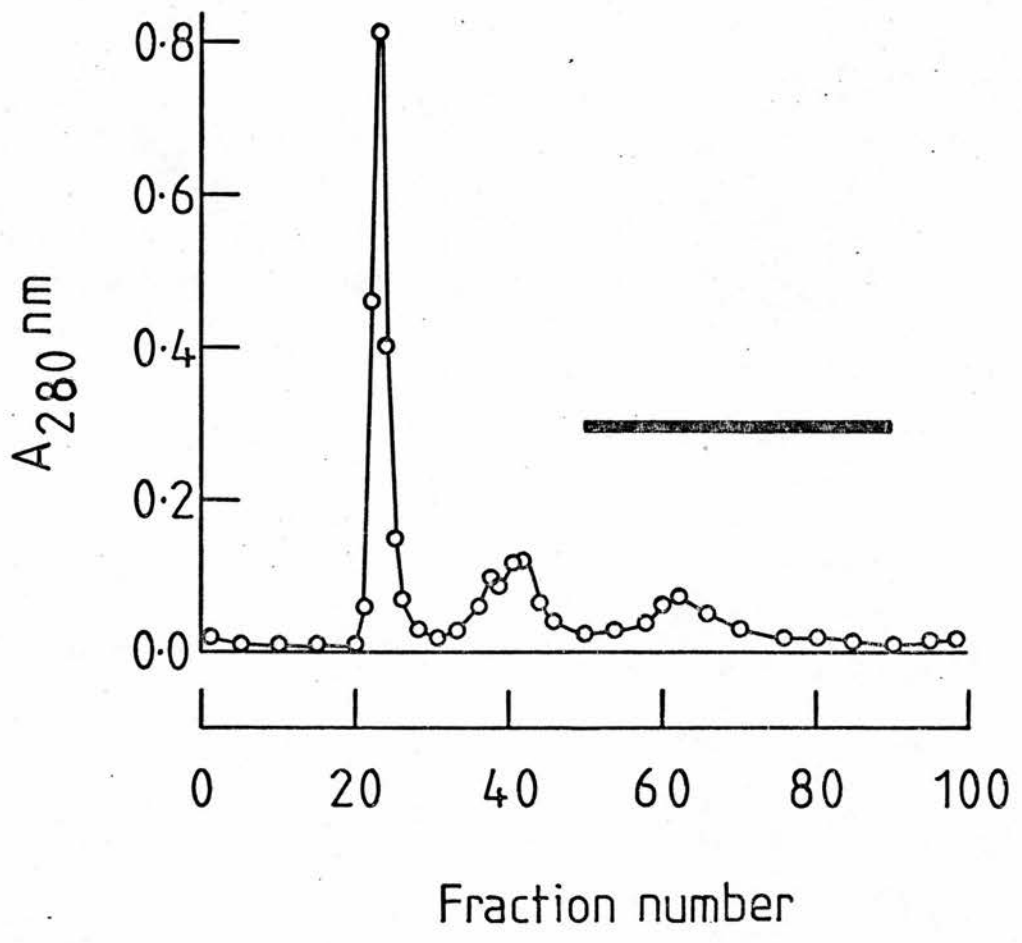
(ii) a smaller peak with a shoulder eluting between 114 and 190 ml, corresponding to proteins of molecular weights 20,000 to 70,000 daltons.

(iii) the smallest and broadest peak eluting between 209 and 285 ml, representing proteins with molecular weights of less than 20,000 daltons.

Cholera toxin, with a molecular weight of 82,000 daltons would be expected to elute with or immediately after the void volume, but in fact the toxin was detected in column fractions 50 to 90 in the very broad peak of apparent molecular weight less than 20,000. Thus in an analogous manner to the elution characteristics of cholera toxin from Agarose A-5m and Sephadex G-75 (Finkelstein and LoSpalluto, 1969), the toxin has also behaved anomalously on Ultrogel AcA-44 indicating that some degree of affinity probably exists between cholera toxin and the agarose matrix. The usefulness of this property for purification purposes is also illustrated in Fig. 4.5. where it can be seen that, had the toxin behaved as would have been expected from a consideration of its molecular weight, it would undoubtedly have been associated with several major contaminants and would not have been obtained in a purified state.

Figure 4.5. <u>Elution of pooled toxin-containing fractions after DEAE-cellulose chromatography from Ultrogel AcA-44.</u>

Cholera toxin fractions from DEAE-cellulose chromatography (Fig. 4.1.) were pooled and dialysed against 0.1M-Tris/HCl, 0.1M-NaCl, pH 7.4. After freeze-drying and resuspending in a small volume of the same buffer, the fractions were applied to a 2 cm x 92 cm column of Ultrogel AcA-44 and elution was carried out overnight at a flow rate of 5.8 cm/hr. 3.8ml - fractions were collected which were analysed for protein by recording the absorbance at 280 nm. Cholera toxin (shaded bar) was detected as described in Ch. 2.2.1.


0·8
0·6
0·4
0·2
0·0
A_{280} nm
0
20
40
60
80
100
Fraction number

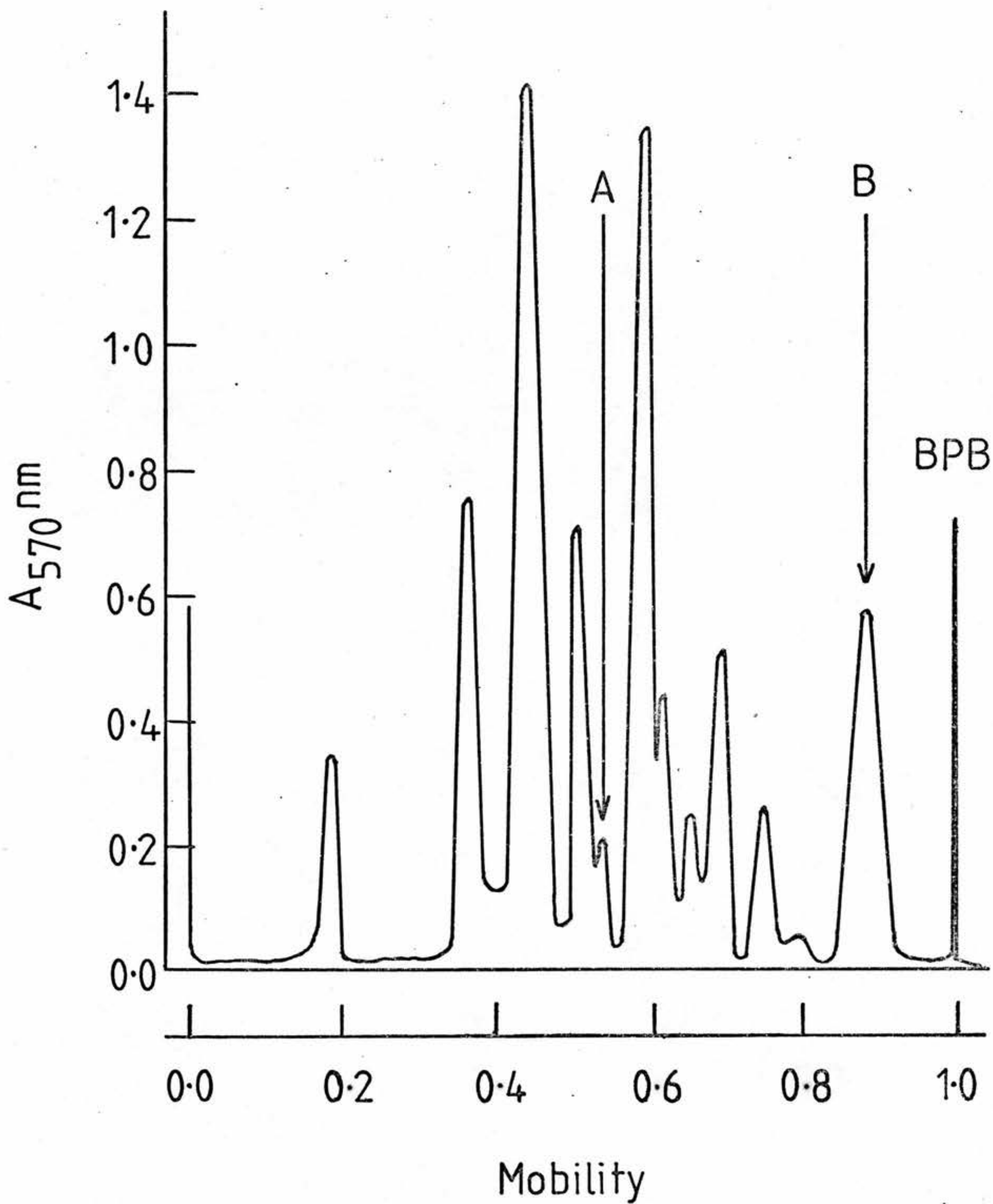
4.2.4. Criteria of purity

Since the exact amount of cholera toxin in the culture filtrates of V. cholerae was not known, it was not possible to calculate the recovery of toxin after each step in the purification. However in a typical preparation, 70 mg of crude starting material yielded about 1 mg of purified toxin product. This is likely to represent a high recovery of cholera toxin during purification since the latter was undoubtedly only a minor component of the culture filtrate. This is illustrated in Figs. 4.6. and 4.7. which show scans of SDS-polyacrylamide gels of the culture filtrate and of the purified toxin respectively. In Fig. 4.7. subunits A and B of the purified toxin migrated with mobilities of 0.55 and 0.87 corresponding to molecular weights of 21,000 and less than 12,000 respectively (see Fig. 2.1.). These values are typical of those for the toxin subunits determined in this system (van Heyningen, 1976a). In Fig. 4.6. the corresponding peaks (marked A and B) constitute only a small fraction of the total protein in the culture filtrate. Similarly on native polyacrylamide gels, pure cholera toxin migrated with a mobility of 0.05 (Fig. 4.9.) and the corresponding peak on a gel of the culture filtrate (Fig. 4.8.) appeared only as a small shoulder on a major slow-moving peak.

Since a study of the detailed structure of cholera toxin was not the prime concern of the present report, the purity of the final product was not investigated in other analytical systems. The toxin appeared to be homogeneous on both SDS and native polyacrylamide gels (Figs. 4.7. and 4.9.) and migrated in an identical fashion to

Figure 4.6. <u>SDS-Polyacrylamide gel electrophoresis of V. cholerae culture filtrate.</u>

50µl of V. cholerae culture filtrate (containing 150µg of protein) were electrophoresed on 10%-polyacrylamide SDS gels as described in Ch. 2.3.1. After staining and destaining, the gel was scanned spectrophotometrically at 570nm. The positions of the cholera toxin subunits (A and B) were determined from Fig. 4.7. BPB indicates the position of the bromophenol blue marker dye.



1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
A570 nm
A
B
BPB
0·0
0·2
0·4
0·6
0·8
1·0
Mobility

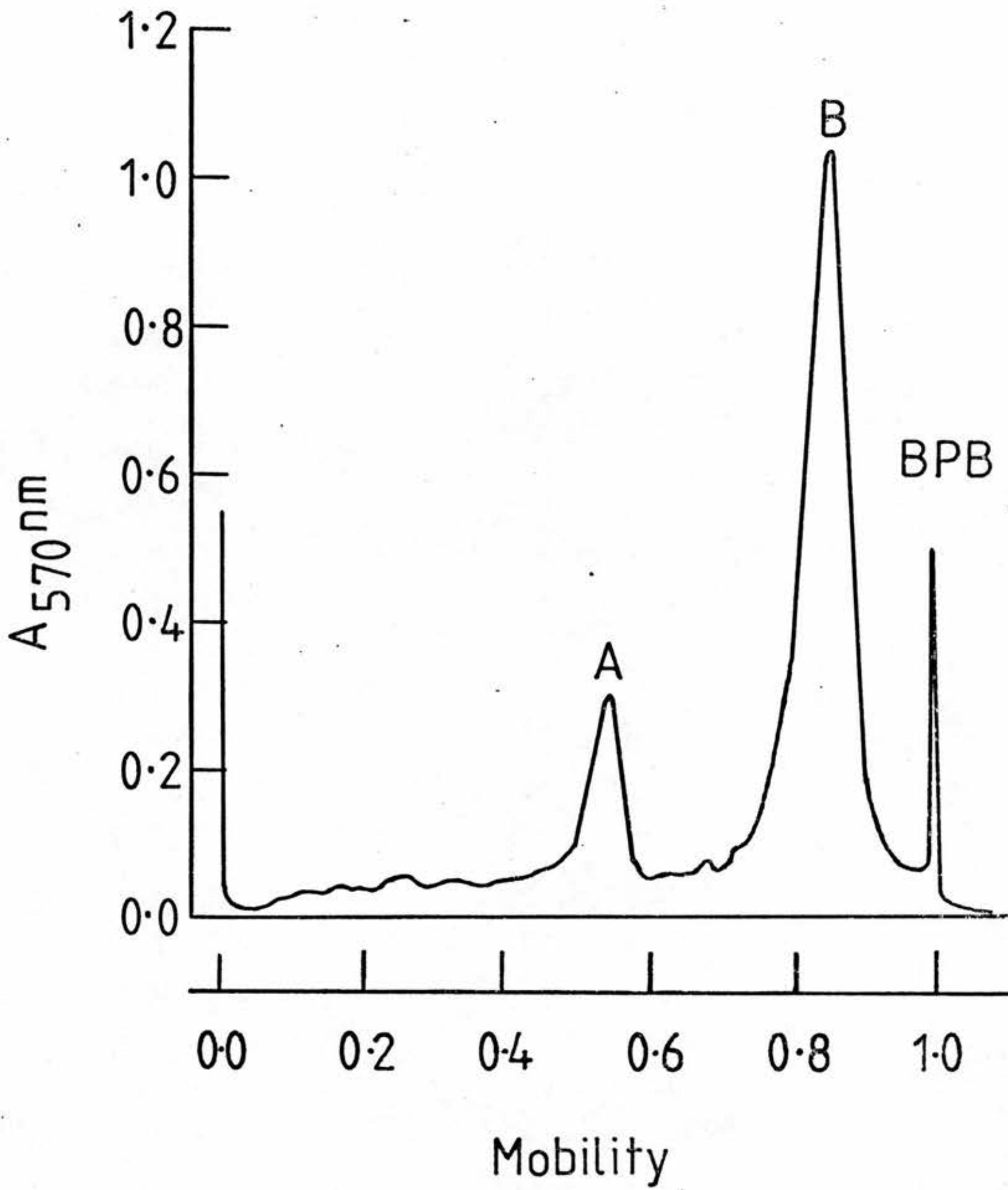
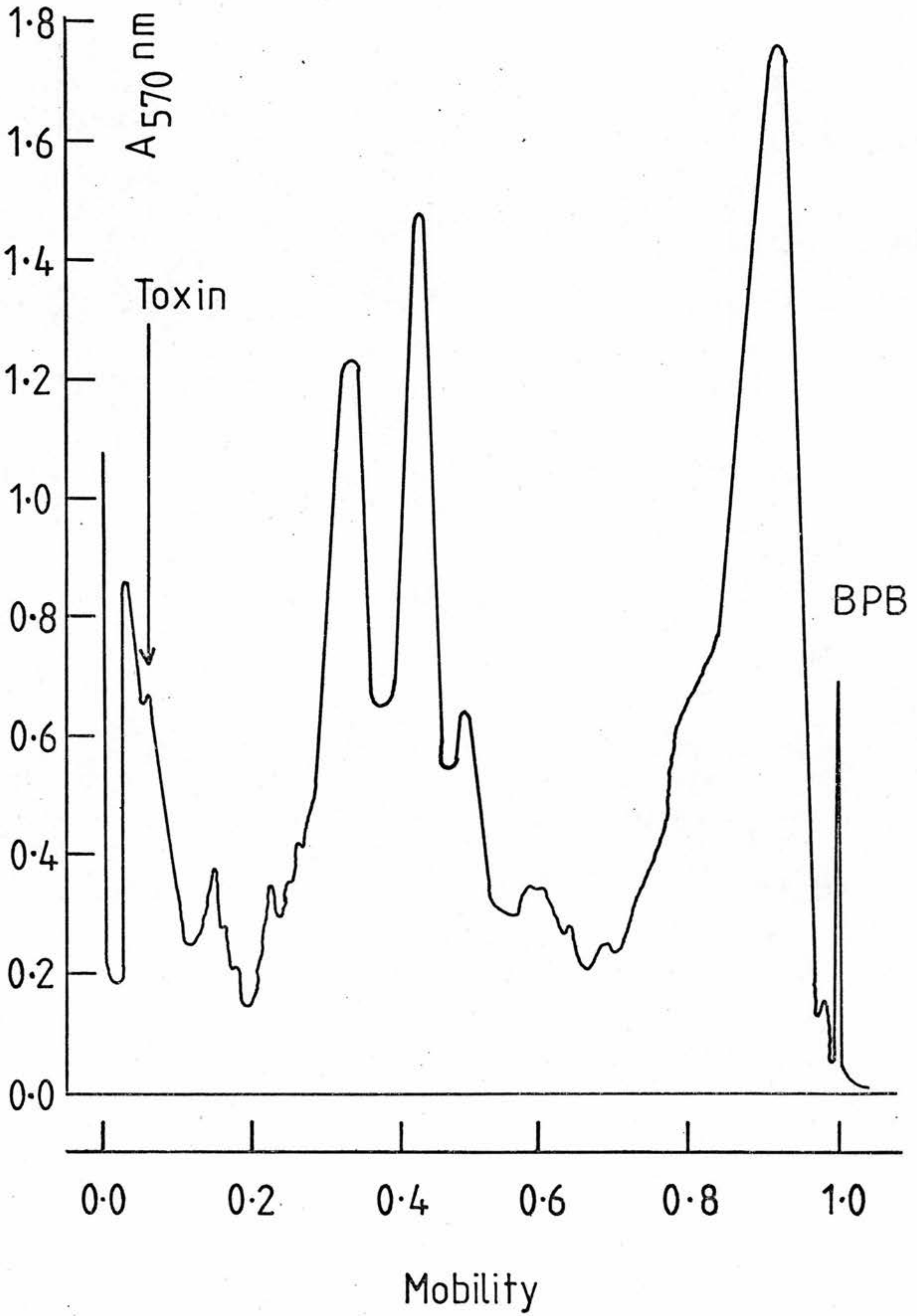


Figure 4.7. SDS-Polyacrylamide gel electrophoresis of cholera toxin purified from V. cholerae culture filtrates.

Experimental details were exactly as described in the legend to Fig. 4.6. except that 50 µl of purified cholera toxin (containing 50µg of protein) were subjected to SDS gel electrophoresis.

Figure 4.8. Native polyacrylamide gel electrophoresis of V. cholerae culture filtrates.

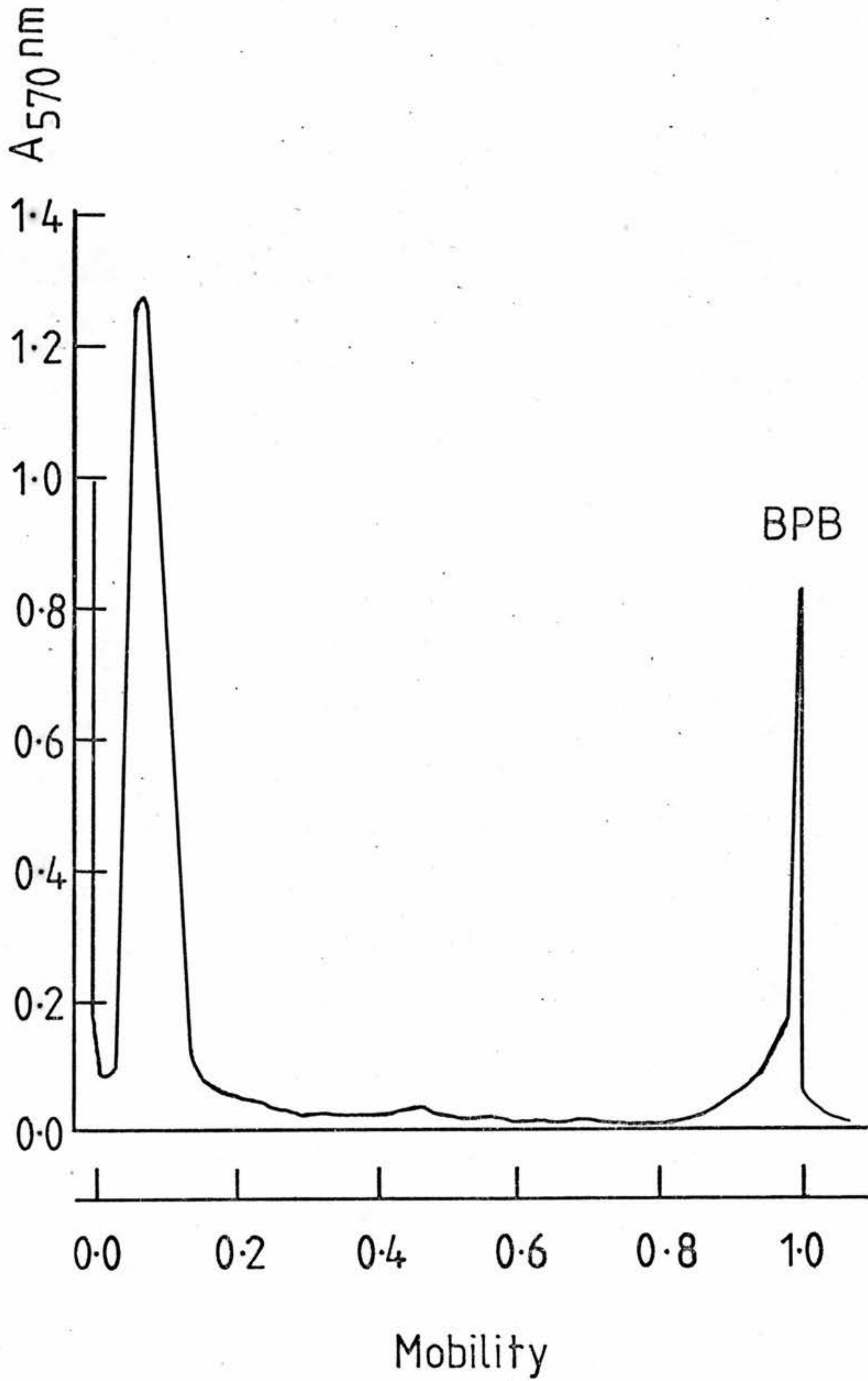
50μl of V. cholerae culture filtrate (containing 150μg of protein) were electrophoresed on native polyacrylamide gels as described in Ch. 2.3.2. After staining and destaining the gel was scanned spectrophotometrically at 570 nm. The position of cholera toxin was determined from the data in Fig. 4.9. BPB indicates the position of the bromophenol blue marker dye.



1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
A_{570} nm
Toxin
BPB
0·0
0·2
0·4
0·6
0·8
1·0
Mobility

Figure 4.9. <u>Native polyacrylamide gel electrophoresis of cholera toxin purified from V. cholerae culture filtrates</u>.

Experimental details were exactly as described in the legend to Fig. 4.8. except that 50 μl of purified cholera toxin (containing 50 μg of protein) were subjected to electrophoresis.



A_{570} nm
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
BPB
0·0
0·2
0·4
0·6
0·8
1·0
Mobility

cholera toxin obtained from Dr. R.A. Finkelstein (results not shown.) These observations were taken as an indication that the above purification method resulted in the production of apparently pure cholera toxin, indistinguishable from a known sample of highly purified material, and the toxin was therefore considered to be suitable for the studies described in this report.

4.3. Discussion

It is clear from the above results that cholera toxin was only a minor component in the culture filtrate of V.cholerae obtained from the Wellcome Research Laboratories. Since the conditions employed for the growth of V. cholerae and the methods of preparation of the culture filtrates were not known, it is not possible to evaluate the extent to which these conditions were optimal. However cholera toxin was readily detectable in the crude material by Ouchterlony immunodiffusion and from about 100mg of starting protein, 1 to 2 mg of toxin were obtained in a purified form, which was sufficient for several months of experimentation. It should be noted however that different batches of culture filtrate varied considerably especially with respect to protease contamination. In the material used for the purification described above, such contamination was not detectable but in some batches, it was not possible to isolate purified cholera toxin by the above method, a problem which was eliminated by including 0.1mM-PMSF in the buffers used throughout the purification procedure. Analysis of the toxin purified from these batches by SDS-polyacrylamide gel electrophoresis after various times of incubation at 37°C prior to electrophoresis, showed no evidence of toxin destruction and the proteases were therefore separated from the toxin at some stage in the purification.

The purification scheme described here is a significant simplification of the procedure of Finkelstein and LoSpalluto,(1970) and can in fact be simplified further by the complete omission of the ammonium sulphate fractionation step. The small amount of material removed from the toxin in this step was subsequently found to be separated from cholera toxin in any case, by DEAE-cellulose chromatography and thus, a two-step procedure consisting of ion-exchange chromatography on DEAE-cellulose followed by gel filtration on Ultrogel AcA-44 was sufficient to obtain purified cholera toxin which was homogeneous on SDS and native polyacrylamide gels. By far the most effective step in the purification scheme was DEAE-cellulose chromatography in which it was possible to separate more than 75% of contaminating proteins from the toxin (Fig. 4.1.). However were it not for the anomalous behaviour of cholera toxin on Ultrogel AcA-44, this would have been of little use in the subsequent gel filtration step since there was still significant contamination by proteins with similar molecular weights to that of the toxin. However since cholera toxin eluted from Ultrogel AcA-44 with the characteristics of a protein with a molecular weight of less than 20,000 daltons, it was efficiently separated from these remaining contaminants (Fig. 4.5.)

Although the purified toxin behaved as a homogeneous protein on SDS and native polyacrylamide gels with identical migration characteristics to those of highly purified material from Dr. R.A. Finkelstein, some of the results presented in Ch. 5.2. indicated that the toxin was not homogeneous and appeared to contain several fragments of cholera toxin as well as some contaminating NADase activity distinct from the toxin and from fragments of the toxin. Presumably, in the

method of Finkelstein and LoSpalluto (1970) these contaminants may be resolved from the intact toxin in their final double gel filtration step.

It seems likely that studies on the mechanism of action of cholera toxin may indicate alternative simplifications for cholera toxin purification methods and for example, the involvement of NAD^+ in toxin action (see Chs. 5. and 6.) has resulted in the demonstration that cholera toxin will bind reversibly to columns of Sepharose covalently linked to NAD^+ (Antoni et al, 1978) and affinity chromatography on such columns may be of considerable use in purification procedures. Similar properties have been described for diphtheria toxin (Montanaro and Sperti, 1967) and have been used to purify the toxin on NAD^+ - Sepharose columns (Cukor et al, 1974).

Chapter 5. Results

5.1. Characterisation of rat liver adenylate cyclase and activation by cholera toxin.

Production of cAMP from ATP in rat liver homogenates was linear with time over the ranges tested both at 25°C and at 37°C (Figs. 5.1. and 5.2. respectively). The specific activity of adenylate cyclase under these conditions was 44 and 86 pmoles cAMP min^{-1} mg^{-1} protein respectively. With a 20 min. assay time at 37°C, cAMP production also increased linearly with homogenate protein concentration up to at least 357 μg protein per 25 μl assay volume (Fig. 5.3.). These data were taken as an indication that the adenylate cyclase assay described in Ch. 3.3. was suitable for determining the activity of this enzyme in rat liver homogenates, and subsequent experiments were conducted within the ranges of time and protein concentration determined above.

When rat liver homogenates were incubated with cholera toxin for 10 min. at 25°C in the presence of NAD^{+}, ATP, DTT and GTP, adenylate cyclase activity upon subsequent assay was elevated to a maximum of about three-fold over basal levels. As shown in Fig. 5.4. activation was detectable with only 0.01 μg/ml cholera toxin and was maximal with 1 μg/ml. Further addition of toxin at concentrations up to 10μg/ml had no further effect on adenylate cyclase activity.

In contradiction to earlier reports in the pigeon erythrocyte system (Gill, 1976b), activation of adenylate cyclase in rat liver membranes by cholera toxin occurred even in the absence of

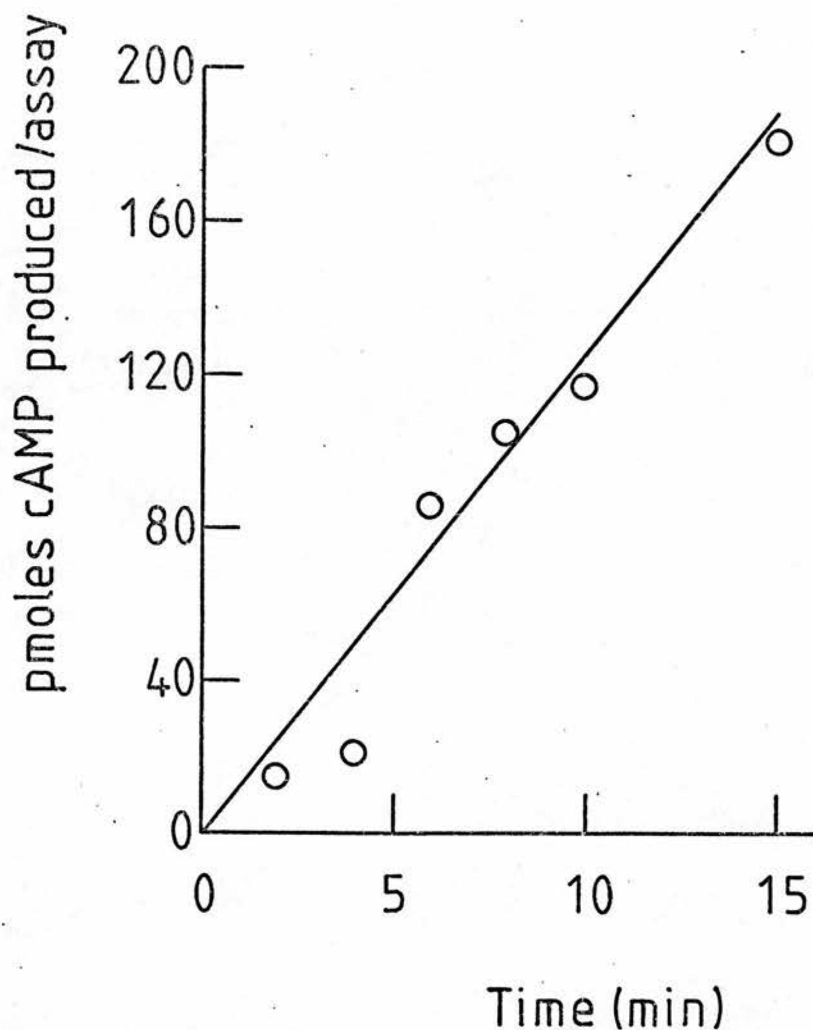


Fig.5.1. Time course of cAMP production by rat liver homogenate at 25°C.

A rat liver homogenate was prepared in Buffer System B as described in Ch.2.5. and 15µl aliquots were added to 10µl of assay medium I (Ch.3.3.2.3) containing 25,000pmoles of ATP and 0.6µCi of [8-^{3}H]ATP. After the indicated times at 25°C, 10µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 5µl of each supernatant taken for estimation of [^{3}H]cAMP. All values were single determinations only.

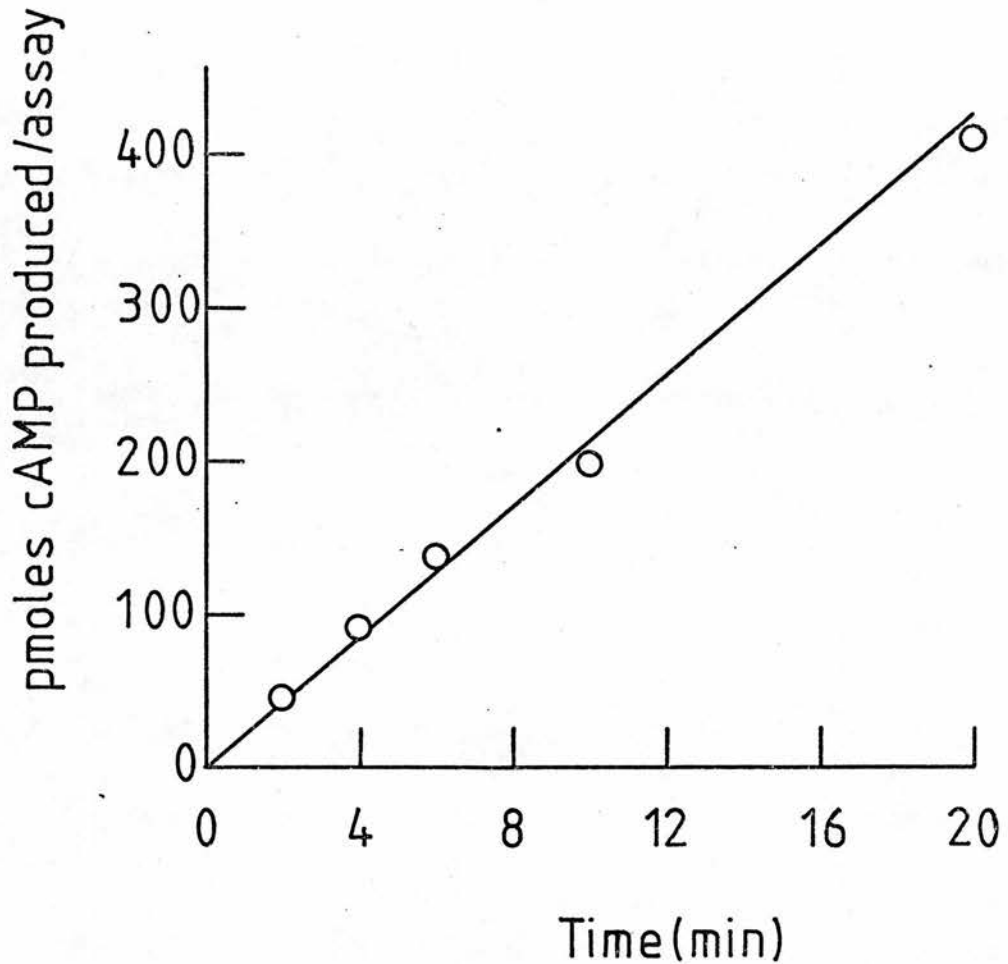


Fig.5.2. Time course of cAMP production by rat liver homogenate at 37°C.

A rat liver homogenate was prepared in Buffer System A as described in Ch.2.5. and 15µl aliquots were added to 10µl of assay medium I (Ch.3.3.2.3) containing 25,000pmoles of ATP and 1µCi of [8-^{3}]ATP. After the indicated times at 37°C, 5µl of 50%-TCA were added, precipitated protein was removed by centrifugation, and 5µl of each supernatant taken for estimation of [^{3}H]cAMP. All values were single determinations only.

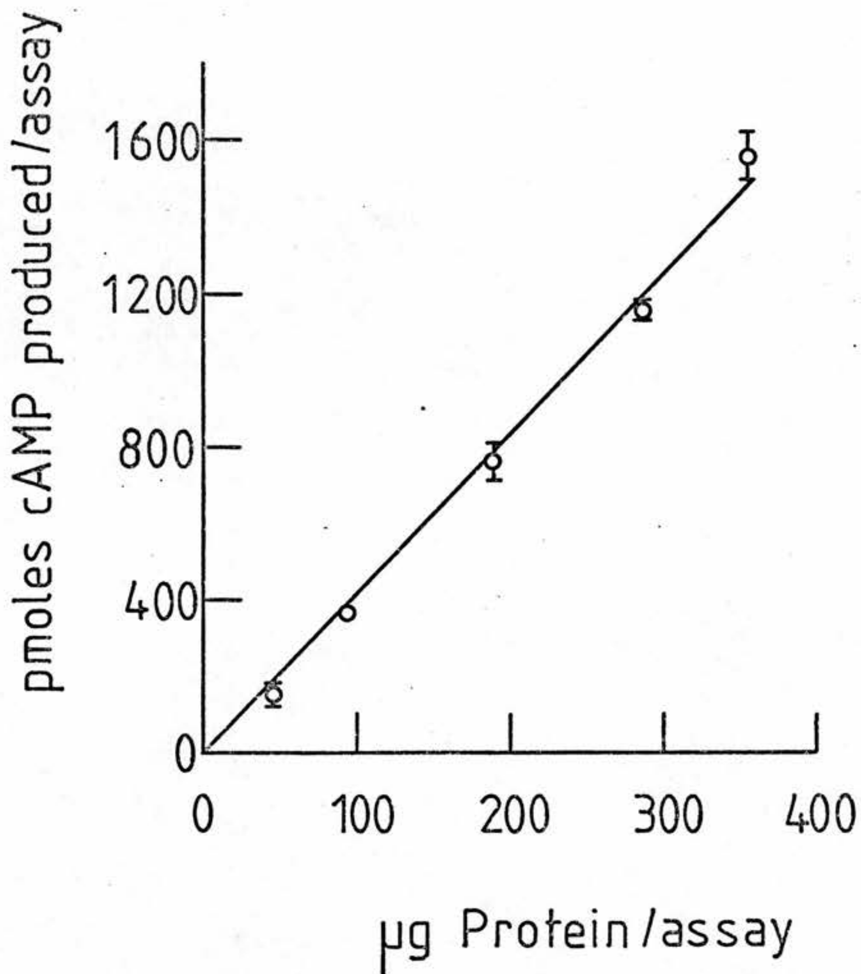


Fig.5.3. Production of cAMP at 37°C by rat liver homogenate as a function of homogenate protein concentration.

A rat liver homogenate was prepared in Buffer System B as described in Ch.2.5. and increasing volumes up to 15µl were added to 10µl of assay medium I (Ch.3.3.2.3) containing 14,500pmoles of ATP and 0.6µCi of [8-^{3}H]ATP. The total volume of each assay was made up to 25µl with buffer. After 20min at 37°C, 10µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{3}H]cAMP. Error bars indicate the range of duplicate determinations.

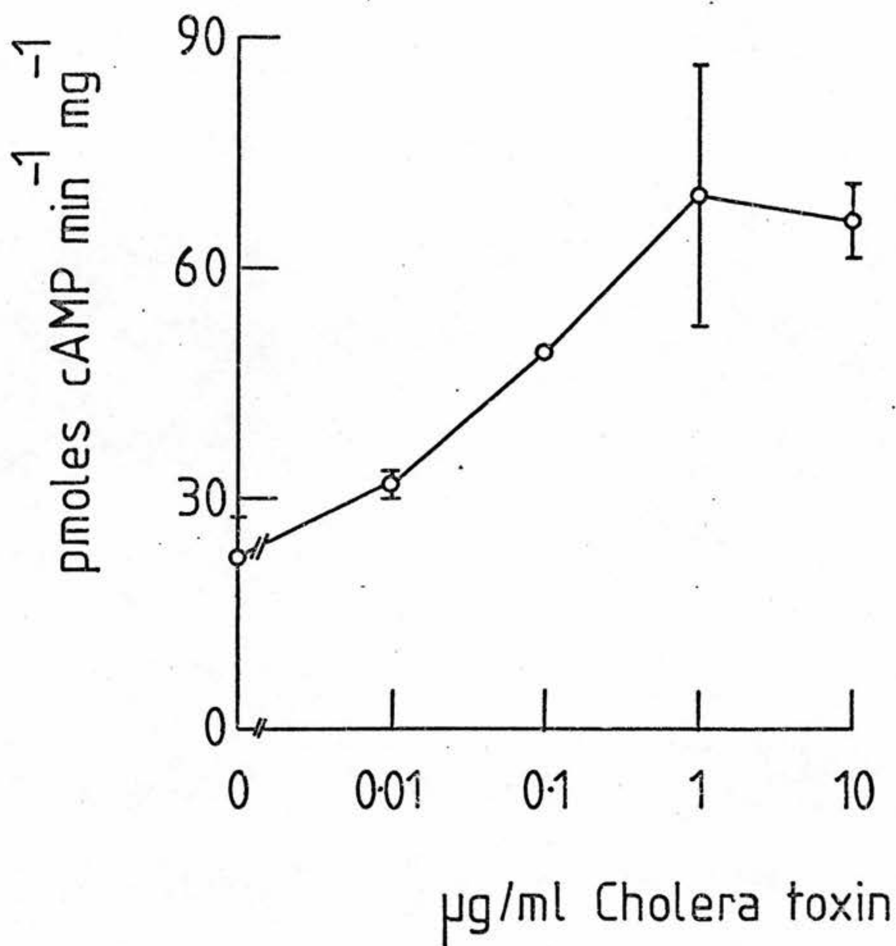


Fig.5.4. Activation of adenylate cyclase by cholera toxin in rat liver.

A rat liver was prepared in Buffer System D as described in Ch.2.5. and 140µl aliquots were incubated in a total volume of 200µl with 2mM-ATP, 2mM-DTT, 1mM-NAD^+,200µM-GTP, and the indicated final concentrations of cholera toxin. After 10min. at 25°C, an excess of cold buffer was added, membranes were precipitated by centrifugation and resuspended in 50µl of buffer. 15µl aliquots were added to 10µl of assay medium II (Ch.3.3.2.3) and after 10min at 25°C, 5µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 10µl aliquots taken for estimation of [^{3}H]cAMP. Error bars indicate the range of duplicate determinations.

cytosol. This is clearly evident from the results shown in Fig. 5.5. in which crude rat liver membranes were washed several times to remove all traces of cytosol, and then incubated in adenylate cyclase assay medium, supplemented with 1mM-NAD^+, in the presence and absence of cholera toxin. At all points on the subsequent time course, production of cAMP was markedly greater in the presence of toxin and activation over basal adenylate cyclase activity was 2.3-fold and 3.3-fold after 5 min. and 30 min. of assay respectively. However, the maximum toxin-stimulated specific activity (125 pmoles cAMP $min.^{-1}$ mg^{-1} protein) was achieved after only 10 min. of assay, and the progressive increase in the extent of cyclase activation over the 30 min. assay period was due to the much greater instability of the unstimulated enzyme, which produced cAMP linearly with time during only the first few minutes of assay. This contrasts with the results obtained in rat liver homogenate where cAMP production was linear with time up to at least 15 min. (Fig. 5.1.). Although the reasons for this were not investigated thoroughly, it may be related to the fact that cytosol was found to contain a factor (s) which elevated basal adenylate cyclase activity two-fold (results not shown), a finding which has already been reported for fat cell adenylate cyclase (Ganguly and Greenough, 1975). It seems possible that such stimulatory factors may also contribute to the stability of the cyclase during prolonged incubations. In contrast to the basal activity of the enzyme, toxin-stimulated activity in the absence of cytosol was not unstable to long incubation times (Fig.5.5.)

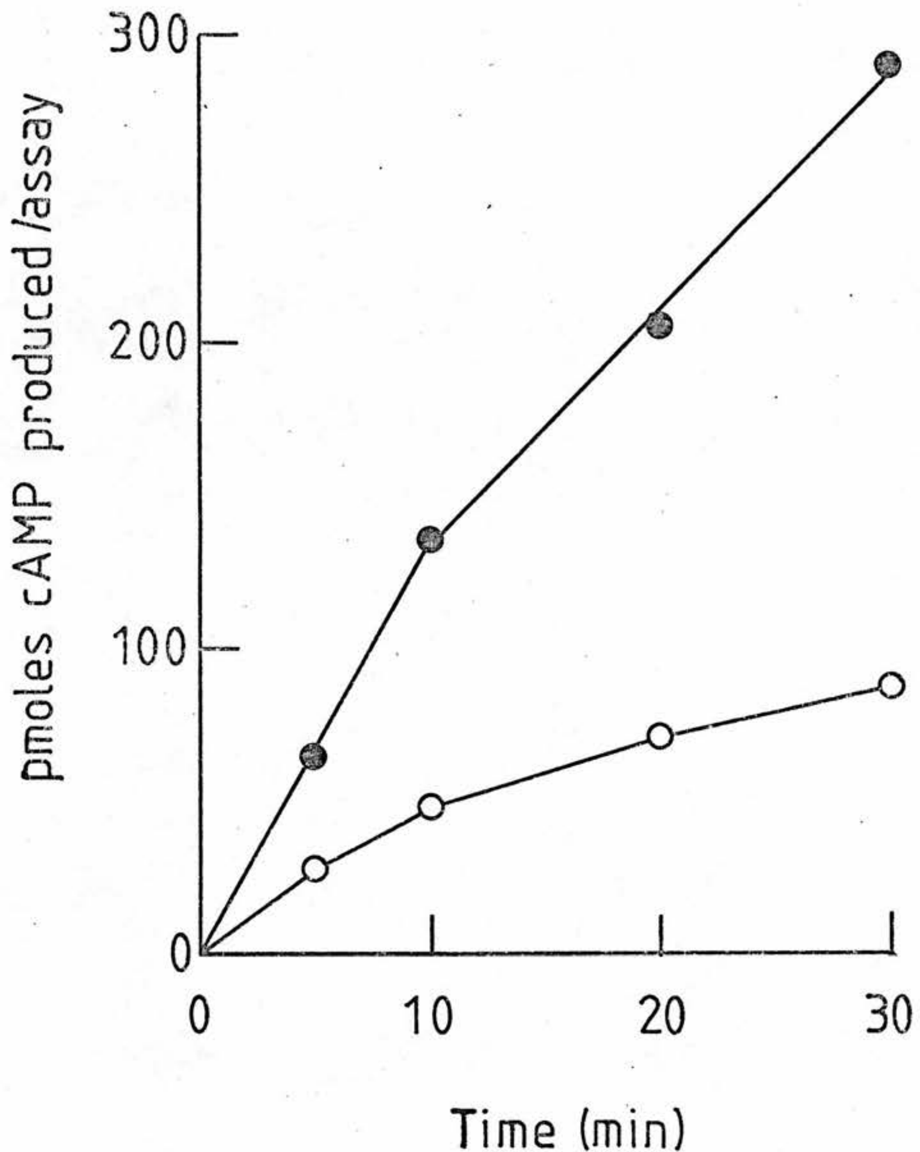


Fig.5.5. Time courses of cAMP production in crude rat liver membranes in the presence and absence of cholera toxin.

Crude rat liver membranes were prepared in Buffer System D as described in Ch.2.5. and 10µl aliquots were added to 10µl of assay medium II (Ch.3.3.2.3) supplemented with NAD^+ to a final concentration of 1mM. 5µl of preactivated cholera toxin (final concentration:2µg/ml) (●—●) or 5µl of activation solution for controls (○—○) were added and after the indicated times at 25°C, 5µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of $[^3H]$cAMP. All values were single determinations only.

5.2. The NADase activity of cholera toxin

5.2.1. Demonstration of activity

Cholera toxin purified as described in Ch. 2.1. was assayed for NADase activity using the assay medium originally described by Moss et al (1976b). As shown in Fig. 5.6. production of nicotinamide from NAD^+ increased linearly with cholera toxin concentration over the range tested (up to 500µg/ml), and the specific activity of the toxin under these conditions was 3.2. nmoles nicotinamide $min.^{-1}$ mg^{-1} toxin. From a comparison of Figs. 5.4. and 5.6. it is immediately apparent that extremely large amounts of cholera toxin are required to demonstrate hydrolysis of NAD^+ in relation to the amounts required for activation of adenylate cyclase in rat liver homogenates. For example in the NADase assay, activity was not readily detectable at toxin concentrations less than 100µg/ml, while adenylate cyclase activation was maximal with 1µg/ml toxin.

Although cytosolic factors, as described in the previous section, did not appear to be essential for the action of cholera toxin in rat liver membranes, the importance of such factors reported in other tissues, such as pigeon erythrocytes (Gill, 1976b), prompted the suggestion that a possible role for these factors may be to facilitate the action of cholera toxin by enhancing its activity towards NAD^+ in an enzymic reaction. However, as shown in Fig. 5.7., the NADase activity of the toxin was essentially unchanged when measured in the presence of freshly-prepared pigeon erythrocyte cytosol. The apparent small

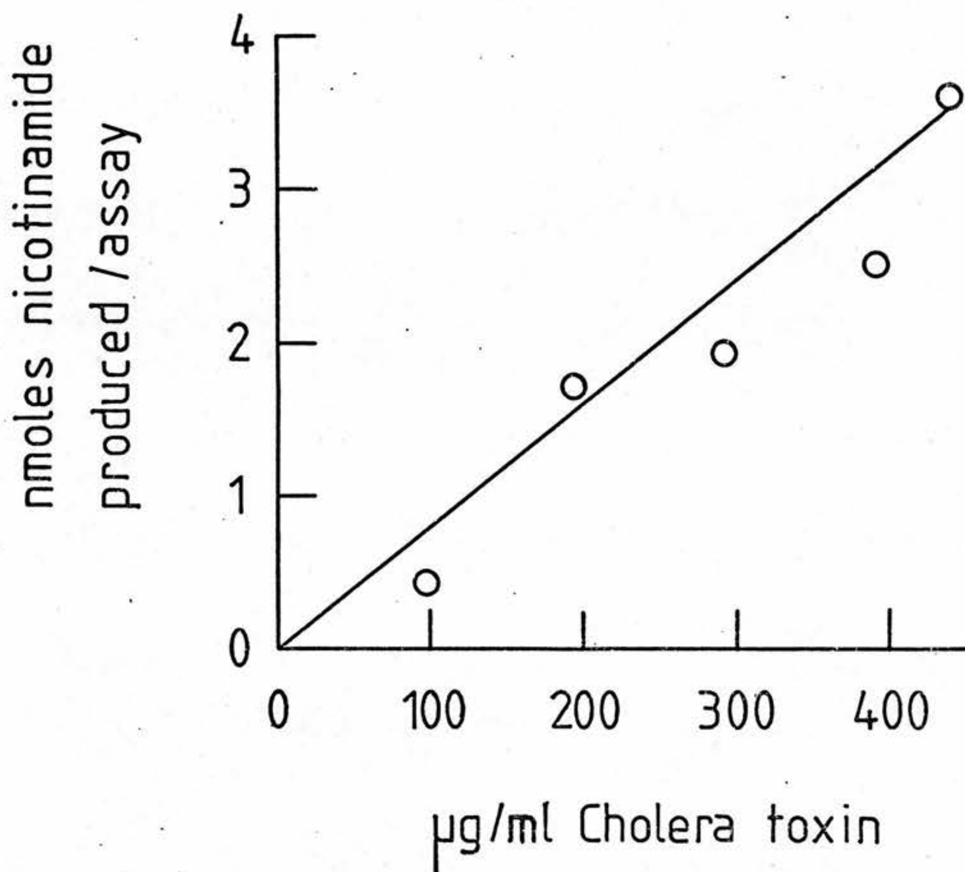


Fig.5.6. Hydrolysis of NAD^+ by purified cholera toxin.

Assays were conducted in a total volume of 25μl containing 200mM-potassium phosphate, 20mM-DTT, 1.5mM-NAD^+, 4nCi of [carbonyl-^{14}C]NAD^+, and the indicated concentrations of cholera toxin, pH 7.0. After 90min at 37°C, 10μl aliquots were taken for estimation of [^{14}C]-nicotinamide. All values were single determinations only.

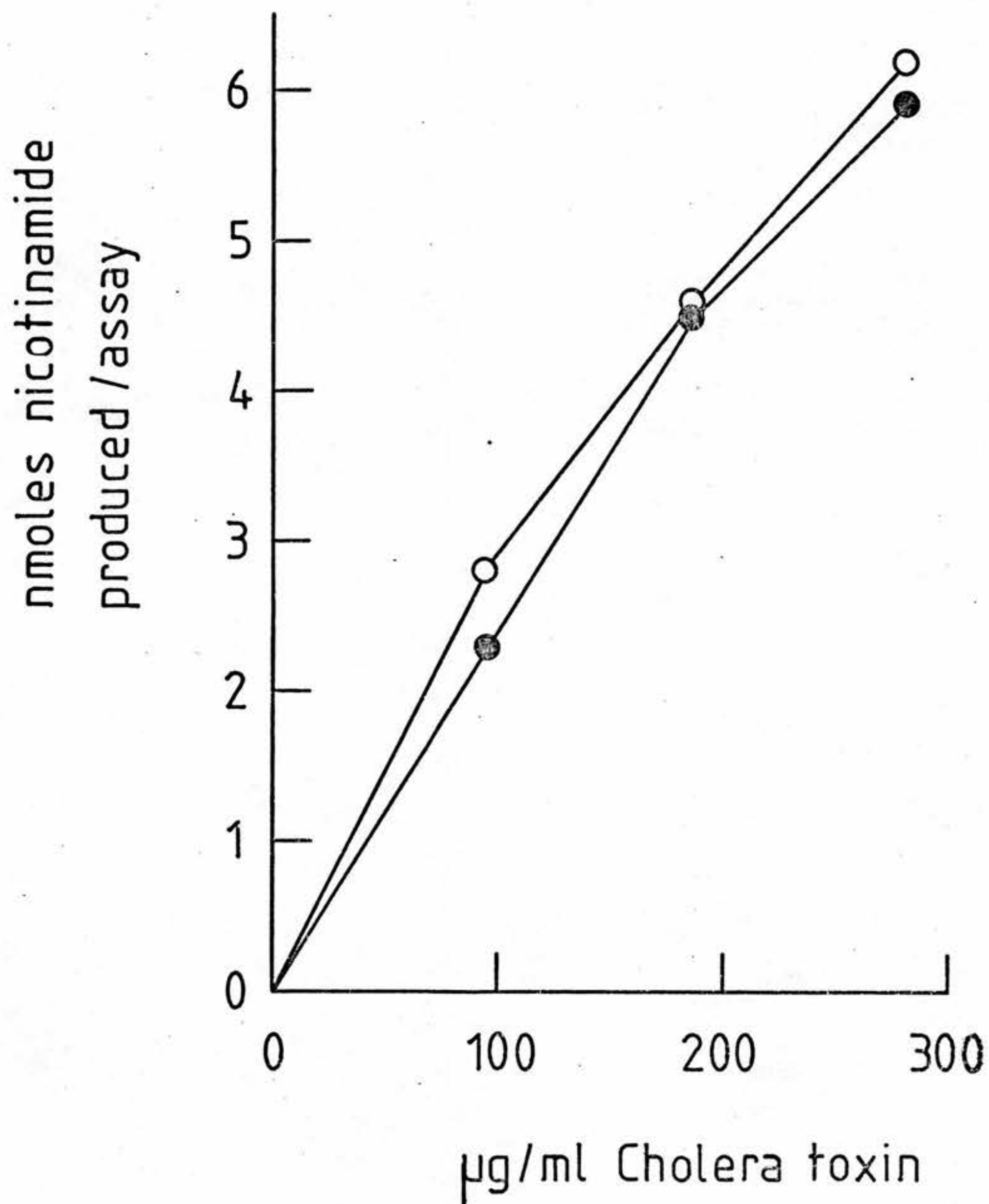


Fig.5.7. Effect of cytosol on the NADase activity of cholera toxin.

Assays were conducted in a total volume of 25µl containing 100mM-potassium phosphate, 10mM-DTT, 1mM-NAD^+, 5nCi of [carbonyl-^{14}C]-NAD^+, the indicated concentrations of cholera toxin, and either 12.5µl of pigeon erythrocyte cytosol (●—●) or buffer for controls (○—○). After 4hr at 37°C, 25µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 25µl of each supernatant taken for estimation of [^{14}C]nicotinamide. All values were the means of duplicate determinations.

decrease in activity in the presence of cytosol probably reflects some degree of isotope dilution by endogenous NAD^+ in the preparation but it is quite clear that even if these endogenous levels were accounted for in the calculation of results, the NADase activity of the toxin would not be significantly elevated above that seen in the absence of cytosol.

5.2.2. The effect of phosphate ions and ionic strength on the NADase activity of cholera toxin

Moss et al (1976 b) and Moss and Vaughan (1977) reported that phosphate buffers stimulated the NADase activity of cholera toxin and an almost six-fold enhancement of activity was observed by increasing the potassium phosphate concentration from 100mM to 400mM. In an attempt to repeat this work, assays were conducted in the presence and absence of cholera toxin in increasing concentrations of potassium phosphate up to 500 mM. In order to ensure that assays in the absence of phosphate were maintained at the correct pH, all solutions were prepared in 10mM-Tris/HCl and adjusted to pH 7.0 with concentrated NaOH.

By increasing the potassium phosphate concentration from zero to 500mM there was an apparent 2.3-fold increase in the NADase activity of cholera toxin (Fig. 5.8.). However since there was an almost equivalent increase in the amount of NAD^+ hydrolysed in the absence of cholera toxin, NAD^+ hydrolysis due to the toxin itself increased only slightly with increasing potassium phosphate concentration (from 4.7 nmoles nicotinamide $min.^{-1}$ mg^{-1} in the absence of phosphate

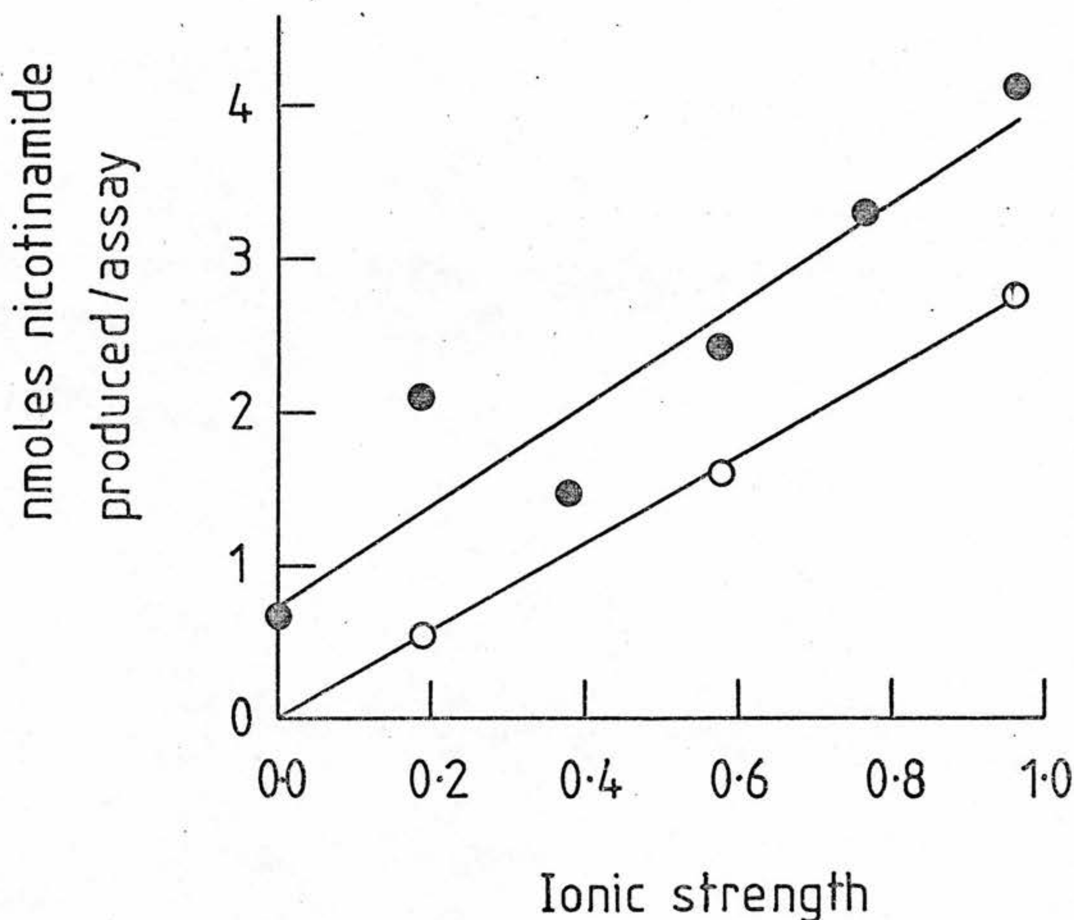


Fig.5.8. Effect of potassium phosphate on the hydrolysis of NAD^+ in the presence and absence of cholera toxin.

Assays were conducted in a total volume of 40µl containing 10mM-Tris/HCl, pH 7.0, 2mM-NAD^+, 20mM-DTT, 50nCi of [carbonyl-^{14}C]NAD^+, the indicated ionic strength supplied as potassium phosphate, and either 5µg of cholera toxin (●—●) or buffer for controls (○—○). After 30min at 37°C, 10µl aliquots were taken for estimation of [^{14}C]-nicotinamide. All values were single determinations only.

to 7.3 nmoles nicotinamide $min.^{-1}$ mg^{-1} at 500mM-potassium phosphate).

A similar experiment testing equivalent ionic strengths supplied as NaCl gave similar results but with several minor differences (Fig. 5.9.). Below an ionic strength of 0.2, NaCl had little effect on NAD^+ hydrolysis either in the presence or absence of cholera toxin and the specific activity of the toxin under these conditions was 6.7 nmoles nicotinamide $min.^{-1}$ mg^{-1}. However when the ionic strength was increased from 0.2 to 1.0, there was a ten-fold increase in NAD^+ hydrolysis under both conditions leading to an apparent ten-fold increase in the specific activity of the toxin, which was about 77 nmoles nicotinamide $min.^{-1}$ mg^{-1} at 1M-NaCl. For reasons discussed in Ch. 6. little significance was attached to this latter result and in subsequent work, NADase assays were conducted either in phosphate buffers of low ionic strength (less than 0.2) or in buffers such as sodium acetate, Tris/HCl, or Hepes/NaOH which did not promote non-specific hydrolysis of NAD^+.

5.2.3. The effect of arginine on the NADase activity of cholera toxin.

The stimulatory effect of arginine on the NADase activity of cholera toxin described by Moss and Vaughan (1977), could not be observed in this work. Arginine at concentrations up to 100mM consistently had no effect on the amount of NAD^+ hydrolysed either by cholera toxin purified as described in Ch. 2.1. (Table 5.1.), or by a sample of purified cholera toxin obtained from Dr. R. Rappaport (Table 5.2.)

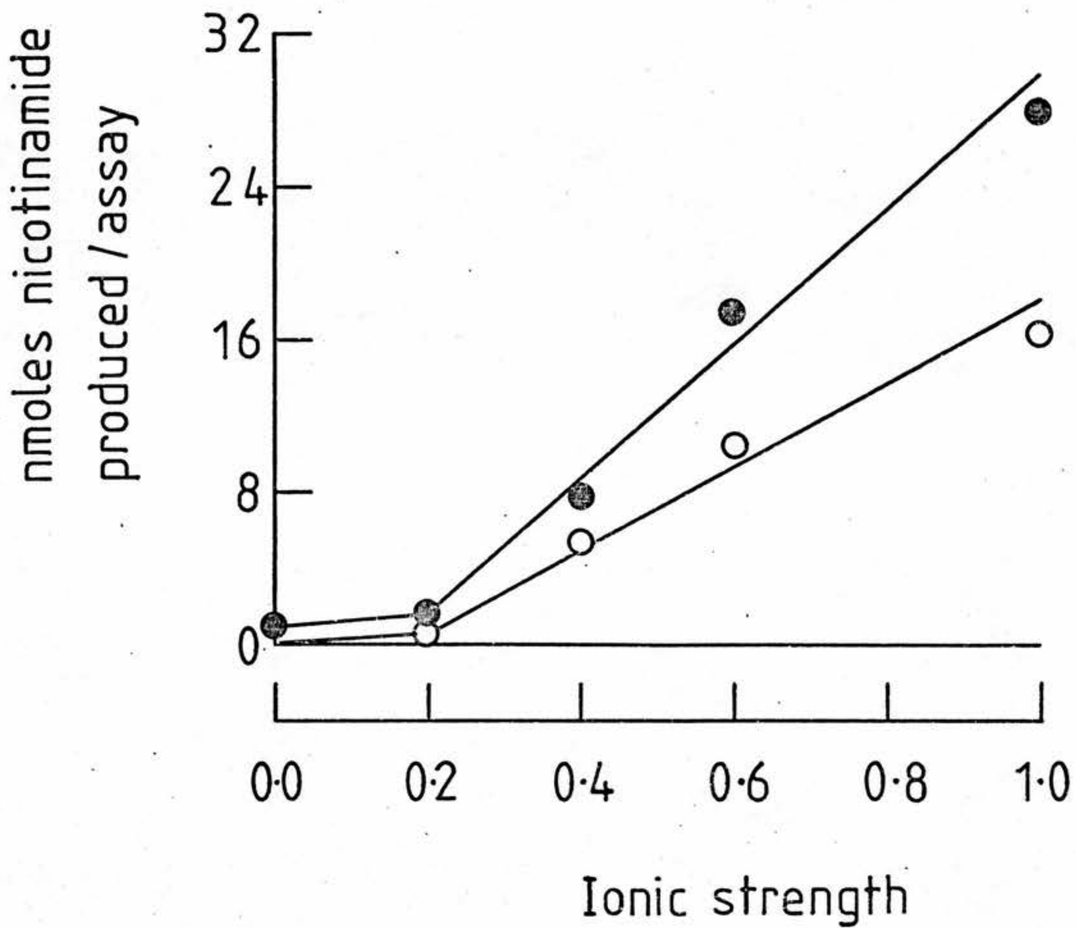


Fig.5.9. Effect of NaCl on the hydrolysis of NAD^+ in the presence & absence of cholera toxin.

Assays were conducted in a total volume of 40µl containing 10mM-Tris/HCl,pH 7.0, 2mM-NAD^+, 20mM-DTT, 50nCi of [carbonyl-^{14}C]NAD^+, the indicated ionic strength supplied as NaCl, and either 5µg of cholera toxin (●—●) or buffer for controls (○—○). After 30min at 37°C, 10µl aliquots were taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.

Table 5.1. Effect of arginine on the NADase activity of cholera toxin purified from V. cholerae culture filtrates as described in Ch. 2.1.

Assays were conducted in a total volume of 20µl containing 200mM-potassium phosphate, 20mM-DTT, 2mM-NAD^+, 4 nCi of [Carbonyl- ^{14}C] NAD^+, 60 µg/ml cholera toxin, and the indicated concentrations of L-arginine. pH 7.0. After 105 min. at 37°C, 10 µl aliquots were taken for estimation of [^{14}C] nicotinamide. All values were single determinations only.

Arginine concentration (mM)	NADase activity (nmoles nicotinamide min^{-1} mg^{-1})
0	5.8
15	5.1
25	4.9
35	6.4
50	7.1
75	5.7

Table 5.2. Effect of arginine on the NADase activity of purified cholera toxin obtained from Dr. R. Rappaport.

Assays were conducted in a total volume of 40µl containing 10mM-Hepes, 0.25M-sucrose, 5mM-$MgCl_2$, 20mM-DTT, 2mM-NAD^+, 50 nCi of [carbonyl - ^{14}C] - NAD^+, 25 µg/ml cholera toxin, and the indicated concentrations of L-arginine, pH 7.4. After 90 min. at 37°C, 10 µl of 25% - TCA were added and 10 µl aliquots taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.

Arginine concentration (mM)	NADase activity (nmoles nicotinamide min^{-1} mg^{-1})
0	10.0
10	8.0
50	10.5
100	8.6

5.2.4. NAD^+ Hydrolysis by crude culture filtrates of *V. cholerae*

Crude culture filtrates of *V. cholerae* were found to catalyse NAD^+ hydrolysis with a specific activity of almost 600 nmoles nicotinamide $min.^{-1}$ mg^{-1} (Fig. 5.10.), which was therefore about 200 times more active than purified cholera toxin (see Fig. 5.6.). Furthermore, as shown in Table 5.3., the majority of this activity could be separated from the toxin during purification. After ammonium sulphate fractionation the highest NADase specific activity was found in the supernatant which contained no cholera toxin as judged by SDS gel electrophoresis and Ouchterlony immunodiffusion. Similarly after DEAE-cellulose column chromatography, a pool of the fractions which did not contain cholera toxin, was enriched in NADase activity. The small amount of activity remaining in the toxin-containing fractions copurified with the toxin again in the final gel filtration step.

Since crude culture filtrates of *V. cholerae* therefore contained a very highly active NADase distinct from cholera toxin, it was important to establish whether the NADase activity associated with purified toxin preparations was a property of the toxin itself, or whether it was due to residual contamination by a small amount of the NADase in the culture filtrate. The relative activities of the purified toxin NADase and of the NADase eluted from DEAE-cellulose (hereafter referred to as 'non-toxin NADase'), can be seen clearly from the time courses of Fig. 5.11. The non-toxin NADase, with a specific activity (measured after 30 min. of assay) of about 2,600 nmoles nicotinamide $min.^{-1}$ mg^{-1}, was at least 150 times more active than the purified toxin NADase. As shown in Table 5.4., both activities were insensitive

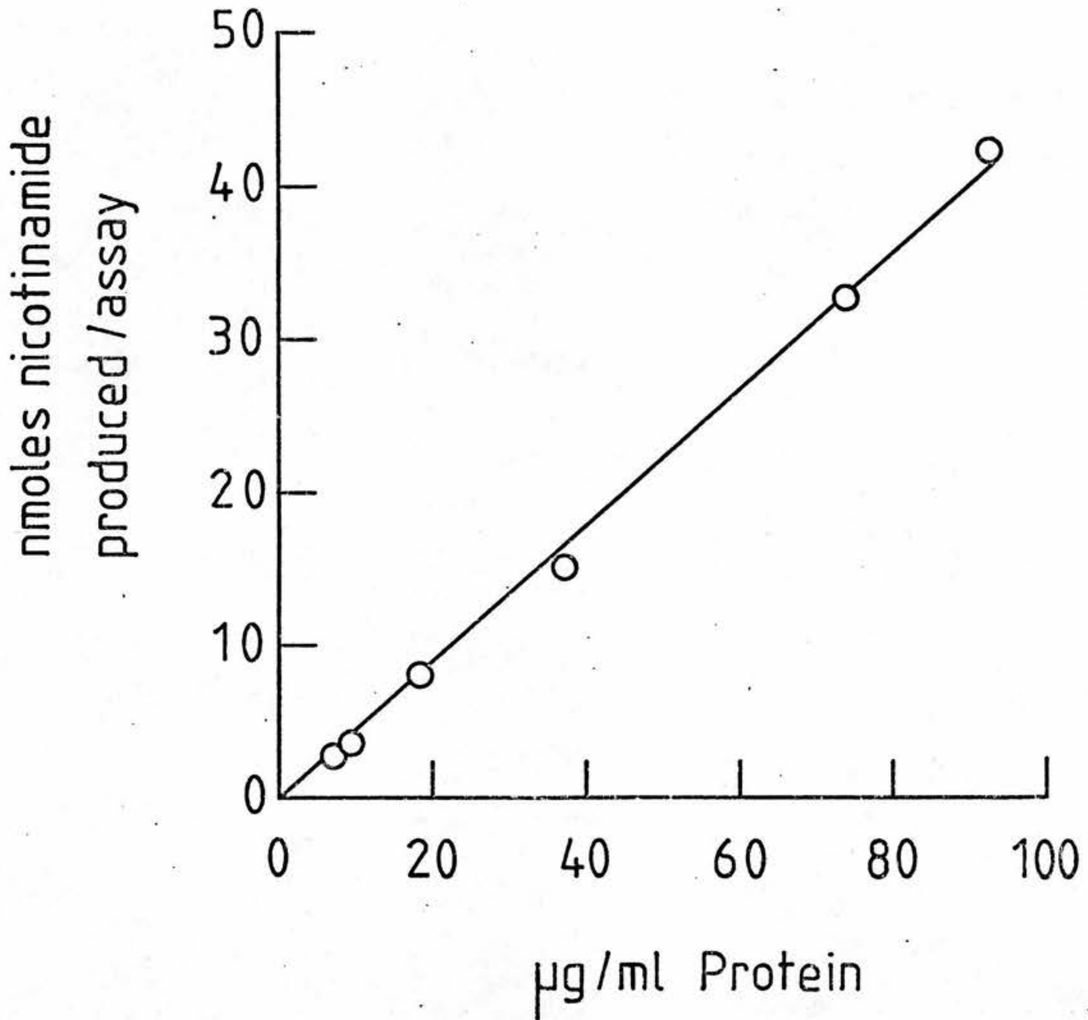


Fig.5.10. Hydrolysis of NAD^+ by crude culture filtrates of V. cholerae.

Assays were conducted in a total volume of 15µl containing 200mM-sodium acetate, 20mM-DTT, 3.3mM-NAD^+, 10nCi of [carbonyl-^{14}C]-NAD^+, and the indicated concentrations of culture filtrate protein, pH 6.2. After 50min at 37°C, 10µl aliquots were taken for estimation of [^{14}C]nicotinamide. All values were the means of duplicate determinations.

Table 5.3. Purification of cholera toxin from V. cholerae culture filtrate - separation from NADase activity.

NADase activities were determined in a total volume of 20μl containing 200mM-potassium phosphate, 20mM-DTT, 2mM-NAD^+ and 10 nCi of [carbonyl-^{14}C]NAD^+, pH 7.0. After 30 min. at 37°C, 10μl aliquots were taken for estimation of [^{14}C]nicotinamide. All values were the means of duplicate determinations and all other techniques were performed as described in Ch. 2.

Fraction	Protein Concentration (mg/ml)	Presence of cholera toxin		NADase activity (nmoles nicotinamide min^{-1} mg^{-1})
		SDS-gel electro-phoresis	Ouchterlony immuno-diffusion	
Culture filtrate	3.03	+	+	538 ± 36
0-60% $AmSO_4$ Fraction	6.45	+	+	215 ± 6
$AmSO_4$ Supernatant	0.27	-	-	951 ±148
Non-toxin DEAE-Fractions	0.77	-	-	1862 ± 8
Toxin DEAE fractions	6.00	+	+	13.8 ± 0.2
Non-toxin AcA-44	2.28	-	-	4.1 ± 0.3
Toxin AcA-44 Fractions	0.84	+	+	15.5 ± 0.4

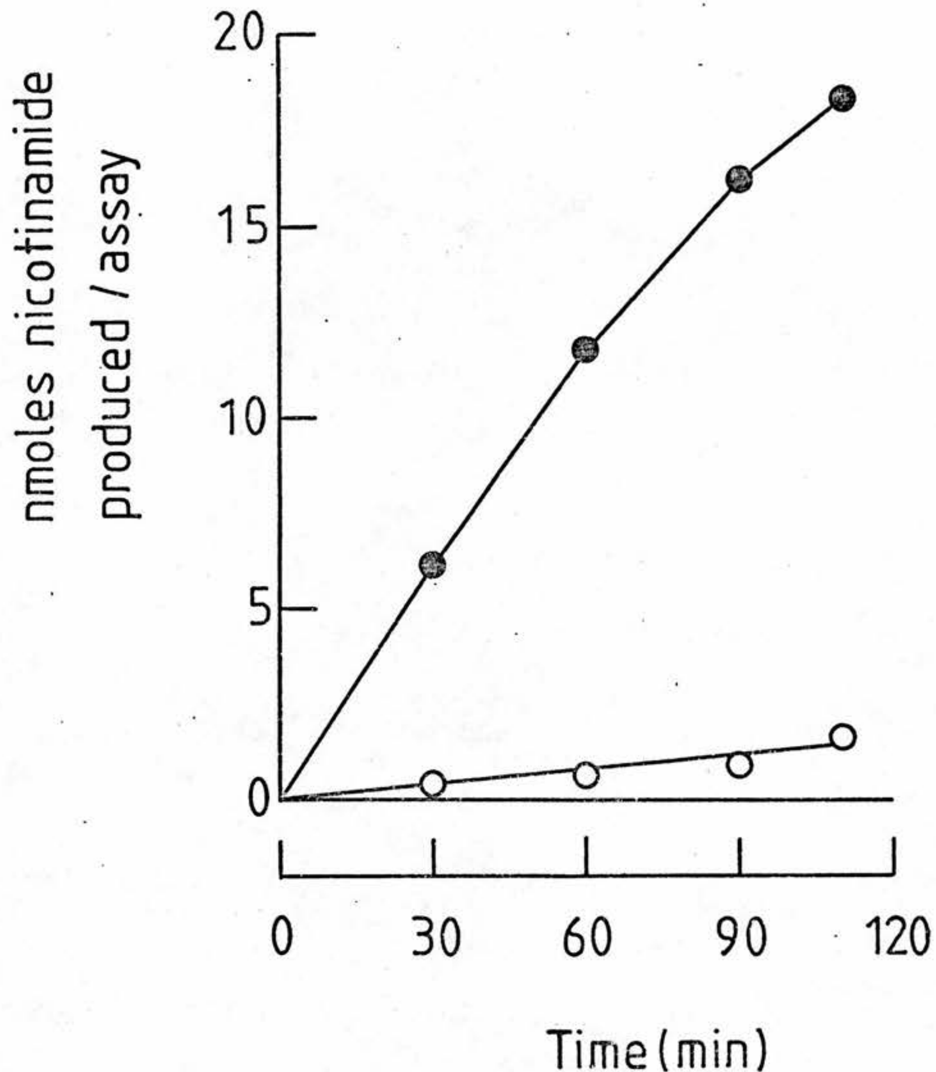


Fig.5.11. Hydrolysis of NAD^+ by purified cholera toxin and by the non-toxin NADase eluted from DEAE-cellulose.

Assays were conducted in a total volume of 20µl containing 200mM-potassium phosphate, 20mM-DTT, 2mM-NAD^+, 10nCi of [carbonyl-^{14}C]-NAD^+, and either 0.75µg of purified cholera toxin (o—o) or 0.08µg of non-toxin NADase (●—●), pH 7.0. After the indicated times at 37°C, 10µl aliquots were taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.

Table 5.4. Effect of INH on the NADase activity of purified cholera toxin and of non-toxin NADase.

Assays were conducted in a total volume of 20µl containing 200 mM-potassium phosphate, 20mM-DTT, 2mM-NAD^+, 4nCi of [carbonyl-^{14}C] NAD^+, and the indicated concentrations of INH, pH 7.0. Assays were initiated by the addition of either purified cholera toxin (7µg protein) or non-toxin NADase (0.08µg protein) and after 90 min. at 37°C, 10µl aliquots were taken for estimation of [^{14}C] nicotinamide. Values were the means of duplicate determinations except where indicated otherwise.

Preparation	INH concentration (mM)	NADase activity (nmoles nicotinamide min^{-1} mg^{-1}).
Non-toxin	0	2134 ± 57
NADase	10	2046 ± 136
Purified Cholera Toxin	0	7.7 ± 1.1
	0.002	8.1*
	0.01	8.5*
	0.1	7.6*
	2	7.5*
	130	7.4*

*Single determinations only.

to inhibition by iso-nicotinic acid hydrazide (INH), an analogue of nicotinamide, and were therefore similar to most other bacterial NADases (seeCh.6.)

The NADase activity of the purified toxin was not stimulated after treatment with dilute mixtures of SDS and DTT, a procedure which enhances the ability of cholera toxin to activate adenylate cyclase in broken cell systems (Gill, 1976 b ; van Heyningen, 1977 b). Instead, this activity and the activity of the non-toxin NADase, were both inhibited markedly after SDS-DTT treatment (Table 5.5.). Conflicting effects were also observed in experiments in which the toxin was preincubated with an excess of antibodies raised against whole toxin in rabbits (see van Heyningen, 1976 a). This treatment, which inhibits the action of cholera toxin in disrupted cell systems (van Heyningen, 1976 b), had no effect on the NADase activity of either purified toxin or of crude culture filtrates of V. cholerae (Table 5.6.). It should be noted that the antitoxin used in this experiment was the purified IgG fraction from crude rabbit antiserum (the crude antiserum itself contained a highly active NADase contaminant which was removed after the purification steps described in Ch. 2.2.2.).

From the quantities of this material and of the purified toxin added to the preincubation mixtures, and assuming molecular weights of 84,000 daltons and 160,000 daltons for cholera toxin and IgG respectively, it can be calculated that IgG was present in an approximately 20-fold molar excess over cholera toxin in this experiment. Although it is unlikely that all of the protein in the IgG fraction was antitoxin antibody, it is probable that sufficient antibody was present to neutralise all of the cholera toxin during preincubation.

Table 5.5. Effect of preincubation with SDS and DTT on the NADase activity of purified cholera toxin and of non-toxin NADase.

Purified cholera toxin or non-toxin NADase were added to a 1/10 volume of either 50mM-Tris/HCl, pH 7.4 or the same buffer containing SDS and DTT to give final concentrations of 0.1% SDS, 1mM-DTT in a total volume of 50μl. After 10 min. at 37°C, 5μl aliquots (5.4μg purified toxin or 0.1μg non-toxin NADase) were transferred to 15μl of assay medium to give final concentrations of 200mM-potassium phosphate, 20mM-DTT, 2mM-NAD^+, and 10 nCi of [carbonyl-^{14}C] NAD^+, pH 7.0. After 90 min. (purified toxin) or 30 min (non-toxin NADase) at 37°C, 10μl aliquots were taken for estimation of [^{14}C] nicotinamide. Values were the means of duplicate (non-toxin NADase) or triplicate (purified toxin) determinations.

Preparation	Preincubation conditions	NADase Activity (nmoles nicotinamide min^{-1} mg^{-1})
Purified toxin	-SDS, DTT	8.6 ± 0.3 (SEM)
	+SDS, DTT	2.7 ± 0.1 (SEM)
Non-toxin NADase	-SDS, DTT	5490 ± 60
	+SDS, DTT	46 ± 4

Table 5.6. Effect of cholera antitoxin on the NADase activity of purified toxin and of V. cholerae culture filtrates.

25µl of anti-cholera toxin IgG (350µg protein), prepared as described in Ch. 2.2.2. were mixed with an equal volume of either culture filtrate (0.75µg protein) or purified toxin (15µg protein) and incubated at $37^{\circ}C$ for 10 min. Control incubations contained the buffer in which the antitoxin was prepared. After 10 min. 10µl aliquots were transferred to 10µl of assay medium to give final concentrations of 200 mM-sodium acetate, 20mM-DTT, 2.5mM-NAD^{+}, and 10nCi of [Carbonyl-^{14}C NAD^{+}], pH 6.2. After 60 min (Culture filtrate) or 120 min (purified toxin) at $37^{\circ}C$, 5µl of 25% -TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{14}C] - nicotinamide. Values were the means of duplicate (culture filtrate) or triplicate (purified toxin) determinations.

Preparation	Preincubation conditions	NADase activity (nmoles nicotinamide min^{-1} mg^{-1}.
culture filtrate	- antitoxin	467 $\pm$ 16
	+ antitoxin	429 $\pm$ 48
Purified toxin	- antitoxin	8.8 $\pm$ 0.9 (SEM)
	+ antitoxin	9.4 $\pm$ 0.7 (SEM)

5.2.5. Separation of NADase and adenylate cyclase activating activity of cholera toxin by polyacrylamide gel electrophoresis

The results presented in the previous section suggested that the NADase activity in purified cholera toxin preparations may not be a property of the toxin itself, but may be due to a contaminant not entirely removed during purification. In order to test this hypothesis directly, samples of crude culture filtrates and of purified cholera toxin were electrophoresed on native polyacrylamide gels which were sliced and eluted as described in Ch. 2.3.3. The eluates, after freeze-drying and resuspension in 30µl of water, were analysed for NADase activity, adenylate cyclase activating activity, and antigenicity against rabbit antiserum. The results are shown in Figs. 5.12.A and B for crude and purified toxin respectively.

Antigenic material was detected in both gels at mobility 0.05 to 0.15 where there was the expected comigrating peak of adenylate cyclase activating activity. Surprisingly the crude toxin displayed a major peak of cyclase activating material, distinct from cholera toxin, at mobility 0.45, and although this material was less evident in the purified toxin, it was replaced by several smaller peaks of activity notably at mobilities 0.20, 0.27, and 0.32. The peak at 0.27 was also present, but to a lesser extent, in crude cholera toxin.

In both gels there was one major peak of NADase activity at mobility 0.55 with a smaller peak at 0.27. An area of very low NADase activity co-migrating with cholera toxin in the crude preparation, was not evident after purification.

Figure 5.12. Separation of NADase activity from adenylate cyclase activating activity of cholera toxin on native polyacrylamide gels.

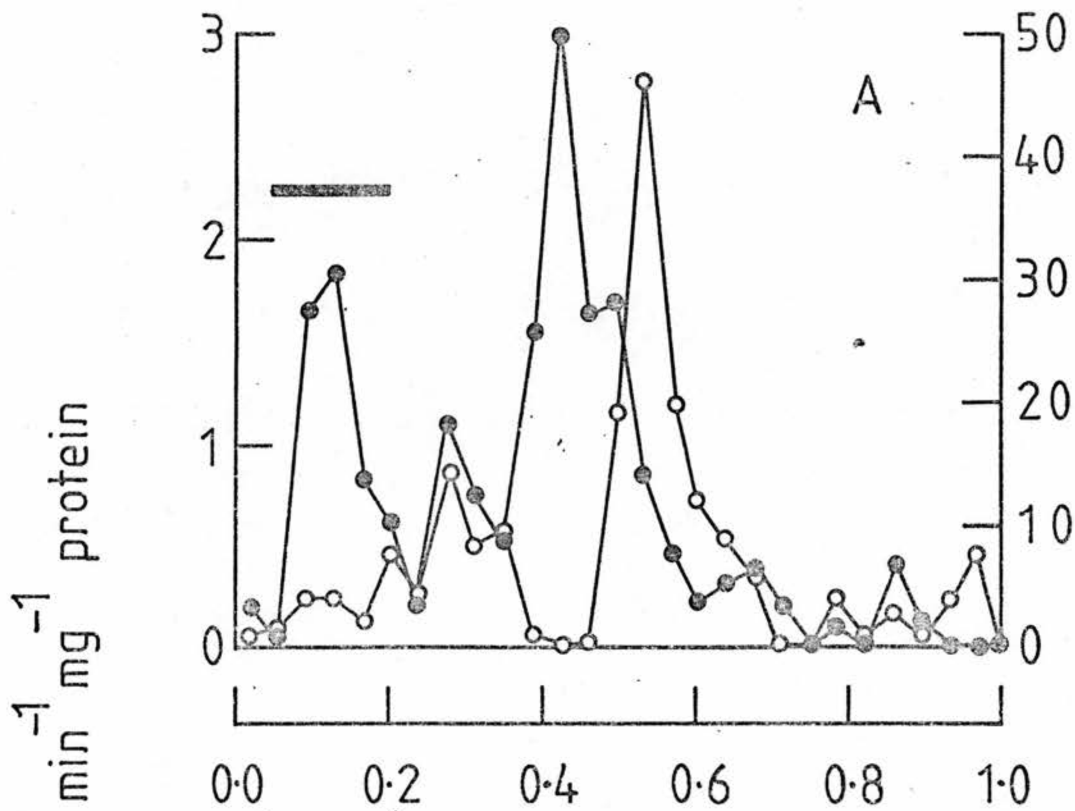
50 µl of either V. cholerae culture filtrate (A) or cholera toxin purified therefrom (B), containing 150 µg and 50 µg of protein respectively, were electrophoresed on native polyacrylamide gels which were sliced and eluted as described in Ch. 2.3.3. After freeze-drying and resuspension in 30 µl of water, 5 µl were taken for Ouchterlony immuno-diffusion against rabbit antitoxin as described in Ch. 2.2.1., 5µl for NADase assay (o——o), and 10 µl for adenylate cyclase activating assay (●——●).

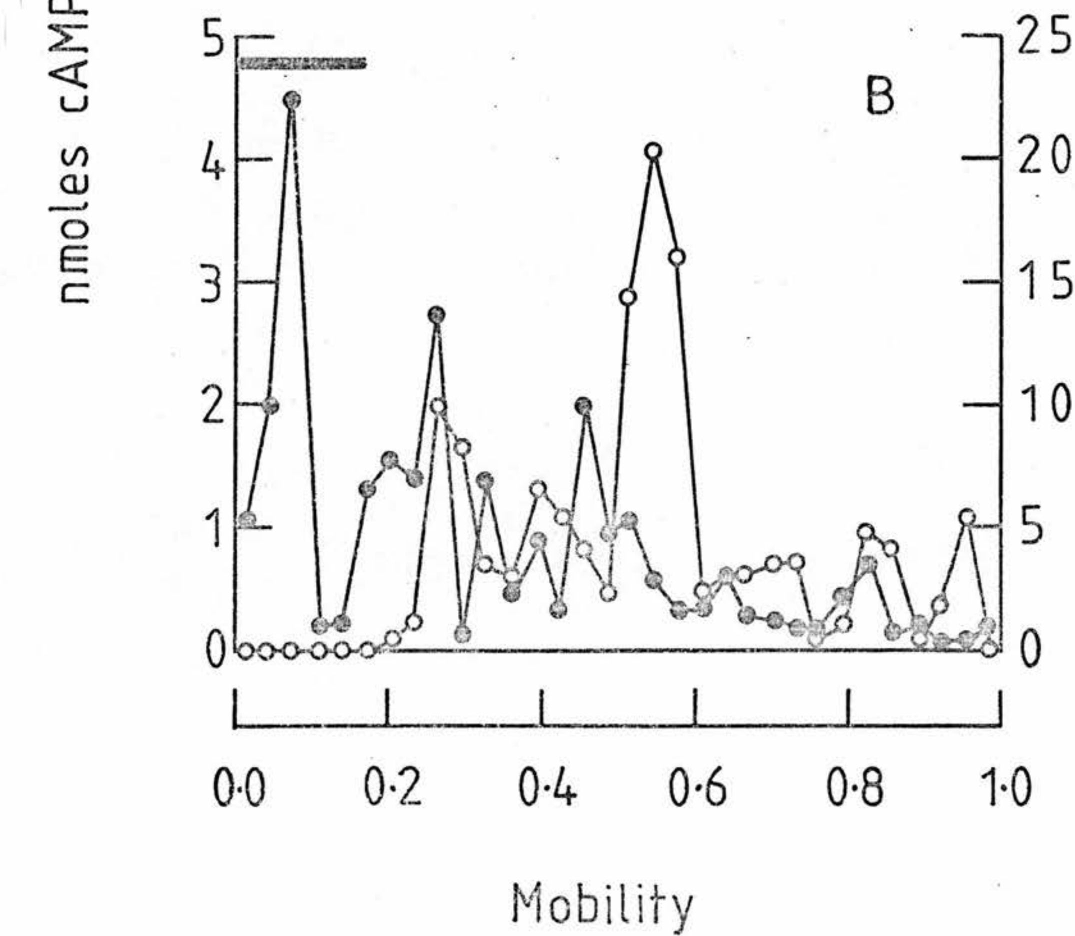
NADase activities were determined in a total volume of 15µl containing 200mM-sodium acetate, 20mM-DTT, 2mM-NAD^+, and 10 nCi of [carbonyl - ^{14}C] NAD^+, pH 6.2. After 50 min. at 37°C, 10 µl aliquots were taken for estimation of [^{14}C]- nicotinamide.

Adenylate cyclase activating assays were carried out by adding 10 µl of each gel slice eluate to 15µl of rat liver homogenate prepared in Buffer System B as described in Ch.2.5., in the presence of 2.5mM-NAD^+, 2.5 mM-ATP, 2.5 mM-DTT, and 2.5mM-INH. After 20 min. at 37°C, 15 µl aliquots were transferred to 10µl of assay medium I (Ch. 3.3.2.3.) containing 14,500 pmoles of ATP and 0.6µCi of [8 - ^3H] ATP, and adenylate cyclase was assayed for 20 min at 37°C. Reactions were terminated by adding 10µl of 25% - TCA, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^3H] cAMP.

Figure 5.12. cont.

All assays were single determinations only. Horizontal bars indicate the mobilities at which cholera toxin was detected by Ouchterlony Immunodiffusion.


A
nmoles cAMP min^{-1} mg^{-1} protein
nmoles nicotinamide produced / 50min / 30µl eluates
0
1
2
3
0
10
20
30
40
50
0·0
0·2
0·4
0·6
0·8
1·0


B
0
1
2
3
4
5
0
5
10
15
20
25
0·0
0·2
0·4
0·6
0·8
1·0
Mobility

Thus although the purified cholera toxin was homogeneous as judged by the presence of one band after staining a corresponding gel for protein (see gel scan of purified toxin in Fig. 4. 9), the enzymatic analysis described here emphasises the extent of heterogeneity in the preparation and supports the hypothesis that the NADase activity of purified cholera toxin is due to a contaminant enzyme, present in small amounts, but of very high activity.

5.2.6. Kinetics of NAD^+ hydrolysis by different preparations of cholera toxin.

In an attempt to determine whether the NADase activity of several different cholera toxin preparations was due to the same contaminant, kinetic rate parameters for the reactions were investigated. Initial rates of NAD^+ hydrolysis in the presence of increasing concentrations of NAD^+ (0.2mM to 1.0 mM) were determined and the results were analysed using the Direct Linear Plot of Eisenthal and Cornish-Bowden (1974), and a later modification of the same method (Cornish-Bowden and Eisenthal, 1978). Analysis of kinetic data on these plots is explained in Appendix II.

As shown in Fig. 5.13. the NADase activity in crude culture filtrates of V. cholerae exhibited a K_m for NAD^+ of 0.24 mM, and a V_{max} of 3.15 µmoles nicotinamide $min.^{-1}$ mg^{-1}. Purified cholera toxin derived from this material displayed a very similar K_m value (0.20mM) with a V_{max} which was only 1/150 of that of the crude material (Fig. 5.14). This is in agreement with the results of the previous experiment where the NADase activity of purified cholera toxin was accounted for by a contaminant which migrated on polyacrylamide gels with the same

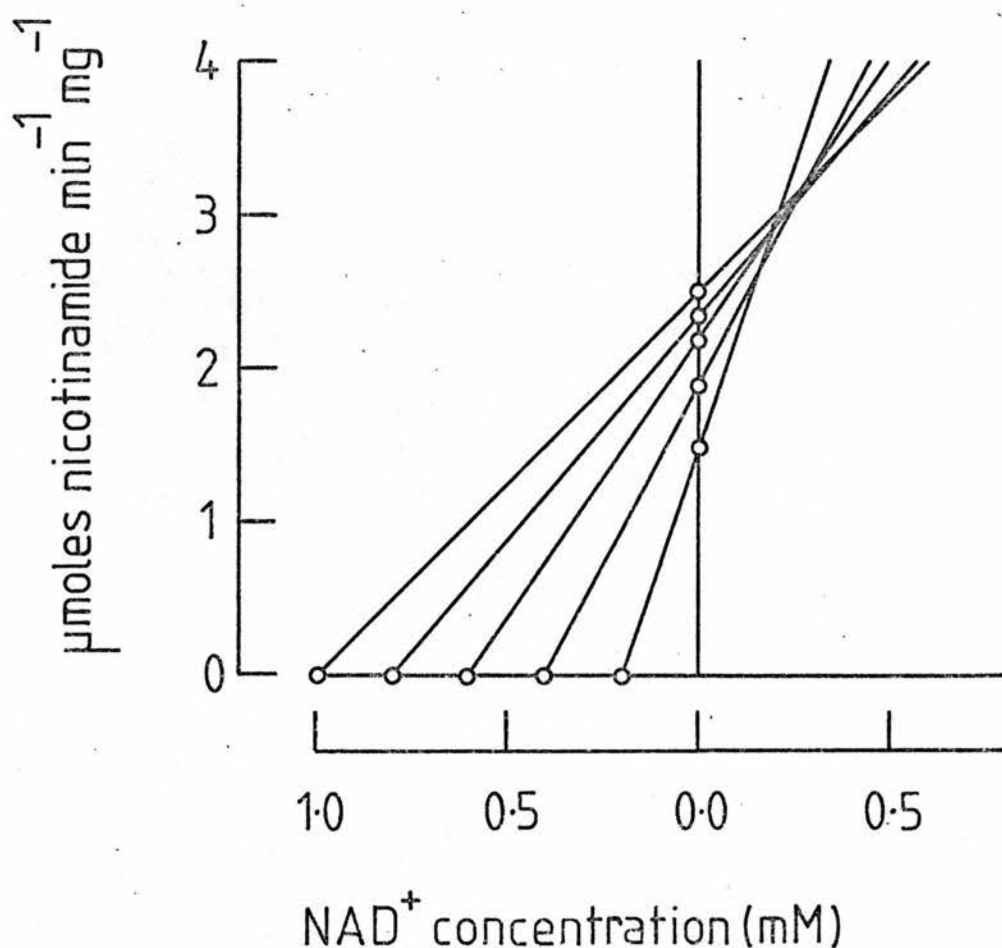


Fig.5.13. Kinetic analysis of the NADase reaction catalysed by crude culture filtrates of *V. cholerae*.

Assays were conducted in a total volume of 40µl containing 10mM-potassium phosphate, 20mM-DTT, 120nCi of [carbonyl-^{14}C]NAD^{+}, 0.31µg of culture filtrate protein, and the indicated concentrations of NAD^{+}, pH 7.0. After 5min at 37°C, 10µl of 25%-TCA were added and 10µl aliquots taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.

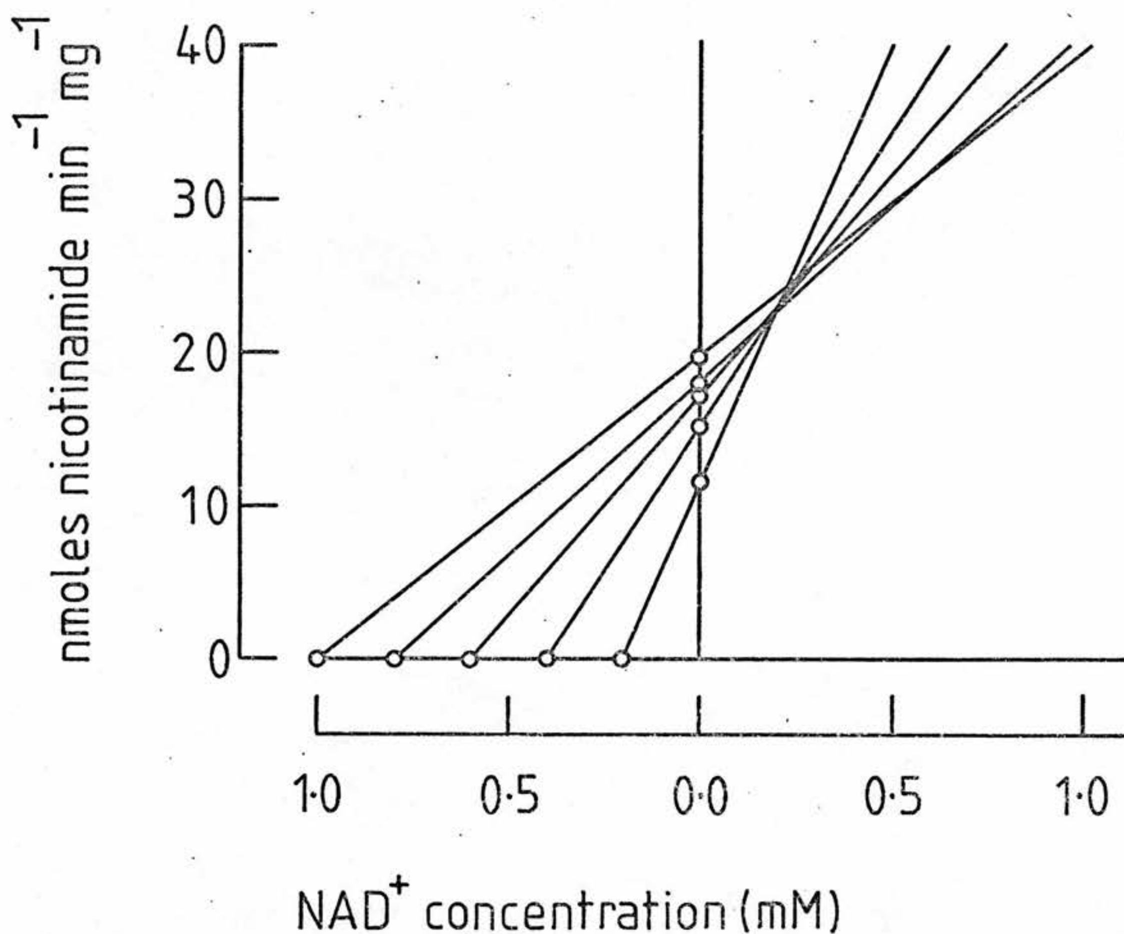


Fig. 5.14. Kinetic analysis of the NADase reaction catalysed by cholera toxin purified from crude culture filtrates of V. cholerae.

Assays were conducted in a total volume of 40µl containing 10mM-potassium phosphate, 20mM-DTT, 100nCi of [carbonyl-^{14}C]NAD$^+$, 1.9µg of cholera toxin, and the indicated concentrations of NAD$^+$, pH 7.0. After 60min at 37°C, 10µl of 25%-TCA were added and 10µl aliquots taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.

mobility as the major peak of NADase activity in the crude toxin, and provides further evidence to support the hypothesis that both activities are due to the same enzyme.

However, when a similar kinetic experiment was conducted with a sample of purified toxin obtained from Dr. R. Rappaport, a much higher K_m value (2.5mM) was calculated (Fig. 5.15), and the V_{max} of this preparation (10.5 nmoles nicotinamide $min.^{-1}$ mg^{-1}) was about half that of the toxin purified from crude culture filtrates in this laboratory. Furthermore, a sample of purified cholera toxin obtained from Dr. R.A. Finkelstein had a similar K_m value of 2.1 mM, and in this preparation the V_{max} was ten-fold lower again at only 1.1 nmoles nicotinamide $min.^{-1}$ mg^{-1} (Fig. 5.16.)

Despite these differences in the K_m values between cholera toxin purified in this laboratory and the other two preparations described above, and despite the fact that in the former preparation, no detectable NADase activity was associated with the toxin itself (Fig. 5.12.B.), this material and the purified toxin from Dr. R.A . Finkelstein activated adenylate cyclase in rat liver membranes to the same extent when added in equal concentrations (Fig. 5.17.)

Since it was not clear from the above results whether or not cholera toxin was capable of hydrolysing NAD^+, and since NADase activity did not appear to correlate with the efficiency of adenylate cyclase activation by the toxin, it was considered that the continued study of this activity could easily result in the formulation of erroneous conclusions, and the role of NAD^+ in toxin action was therefore studied directly in crude preparations of adenylate cyclase.

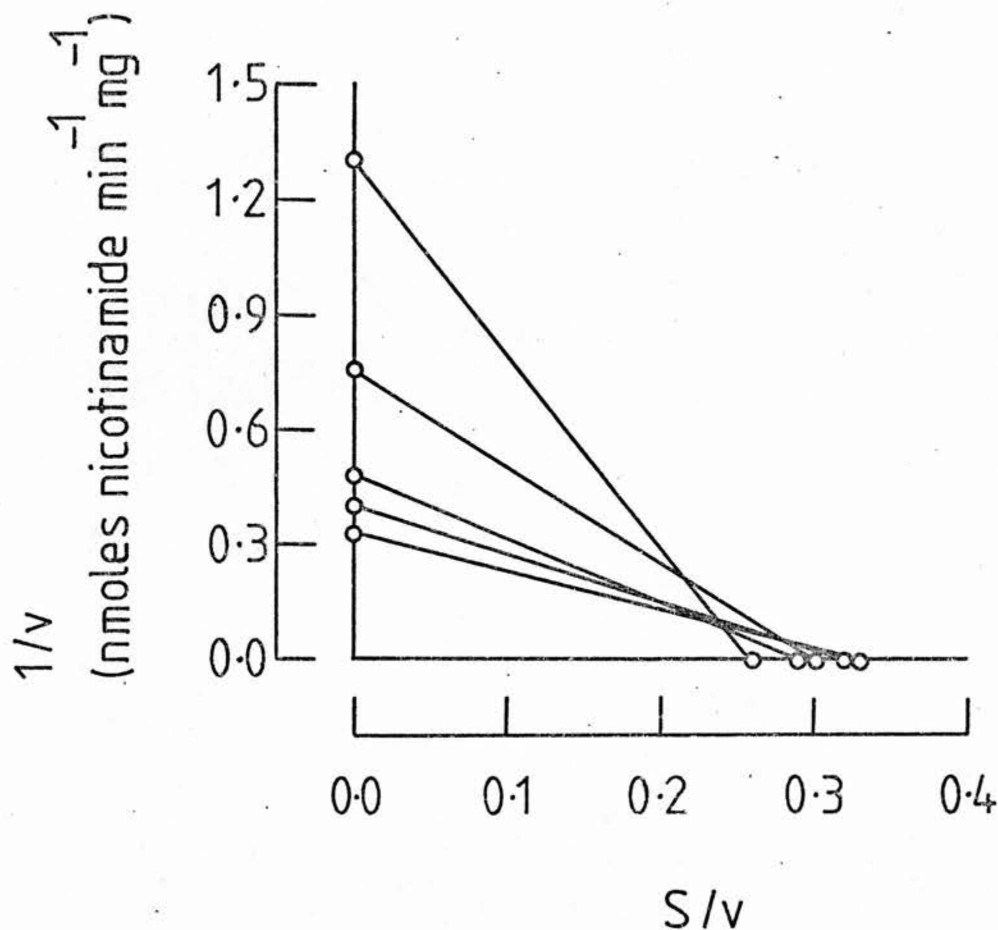


Fig.5.15. <u>Kinetic analysis of the NADase reaction catalysed by cholera toxin obtained from Dr. R. Rappaport</u>.

Assays were conducted in a total volume of 40μl containing 10mM-potassium phosphate, 20mM-DTT, 100nCi of [carbonyl-^{14}C]NAD^+, 4.6μg of cholera toxin, and the indicated concentrations of NAD^+, pH 7.0. After 60min at 37°C, 10μl of 25%-TCA were added and 10μl aliquots taken for estimation of [^{14}C]nicotinamide. All values were the means of duplicate determinations.

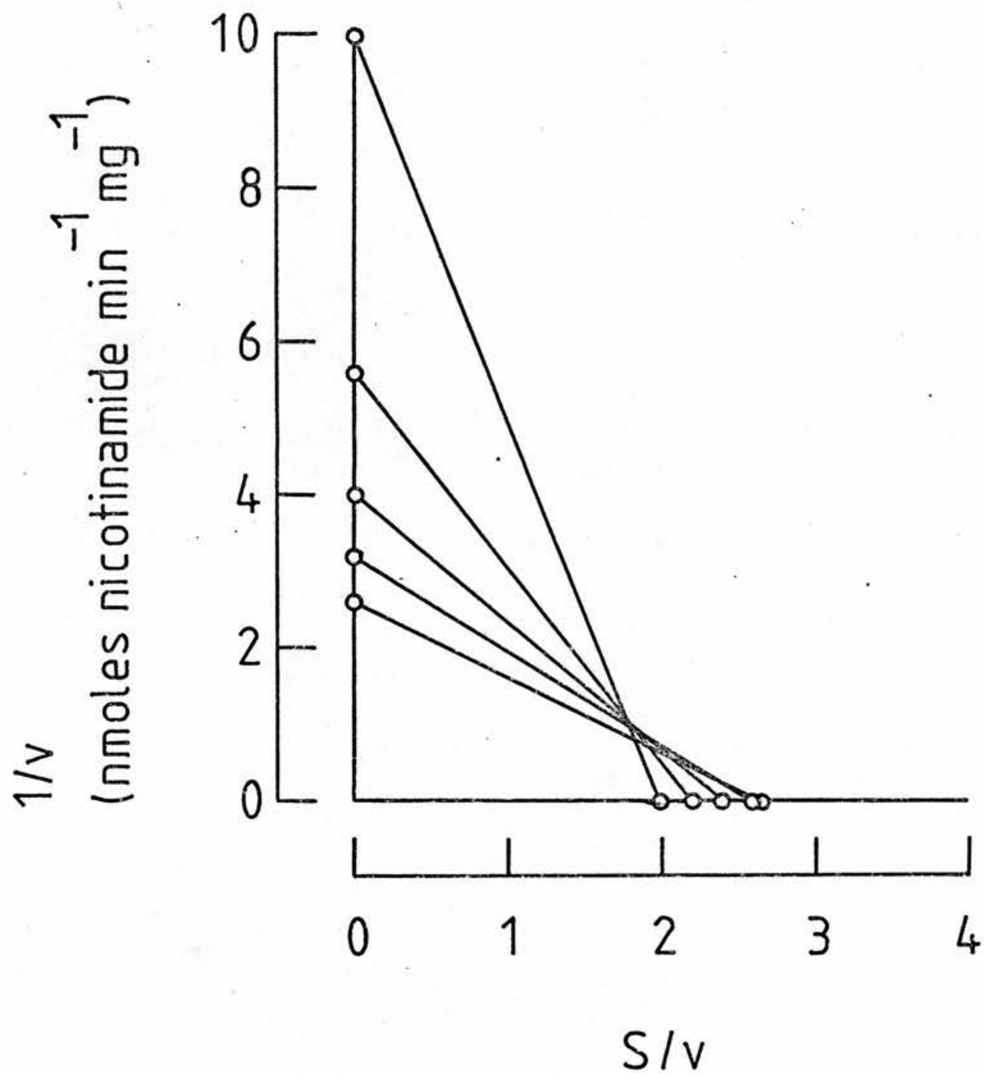


Fig.5.16. Kinetic analysis of the NADase reaction catalysed by cholera toxin obtained from Dr. R.A.Finkelstein.

Assays were conducted in a total volume of 40µl containing 10mM-potassium phosphate, 20mM-DTT, 100nCi of [carbonyl-^{14}C]NAD$^+$, 10µg of cholera toxin, and the indicated concentrations of NAD$^+$, pH 7.0. After 60min at 37°C, 10µl of 25%-TCA were added and 10µl aliquots taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.

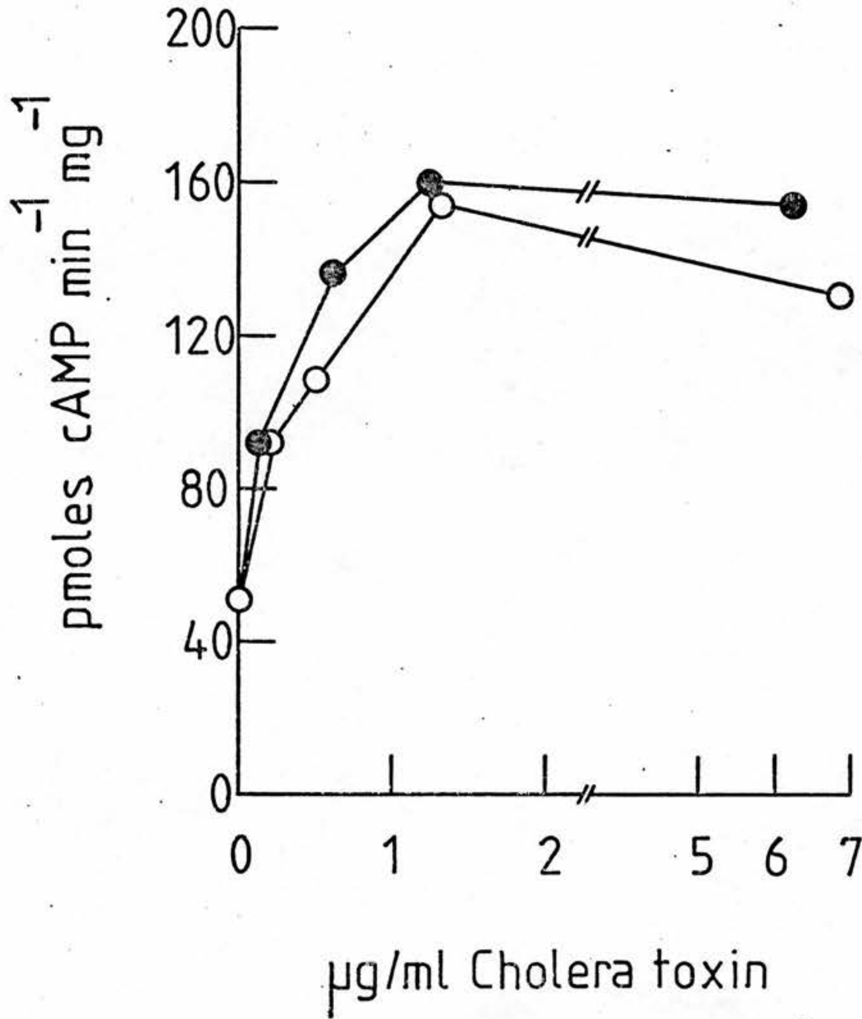


Fig.5.17. Activation of rat liver adenylate cyclase by two different preparations of cholera toxin.

A rat liver homogenate was prepared in Buffer System D as described in Ch.2.5. and aliquots incubated in a total volume of 40µl with 5mM-ATP, 5mM-DTT, 2mM-NAD^+, and either cholera toxin purified in this laboratory (o—o), or purified and obtained from Dr.R.A.Finkelstein (●—●). After 15min at 37°C, an excess of cold buffer was added, membranes were precipitated by centrifugation, resuspended in 40µl of buffer, and 15µl aliquots added to 10µl of assay medium II (Ch.3.3.2.3). After 10min at 37°C, 5µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 5µl of each supernatant taken for estimation of [^{3}H]-cAMP. All values were single determinations only.

5.3. The role of NAD^+ in the activation of adenylate cyclase in particulate fractions of rat liver by cholera toxin.

5.3.1. Dependence of cholera toxin action on the presence of NAD^+.

When crude rat liver membranes were incubated in adenylate cyclase assay medium in the presence of cholera toxin, no activation of adenylate cyclase occurred unless NAD^+ was also present (Fig. 5.18.) However, 10μM-NAD^+ was completely ineffective in supporting cyclase activation, 100μM-NAD^+ supported some activation only after a 10 min. incubation time, and maximum toxin action was observed with 1mM-NAD^+ when cyclase activation first became apparent after 6 min. of assay and was maximal (2.5-fold) after 8 to 10 min. Addition of 5mM-NAD^+ did not result in any greater cyclase activation and indeed reduced the maximum adenylate cyclase specific activity attained.

Thus, although there was an obligatory requirement for NAD^+ in cholera toxin action, the results in Fig. 5.18. were not entirely consistent with the hypothesis that NAD^+ is a substrate in a toxin-catalysed reaction since if this were the case, the extent of cyclase activation would be expected to increase linearly with substrate (NAD^+) concentration until the latter achieved saturating concentrations. These results however indicate that there is a definite 'threshold' concentration of NAD^+ below which adenylate cyclase activation by cholera toxin cannot be supported. Nevertheless, in crude membrane preparations of the type employed here, there may be a number of factors which could interfere in a directly linear response of toxin action with NAD^+ concentration.

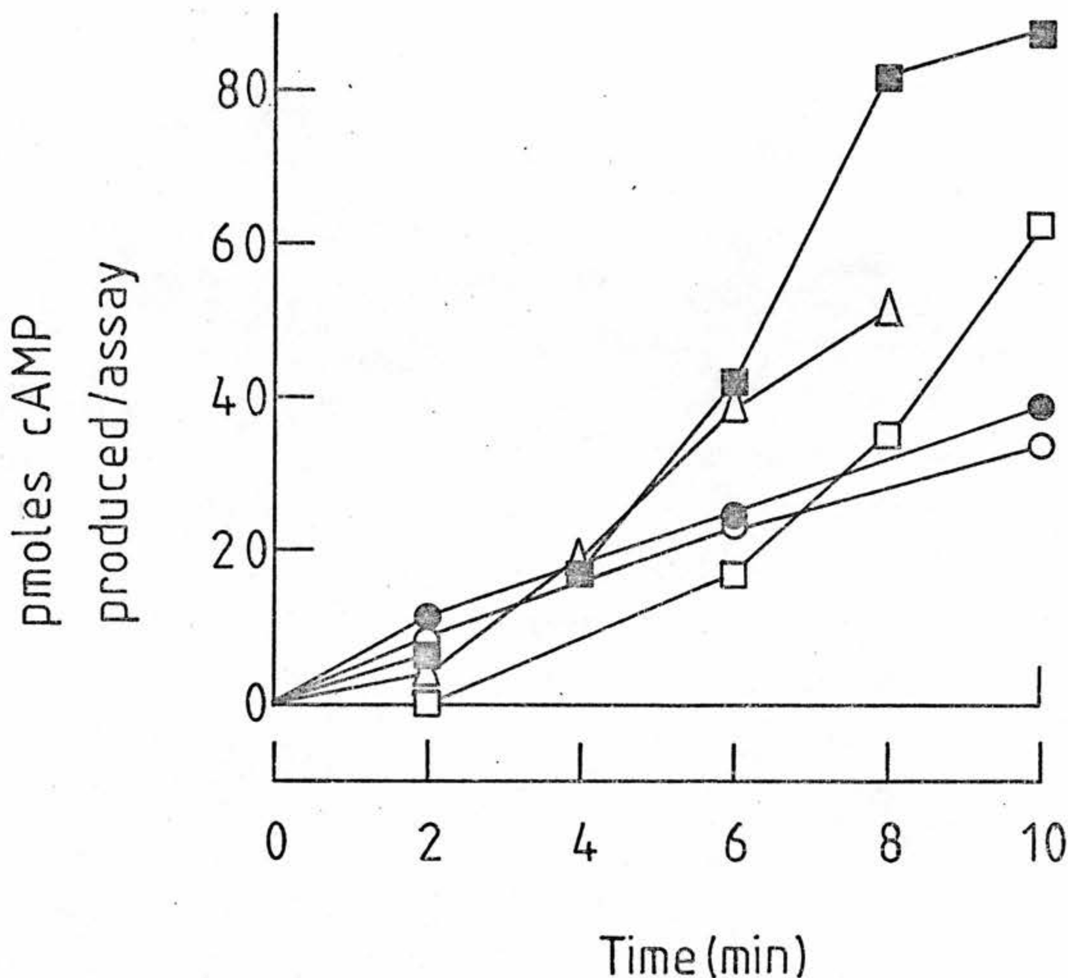


Fig.5.18. Effect of NAD^+ on adenylate cyclase activation by cholera toxin,

Crude rat liver membranes were prepared in Buffer System D as described in Ch.2.5. and 10μl aliquots were incubated in a total volume of 25μl in assay medium II (Ch.3.3.2.3), Assays contained preactivated cholera toxin at a final concentration of 2μg/ml in the presence of 0 (●—●), 100μM (□—□), 5mM (△—△), and 1mM (■—■) NAD^+. Basal activity (○—○) was determined by substituting activation solution for cholera toxin. After the indicated times at 25°C, 5μl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 10μl of each supernatant taken for estimation of [^{3}H]cAMP. All values were single determinations only.

A major source of potential interference was recognised when rat liver fractions were assayed for endogenous NADase activity as described in the following section.

5.3.2. NAD^+ Hydrolysis by rat liver preparations.

As shown in Fig. 5.19., rat liver homogenates were extremely effective in catalysing the release of nicotinamide from NAD^+. When portions of homogenate were incubated in the presence of 2mM-NAD^+, 50% of the NAD^+ was hydrolysed after only 5 min. and after about 20 min, all added NAD^+ was destroyed. Although the release of nicotinamide from NAD^+ can be accomplished by more than one type of enzyme (see Ch. 6.), the activities are referred to here collectively as endogenous rat liver NADase.

Almost all of the endogenous NADase activity was associated with the crude membrane fraction and was found at a very high level in a purified plasma membrane fraction prepared earlier (Table 5.7.) This activity, by catalysing the rapid destruction of NAD^+, may be responsible for the failure of low concentrations of NAD^+ to support adenylate cyclase activation by cholera toxin, and in order to study the mechanism of the toxin's action in the absence of such interference, several attempts were made to selectively inhibit the activity of these enzymes.

Fig. 5.20. illustrates the effects of temperature and of INH on the endogenous NADases. Like most enzymic reactions, NAD^+ hydrolysis was very slow at 4^oC and the activity at 25^oC was about half

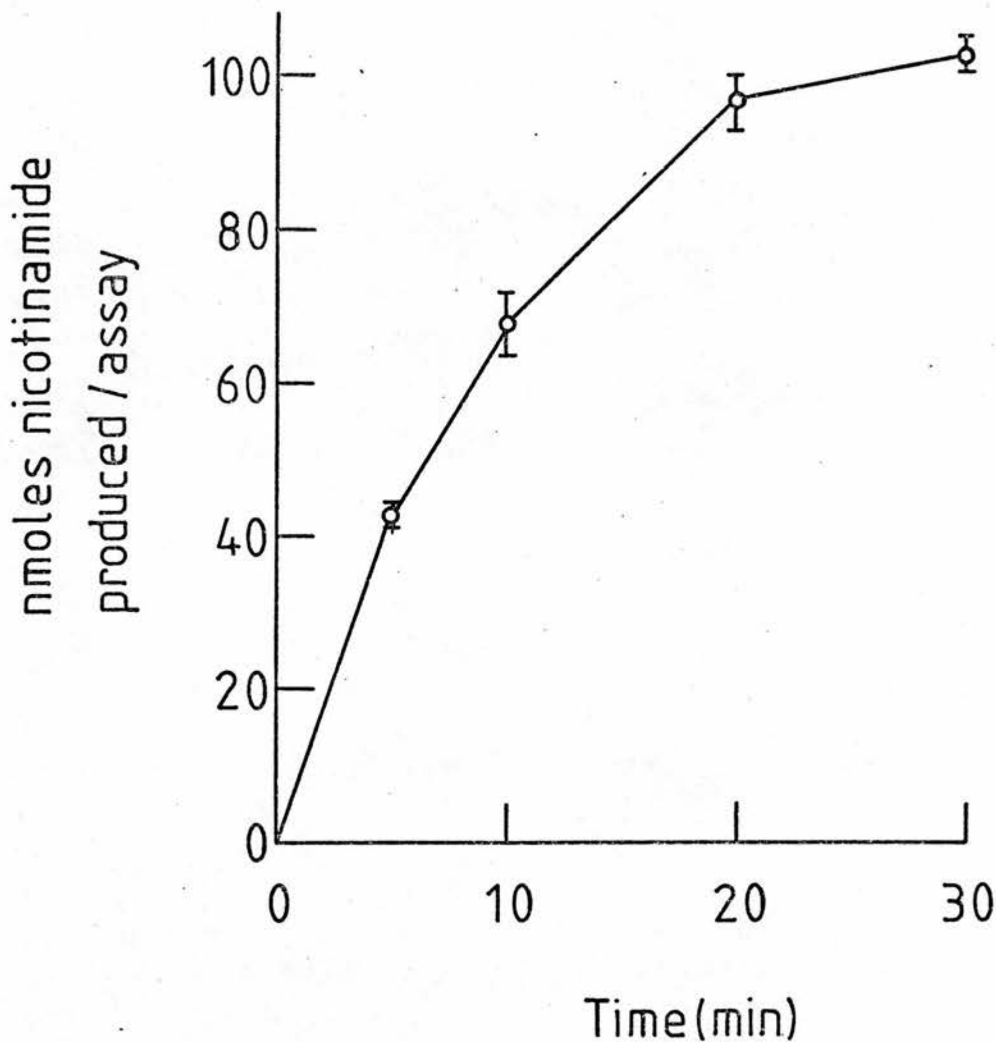


Fig.5.19. Hydrolysis of NAD^+ by rat liver homogenates.

A rat liver homogenate was prepared in Buffer System B as described in Ch.2.5. and 30µl aliquots were incubated in a total volume of 50µl containing 2mM-NAD^+ and 25nCi of [carbonyl-^{14}C]NAD^+. After the indicated times at 37°C, 50µl of 5%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{14}C]nicotinamide. Error bars indicate the range of duplicate determinations.

Table 5.7. NADase activity in subcellular fractions of rat liver.

Crude membranes and cytosol fractions (prepared in Buffer System B) and purified plasma membranes from rat liver were prepared as described in Ch. 2.5. 30µl aliquots of each fraction were incubated in a total volume of 50µl with 2mM-NAD^+ and 25 nCi of [Carbonyl-^{14}C] NAD^+. After 10 min (crude membranes and cytosol) or 5 min. (plasma membranes) at $37^{o}C$, 50µl of 5%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{14}C] nicotinamide. All values were the mean of triplicate determinations.

FRACTION	NADase ACTIVITY (n moles nicotinamide min^{-1} mg^{-1})
Crude membranes	40.0 ± 1.1 (SEM)
Cytosol	6.4 ± 0.4 (SEM)
Plasma membranes	151.6 ± 0.2 (SEM)

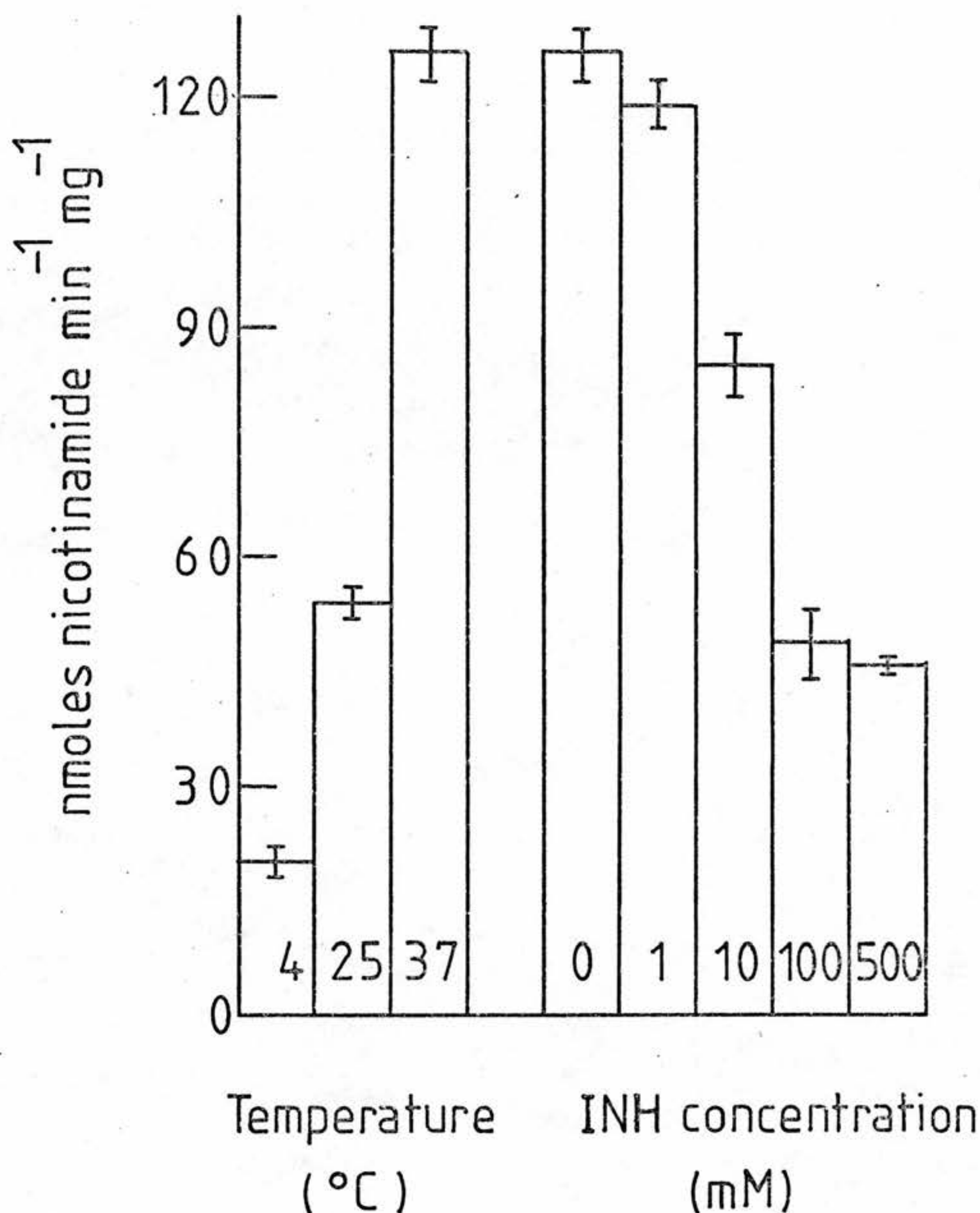


Fig.5.20. Effect of temperature and of INH on rat liver NADases.

Crude rat liver membranes were prepared in Buffer System B as described in Ch.2.5. and 10µl aliquots were incubated in a total volume of 50µl with 2mM-NAD^+ and 25nCi of [carbonyl-^{14}C]NAD^+, equilibrated at the indicated temperatures. Assays were also conducted at 37°C only, in the presence of increasing concentrations of INH. After 5min, 50µl of 5%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{14}C]nicotinamide. Error bars indicate the range of duplicate determinations.

of that at 37°C. At the latter temperature some inhibition was observed with INH, but only when high concentrations were employed. Maximal inhibition (about 60%) was achieved with 100mM-INH and addition of 500mM-INH produced no further effect. It therefore appears that some of the activities contributing to total NAD^+ hydrolysis are sensitive to INH at high concentrations, but that the remainder of the activities are of the 'INH-insensitive' type (see Ch. 6.).

Methylxanthines and Imidazole derivatives have been reported to inhibit NADase and poly (ADP-ribose) polymerase activities (Gill, 1972; Levi et al, 1978).

However, as shown in Fig. 5.21., isobutyl-methylxanthine (BMX), at concentrations approaching the limits of its solubility in water, had only a slight inhibitory effect on the endogenous rat liver NADases. Similarly, Green and Dobrjansky (1971a,b.) have reported the inactivation of NADases from mammalian species by incubation at pH 8.0 with their substrate, NAD^+, but again this treatment was only partially effective in inhibiting endogenous rat liver NADase activities. As shown in Fig. 5.22. maximal inhibition (about 50%) was observed after preincubation with 1mM-NAD^+ for 10 min. at 0°C, and higher NAD^+ concentrations were without further effect. Like INH therefore, preincubation with NAD^+ affected only a certain proportion of the total NAD^+-utilising activities, and since a combination of NAD^+-preincubation and subsequent assay in the presence of 100mM-INH only resulted in the same degree of inhibition (results not shown), both treatments apparently affected the same activities. A similar experiment involving preincubation with nicotinamide was completely ineffective as an inhibitor of endogenous NADase activities (Fig. 5.23.)

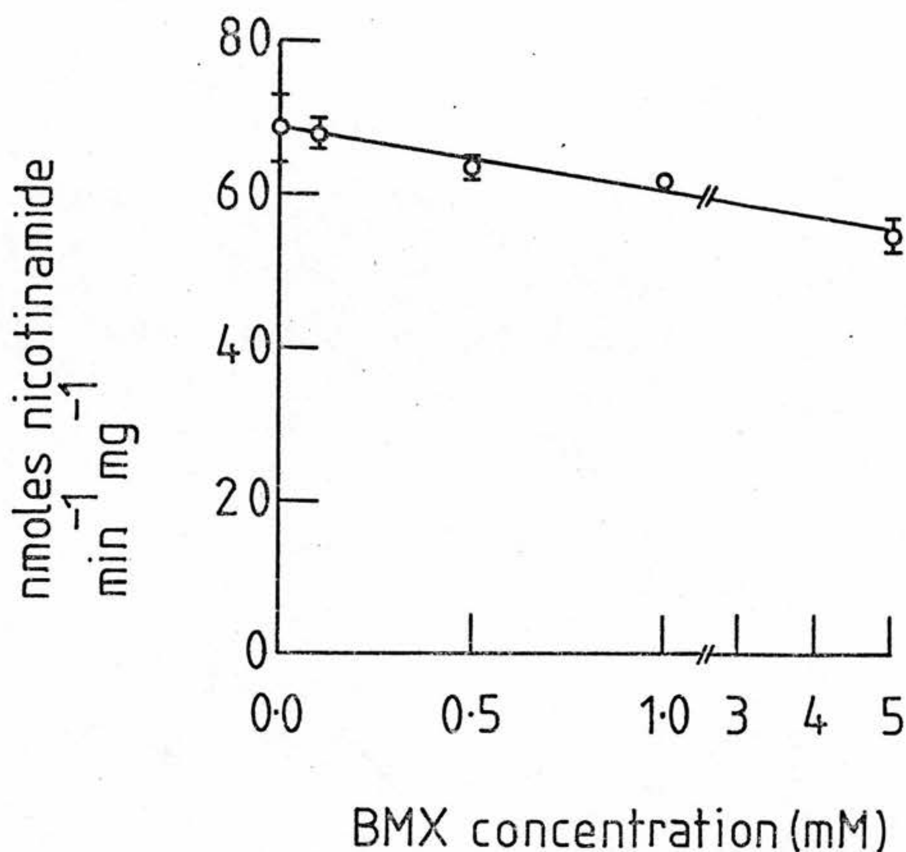


Fig.5.21. Effect of BMX on rat liver NADases.

Crude rat liver membranes were prepared in Buffer System B as described in Ch.2.5. except that the pH was increased to 8.0. 20µl aliquots were incubated in a total volume of 80µl with 1.25mM-NAD^+, 25nCi of [carbonyl-^{14}C]NAD^+, and the indicated concentrations of BMX. After 5min at 37°C, 20µl of 5%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{14}C]nicotinamide. Error bars indicate the range of duplicate determinations.

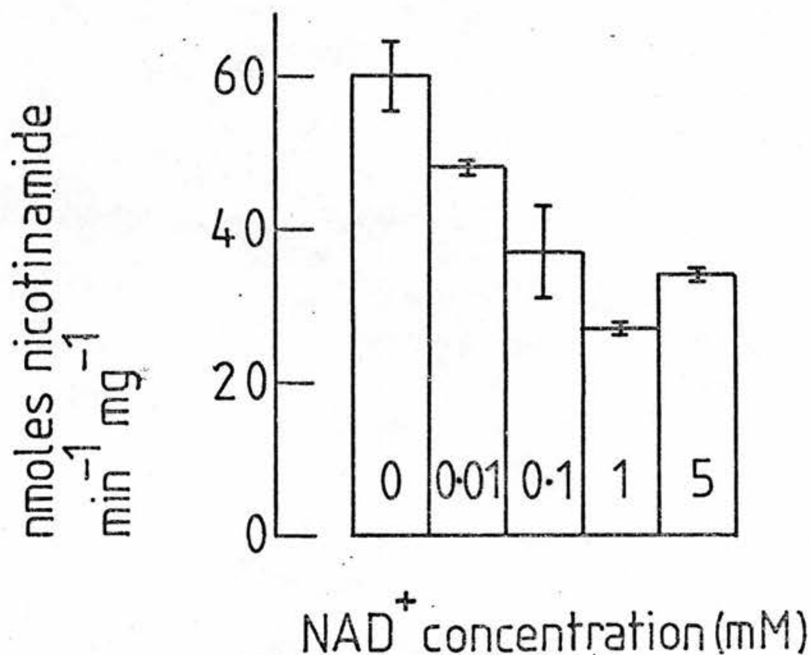


Fig.5.22. Effect of preincubation with NAD^+ on rat liver NADases.

Crude rat liver membranes were prepared in Buffer System B as described in Ch.2.5. except that the pH was increased to 8.0, and 50µl aliquots were incubated in a total volume of 100µl with the indicated concentrations of NAD^+. After 10min at $0^{\circ}C$, an excess of cold 10mM-Tris/HCl, pH 8.0 was added, membranes were precipitated by centrifugation, and after a further wash and centrifugation, were finally resuspended in 100µl of 10mM-Tris/HCl, pH 8.0. 30µl aliquots of each suspension were incubated in a total volume of 50µl with 2mM-NAD^+ and 25nCi of [carbonyl-^{14}C]NAD^+. After 20min at $37^{\circ}C$, 50µl of 5%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{14}C]nicotinamide. Error bars indicate the range of duplicate determinations.

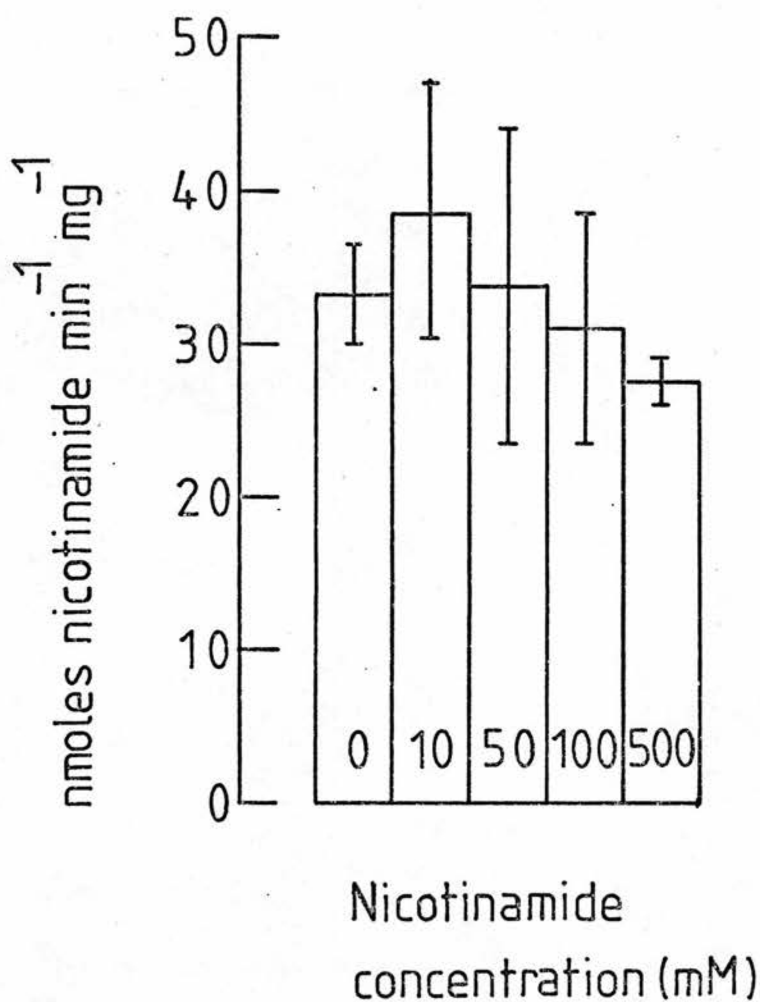


Fig.5.23. Effect of preincubation with nicotinamide on rat liver NADases.

Experimental details were exactly as described in the legend to Fig.5.22. except that 200µl aliquots of homogenate were preincubated in a total volume of 400µl with the indicated concentrations of nicotinamide at 25°C. Error bars indicate the range of duplicate determinations.

The direct use of nicotinamide in the assay medium was initially avoided because of its reported inhibitory effect on the action of cholera toxin (Gill and Meren, 1978). However in the experiments reported in the following section, where adenylate cyclase activation by cholera toxin was studied in relation to the incorporation of ADP-ribose into rat liver membrane proteins, the use of nicotinamide proved extremely useful in investigating the mechanism of toxin action.

A procedure was also devised which enabled almost complete separation of adenylate cyclase from endogenous NADase activities and results obtained with this system are reported in Ch. 5.5.

5.4. Activation of adenylate cyclase and incorporation of ADP-Ribose in rat liver homogenates and membrane preparations.

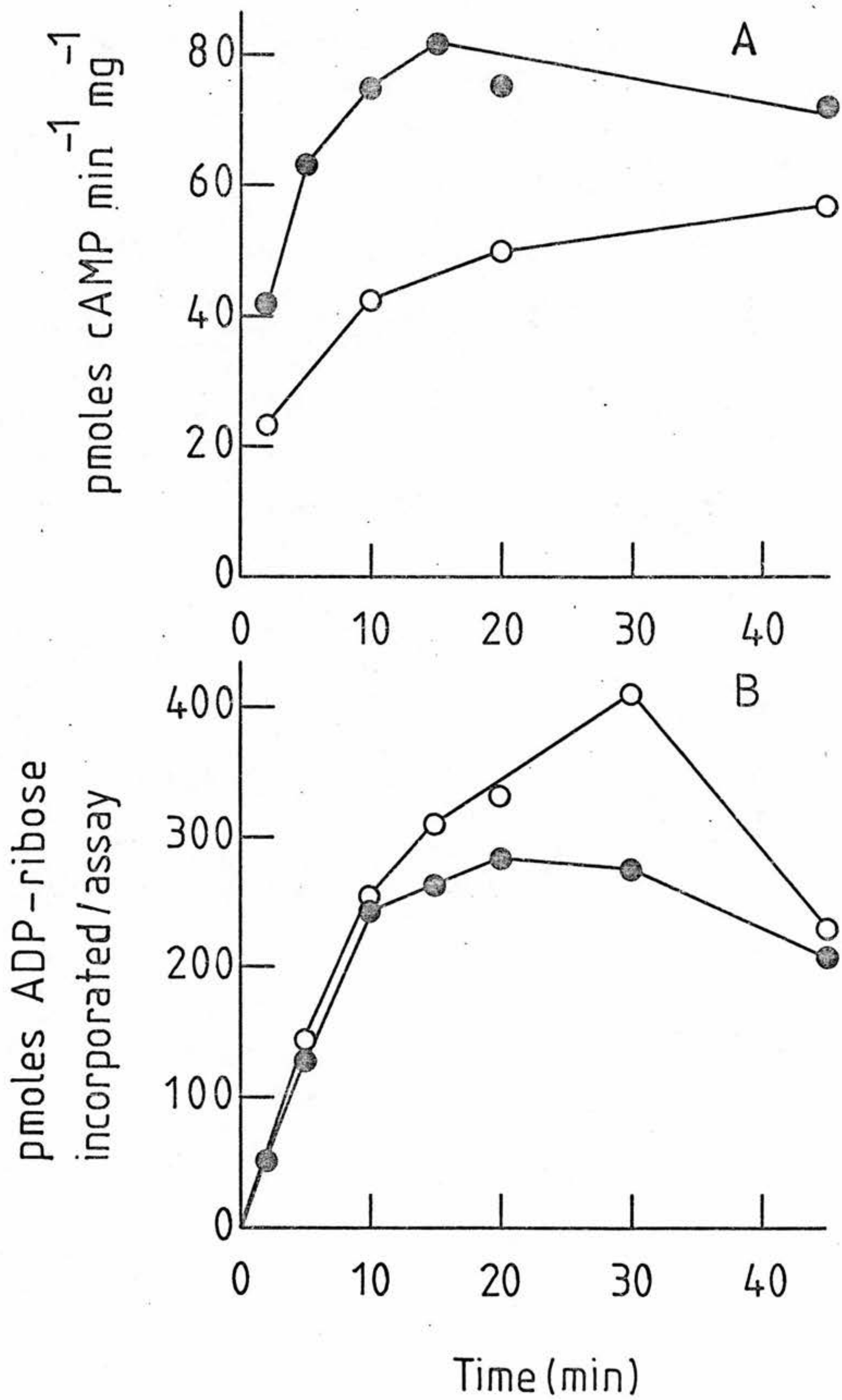
Fig.5.24.A shows an extended time course of adenylate cyclase activation by cholera toxin in rat liver homogenates. In this experiment, aliquots of homogenate were incubated with or without cholera toxin in the presence of NAD^+, ATP, DTT and GTP for increasing times up to 45 min. Membranes were then isolated by centrifugation, resuspended, and assayed for cyclase activity. Activation of the enzyme was apparent after 2 min. of preincubation with toxin, was maximal after 15 min, and remained in an activated state over the entire 45 min. period. The slight decrease in activity towards the end of the time course is probably due to the instability of adenylate cyclase after incubation for prolonged periods. In the absence of cholera toxin, basal adenylate cyclase activity also increased during

Fig.5.24. Time courses of adenylate cyclase activity and of ADP-ribose incorporation in rat liver homogenates in the presence and absence of cholera toxin.

A rat liver homogenate was prepared in Buffer System B as described in CH.2.5. and 40μl aliquots were incubated in a total volume of 60μl with 5mM-ATP, 5mM-DTT, 1mM-NAD^+, 100μM-GTP, 50nCi of [adenine U-^{14}C]NAD^+, and either 10μg/ml cholera toxin (●—●) or buffer for controls (○—○). After the indicated times at 25°C, an excess of cold buffer was added, membranes were precipitated by centrifugation, and resuspended in 40μl of buffer. 15μl of each suspension were added to 10μl of assay medium II (Ch.3.3.2.3) and adenylate cyclase was assayed for 10min at 25°C. 25μl of 5%-TCA were added to the remainders of the preincubation mixtures and 50μl aliquots were taken for estimation of [^{14}C]ADP-ribose incorporation. All values were single determinations only.

A: Adenylate cyclase activities.

B: ADP-ribose incorporation.



A
pmoles cAMP min^{-1} mg^{-1}
80
60
40
20
0
0
10
20
30
40
B
pmoles ADP-ribose incorporated/assay
400
300
200
100
0
0
10
20
30
40
Time (min)

the first few minutes of incubation, an observation which may be related to the presence of GTP in the preincubation mixtures.

Aliquots of the same preincubation mixtures were also assayed for ADP-ribose incorporation and the results are shown in Fig. 5.24.B. During the first 10 min. when adenylate cyclase was undergoing rapid activation, there was little difference in the amount of ADP-ribose incorporated in the presence or absence of cholera toxin, and incorporation was in fact slightly less in the presence of toxin. Incorporation of ADP-ribose in the absence of cholera toxin continued to increase up to 30 min. after which time the net amount of incorporation began to decease. With cholera toxin on the other hand, incorporation of ADP-ribose began to decrease much sooner and never attained the high levels seen in the absence of toxin after 30 min. After 45 min. the amount of ADP-ribose incorporated was again comparable under both conditions. The great majority of the endogenous ADP-ribosyltransferase activity was located in the membrane fraction (Fig. 5.25.) Cytosol contained very little activity, an observation which could be accounted for either by a lack of transferase enzymes or by a lack of the appropriate substrates. However, addition of membranes and cytosol in equal volumes did not result in an increase in the amount of ADP-ribose incorporated and indeed there was a surprising, but significant, inhibition of incorporation on addition of cytosol to membranes. Cholera toxin had little effect on the amount of ADP-ribose incorporated under all three conditions.

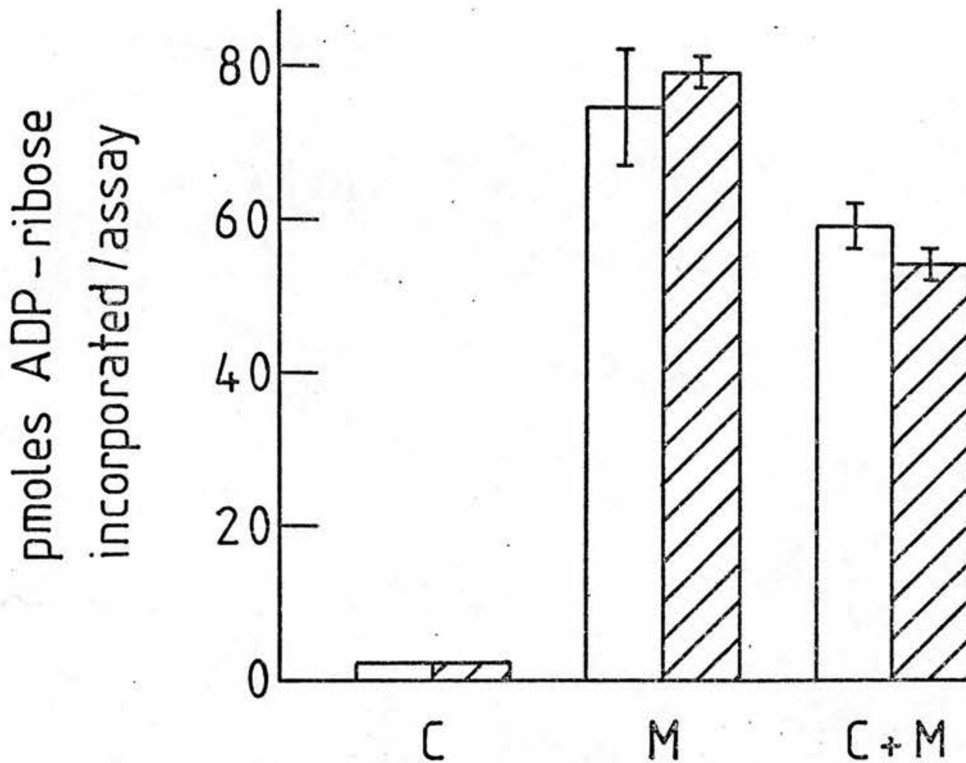


Fig.5.25. ADP-ribose incorporation in membrane and cytosol fractions of rat liver and the effect of cholera toxin.

Crude rat liver membranes and cytosol were prepared in Buffer System B as described in Ch.2.5. and 30µl of either preparation or 30µl of a mixture of equivalent amounts of each, were incubated in a total volume of 50µl with 2mM-ATP, 2mM-DTT, 100µM-NAD^+, 100nCi of [adenine U-^{14}C]NAD^+, 200µM-GTP, and either 10µg/ml cholera toxin(shaded bars) or buffer for controls (clear bars). After 10min at 25°C, 15µl of 25%-TCA were added and 50µl of each suspension taken for estimation of [^{14}C]-ADP-ribose incorporation. Error bars indicate the range of duplicate determinations.

Since no toxin-specific ADP-ribosylation could be detected in the crude system described above, the possibility that the toxin was an ADP-ribosyltransferase was investigated indirectly by studying the effects of nicotinamide on the action of cholera toxin. In the preliminary experiment shown in Fig. 5.26, aliquots of crude membranes were incubated with or without cholera toxin in the presence of increasing concentrations of nicotinamide up to 10mM. Membranes were then washed free of nicotinamide and toxin, and adenylate cyclase activity was determined. Nicotinamide had no effect on the basal activity of adenylate cyclase over the range of concentrations tested, but in the presence of cholera toxin, the compound had two distinct effects. At low concentrations (up to 1mM) the toxin was apparently able to activate adenylate cyclase to a greater extent, and only with 10mM-nicotinamide was the action of toxin inhibited.

As noted earlier from the results in Fig. 5.18, the action of cholera toxin in rat liver membranes was not supported unless the NAD^+ concentration was at fairly high levels. When the ability of NAD^+ to support toxin action was studied at a variety of nicotinamide concentrations, the results shown in Figs. 5.27 and 5.28 were obtained. In these experiments, time courses of adenylate cyclase activity were conducted in the presence of cholera toxin with the NAD^+ and nicotinamide concentrations indicated in the figures and the membrane preparation was the same as that used in the experiment of Fig.5.18.

When the NAD^+ concentration was 100μM (Fig.5.27), cholera toxin increased adenylate cyclase activity almost 2-fold after a 10 min. incubation in the absence of nicotinamide. 10μM-nicotinamide had little effect on this activation but with 100μM-nicotinamide,

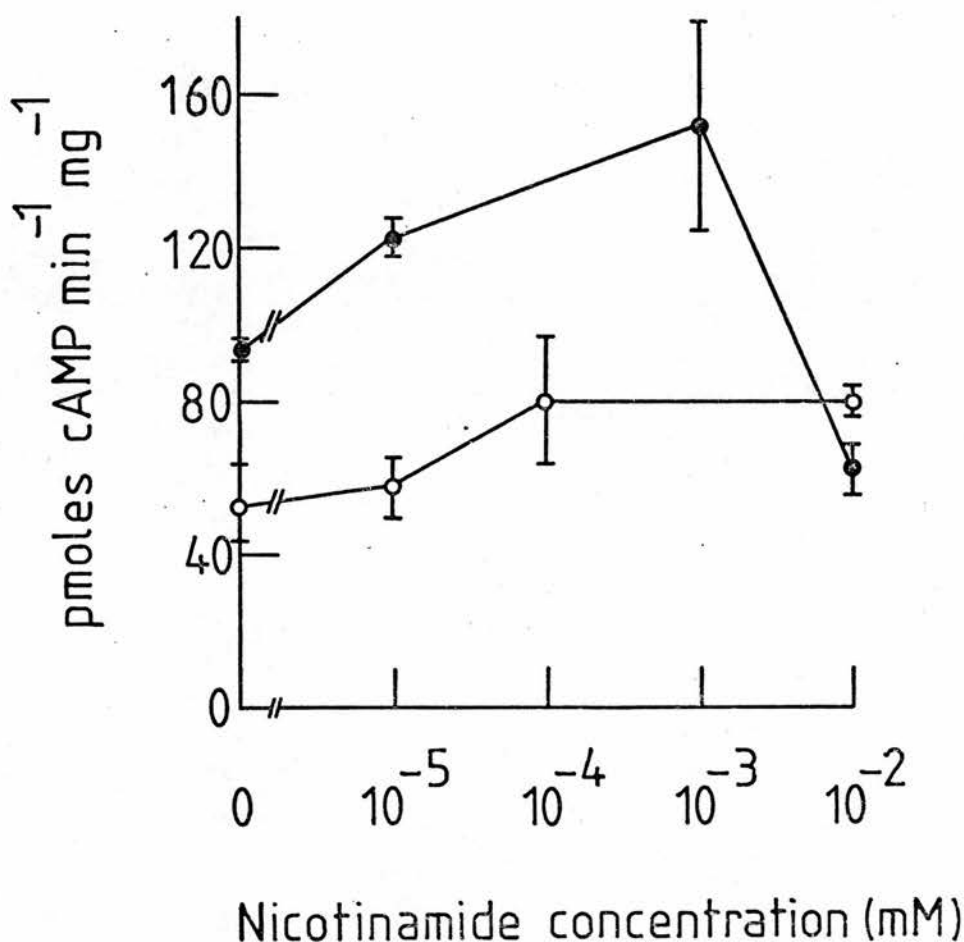


Fig.5.26. Effect of nicotinamide on adenylate cyclase activation by cholera toxin.

Crude rat liver membranes were prepared in Buffer System D as described in Ch.2.5. and 100µl aliquots were incubated in a total volume of 200µl with 5mM-ATP, 5mM-DTT, 200µM-GTP, 500µM-NAD^+, the indicated concentrations of nicotinamide, and either 9µg/ml of preactivated cholera toxin (●—●) or activation solution for controls (○—○). After 5min at 25°C, an excess of cold buffer was added, membranes were precipitated by centrifugation, and resuspended in 100µl of buffer. 15µl aliquots were added to 10µl of assay medium II (Ch.3.3.2.3), and adenylate cyclase was assayed for 10min at 25°C. 5µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{3}H]cAMP. Error bars indicate the range of duplicates.

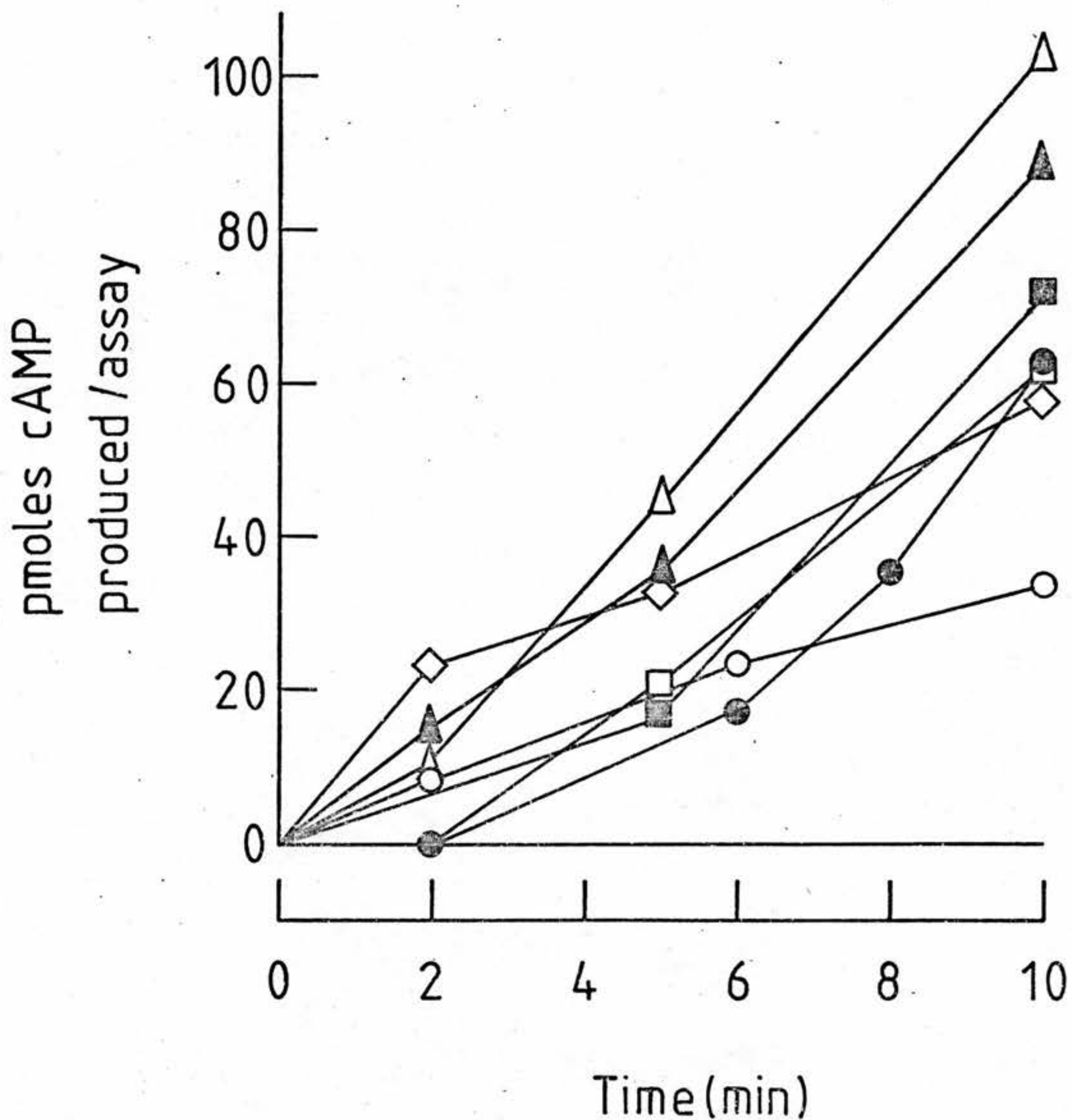


Fig.5.27. Effect of nicotinamide on adenylate cyclase activation by cholera toxin in the presence of 100μM-NAD^+.

Crude rat liver membranes were prepared in Buffer System D as described in Ch.2.5. and 10μl aliquots were incubated in a total volume of 25μl in assay medium II (Ch.3.3.2.3) supplemented with 100μM-NAD^+. Preactivated cholera toxin was added to a final concentration of 2μg/ml and time courses were conducted in the presence of 0 (●—●), 10μM (□—□), 100μM (■—■), 1mM (△—△), 10mM (▲—▲), and 25mM (◇—◇) nicotinamide. Control incubations contained activation solution in place of cholera toxin (○—○). After the indicated times at 25°C, 5μl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 10μl of each supernatant taken for estimation of [^{3}H]cAMP.

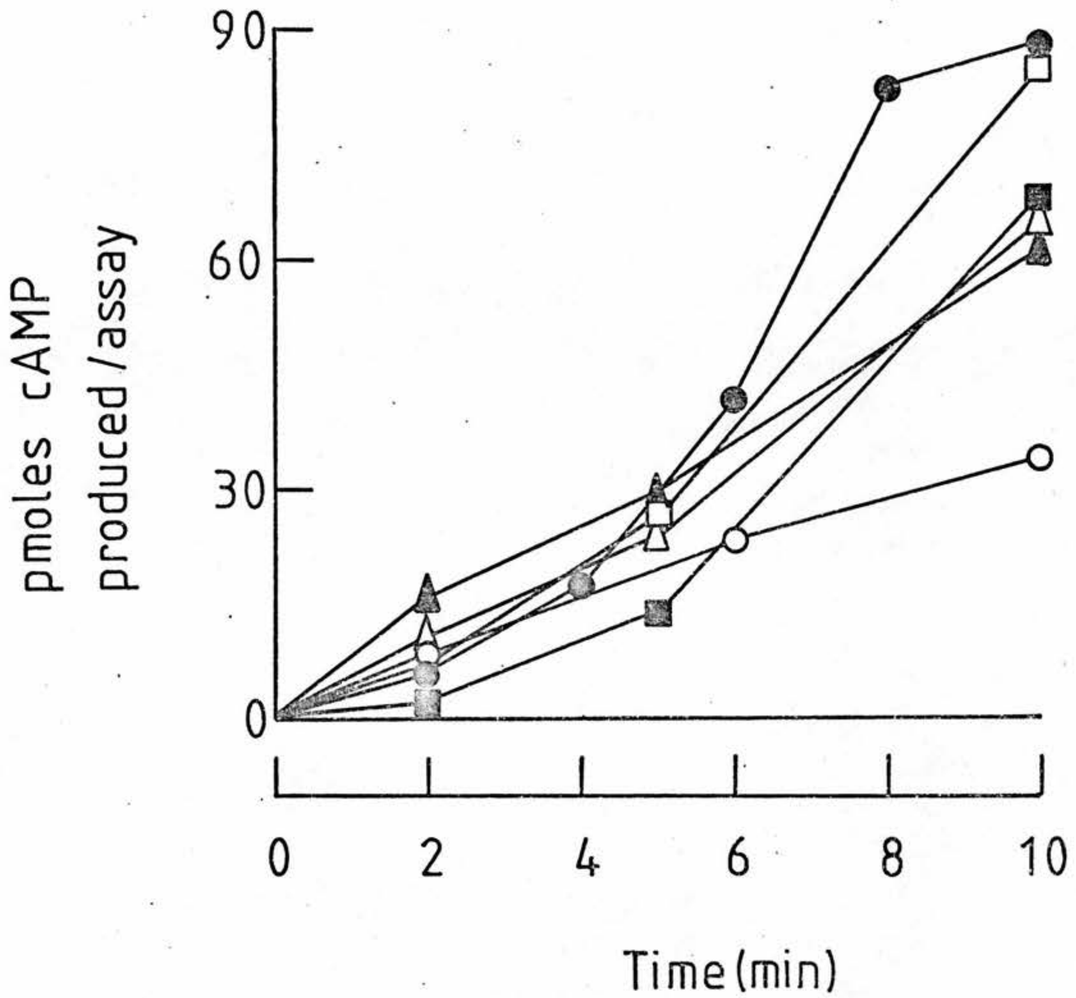


Fig.5.28. Effect of nicotinamide on adenylate cyclase activation by cholera toxin in the presence of 1mM-NAD^+.

Experimental details were exactly as described in the legend to Fig.5.27. except that the NAD^+ concentration was raised to 1mM. Time courses were conducted in the presence of 0 (●—●), 10µM (□—□), 100µM (■—■), 1mM (△—△), and 10mM (▲—▲) nicotinamide. Control incubations contained activation solution in place of cholera toxin (○—○).

the final extent of cyclase activation was increased significantly. The presence of 1mM-nicotinamide provided optimal conditions for cyclase activation and after 10 min. the cholera toxin-stimulated activity was 3-fold above basal levels. Further addition of nicotinamide to a concentration of 10mM reduced the extent of activation seen after 10 min, and with 25mM-nicotinamide this was reduced still further. It should be noted however that with high concentrations of nicotinamide (1mM and above), a more rapid activation of adenylate cyclase was possible and after a 2 min. incubation time, toxin-stimulated cyclase activity increased with increasing nicotinamide concentration.

When the NAD^+ concentration was 1mM (the concentration at which maximum toxin action was supported: see Fig. 5.18), the pattern of nicotinamide effects was rather different (Fig. 5.28). Addition of nicotinamide in increasing concentrations up to 10mM caused a progressive inhibition of adenylate cyclase activation attained after a 10 min. incubation time with cholera toxin. Again, however, with high nicotinamide concentrations, initial activation of the enzyme was more rapid.

The complex effects of nicotinamide on the kinetics and extent of adenylate cyclase activation by cholera toxin are illustrated more clearly in Fig. 5.29 where cyclase activities after 2 min. and after 10 min. have been plotted as a function of nicotinamide concentration for the two different NAD^+ concentrations employed. The results can be summarised as follows:

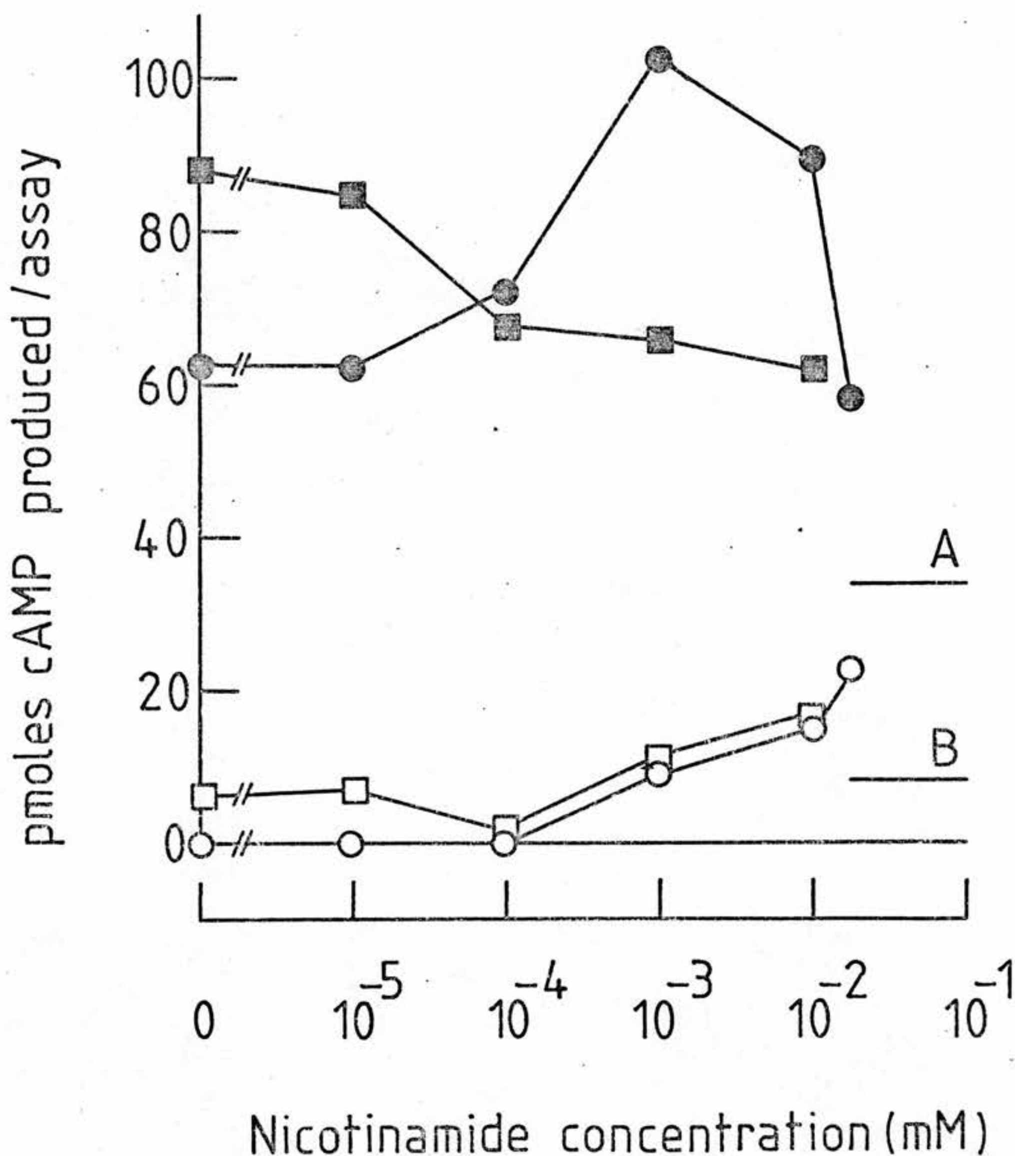


Fig.5.29. Effect of nicotinamide on adenylate cyclase activation by cholera toxin at two different NAD^+ concentrations.

Experimental data are taken from Figs.5.27. and 5.28. Adenylate cyclase activities after 2min (○, □) and 10min (●, ■) of incubation with cholera toxin in the presence of 100μM (○, ●) and 1mM (□, ■) NAD^+, are plotted as a function of nicotinamide concentration. A and B represent basal adenylate cyclase activity after 10min and 2min of incubation respectively.

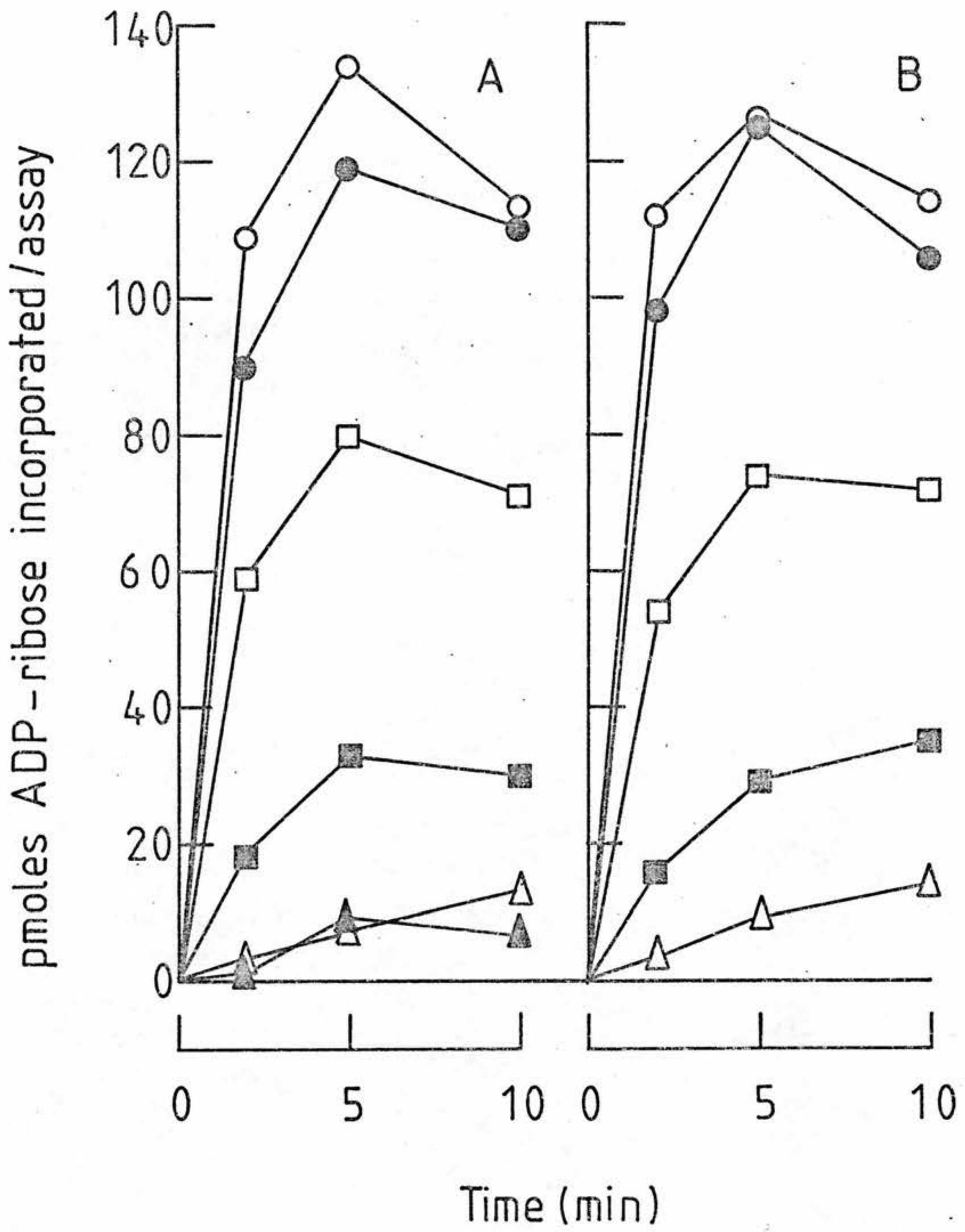
(i) With 100μM-NAD^+ and a 10 min. incubation time, toxin-stimulation of adenylate cyclase is seen in the absence of nicotinamide but the extent of activation can be increased signficantly by adding nicotinamide up to a concentration of 1mM. Higher concentrations result in progressive inhibition of toxin action. With a 2 min. incubation time, addition of nicotinamide causes an initial reduction in the rate of cAMP accumulation but between 1mM and 25mM-nicotinamide, a progressively more rapid activation of adenylate cyclase occurs.

(ii) With 1mM-NAD^+ and a 10 min. incubation time, cholera toxin action is maximal in the absence of nicotinamide, but the extent of cyclase activation is less than is seen with 100μM-NAD^+ and 1mM-nicotinamide. Addition of nicotinamide then progressively inhibits the action of cholera toxin but again the initial rate of cyclase activation observed after a 2 min. incubation time increases with increasing nicotinamide concentration.

Identical incubations to those described above were also conducted in which [adenine U-^{14}C] NAD^+ was included in the assay medium and ADP-ribose incorporation after 2,5, and 10 min. was determined in the presence and absence of cholera toxin. The results are shown in Figs. 5.30 and 5.31 for the experiments with 100μM-NAD^+, and 1mM-NAD^+ respectively. With 100μM-NAD^+, ADP-ribose incorporation under all conditions was maximal after 5 min. of incubation, and after 10 min. some of the incorporated ADP-ribose was apparently removed. Addition of nicotinamide caused a progressive inhibition of incorporation with 50% inhibition occurring with about 100μM-nicotinamide. Maximal inhibition (94%) was achieved with 10mM

Fig.5.30. Effect of nicotinamide on ADP-ribose incorporation with $100\mu M$-NAD^+ in the presence and absence of cholera toxin.

Crude rat liver membranes were prepared inBuffer System D as described in Ch.2.5. and 25µl aliquots were incubated in a total volume of 50µl with 1mM-ATP, 1mM-DTT, $100\mu M$-NAD^+, 80µM-GTP, 75nCi of [adenine-U-^{14}C]NAD^+, and either 2µg/ml of preactivated cholera toxin (B), or activation solution for controls (A). Time courses were conducted at $25^{o}C$ in the presence of 0 (○—○), 10µM (●—●), 100µM (□—□), 1mM (■—■), 10mM (Δ—Δ), and 25mM (▲—▲) nicotinamide. Reactions were terminated by the addition of 10µl of 25%-TCA and 50µl of each suspension were taken for estimation of [^{14}C]ADP-ribose incorporation. All values were single determinations only.



pmoles ADP-ribose incorporated/assay
140
120
100
80
60
40
20
0
A
B
0
5
10
0
5
10
Time (min)

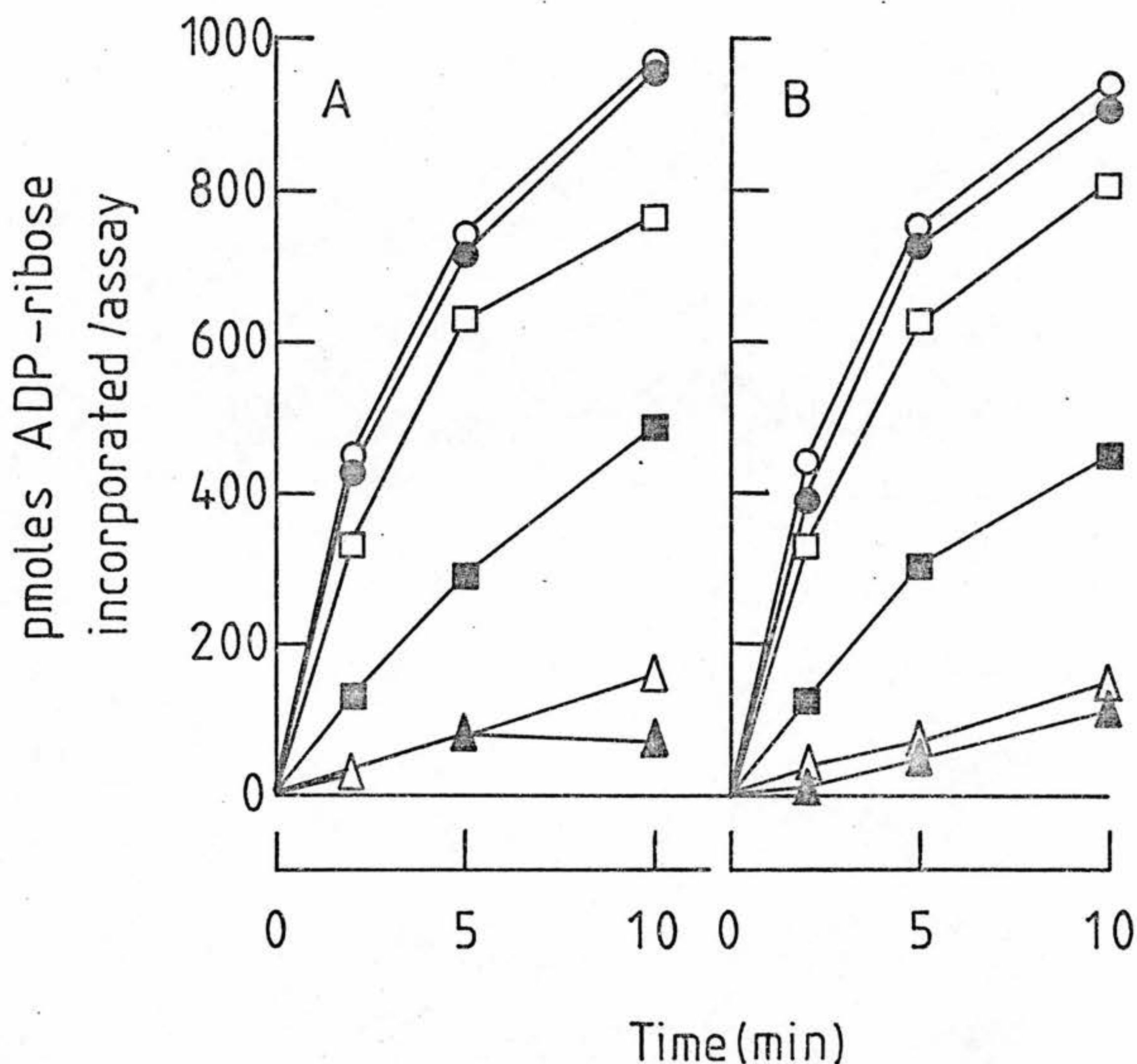


Fig.5.31. Effect of nicotinamide on ADP-ribose incorporation with 1mM-NAD^+ in the presence and absence of cholera toxin.

Experimental details were exactly as described in the legend to Fig.5.30. except that the NAD^+ concentration was 1mM. Time courses were conducted in the presence of 0 (○—○), 10µM (●—●), 100µM (□—□), 1mM (■—■), 10mM (△—△), and 25mM (▲—▲) nicotinamide, in the presence (B) and absence (A) of cholera toxin.

to 25mM-nicotinamide. The pattern of ADP-ribose incorporation in the presence of cholera toxin was essentially identical and there was no detectable toxin-specific ADP-ribosylation under any conditions.

With 1mM-NAD^+ (Fig. 5.31.), ADP-ribose incorporation was always about 10-fold greater than with 100μM-NAD^+ and the time course of incorporation was also different from that seen with 100μM-NAD^+. The amount of incorporation increased throughout the 10 min. incubation period with no indication of any net removal of ADP-ribose already incorporated. Again nicotinamide caused a progressive inhibition of ADP-ribose incorporation but the concentrations required to achieve the same degree of inhibition were greater. For example 50% inhibition was not observed until the nicotinamide concentration was almost 1mM and maximal inhibition, with 10mM to 25mM-nicotinamide, was 89%. Again the extent of incorporation in the presence of cholera toxin was identical under all conditions.

From the above results therefore it is apparent that maximum adenylate cyclase activation by cholera toxin is supported in the simultaneous presence of 100μM-NAD^+ and 1mM-nicotinamide and that activation under these conditions is greater than with 1mM-NAD^+ alone. Despite this, a toxin-specific ADP-ribosylation was still not detectable. Therefore an experiment was conducted using the optimal conditions of NAD^+ and nicotinamide in the presence of 20mM-thymidine. The latter compound is an inhibitor of the enzyme poly (ADP-ribose) polymerase which is likely to be present in the crude membrane preparations used in these experiments (see Ch. 6.). The results are shown in Fig. 5.32. The 75% inhibition of endogenous ADP-ribose incorporation caused by

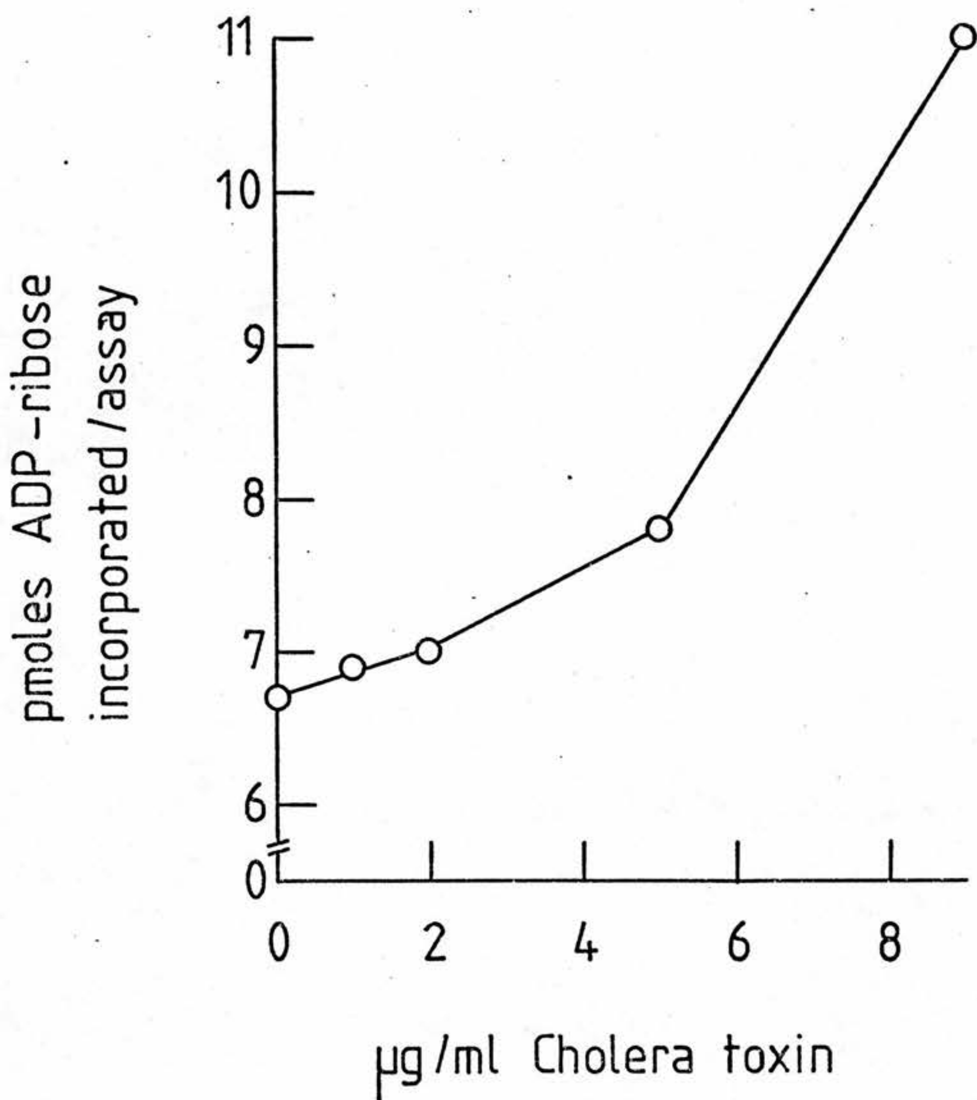


Fig.5.32. ADP-ribosylation catalysed by cholera toxin.

Crude rat liver membranes were prepared in Buffer System D as described in Ch.2.5. and 25μl aliquots were incubated in a total volume of 50μl of 1mM-ATP, 1mM-DTT, 100μM-NAD^+, 80μM-GTP, 20mM-thymidine, 1mM-nicotinamide, 100nCi of [adenine U-^{14}C]NAD^+, and the indicated concentrations of preactivated cholera toxin. After 5min at 25°C, 10μl of 25%-TCA were added and 50μl of each suspension taken for estimation of [^{14}C]ADP-ribose incorporation. All values were single determinations only.

1mM-nicotinamide (Fig. 5.30.) was inhibited by a further 80% by 20mM-thymidine, reducing the rate of ADP-ribose incorporation to barely detectable levels (6 to 7 pmoles ADP-ribose incorporated per 5 min. incubation time). Addition of cholera toxin to these maximally inhibited preparations resulted in a small, but quite distinct, increase in incorporation of ADP-ribose with increasing concentrations of cholera toxin. The maximum amount of ADP-ribose incorporated by the toxin (at a concentration of 9µg/ml) was about 4 pmoles per 5 min. assay period, and with 2 µg/ml cholera toxin (the concentration employed in all of the experiments described above), toxin-specific ADP-ribosylation only amounted to about 0.3 pmoles per 5 min. If these results represent a genuine toxin-specific ADP-ribosylation then the specific activity of the toxin was about 750 pmoles ADP-ribose incorporated min^{-1} mg^{-1} at 1,2 and 5 µg/ml toxin, and 1,900 pmoles ADP-ribose $min.^{-1}$ mg^{-1} at 9µg/ml toxin.

It has been noted earlier that maximum adenylate cyclase activation in rat liver membranes occurs with about 1µg/ml cholera toxin (Fig. 5.4.) However, when a similar experiment was performed in the presence of 100µM NAD^{+}, 1mM-nicotinamide, and 20mM-thymidine, the results presented in Fig. 5.33. were obtained. 0.1µg/ml caused a 1.5-fold stimulation of activity and 1µg/ml toxin produced almost 2-fold activation. However in contrast to earlier results (fig. 5.4.) adenylate cyclase activity continued to increase markedly with increasing toxin concentration and the enzyme was activated 3.5-fold with 10µg/ml toxin and over 4-fold with 20µg/ml toxin. It therefore appears that the conditions established above which enabled the observation of a detectable toxin-specific ADP-ribosylation also enabled the toxin to activate adenylate cyclase to a greater extent over a much wider range of toxin concentrations.

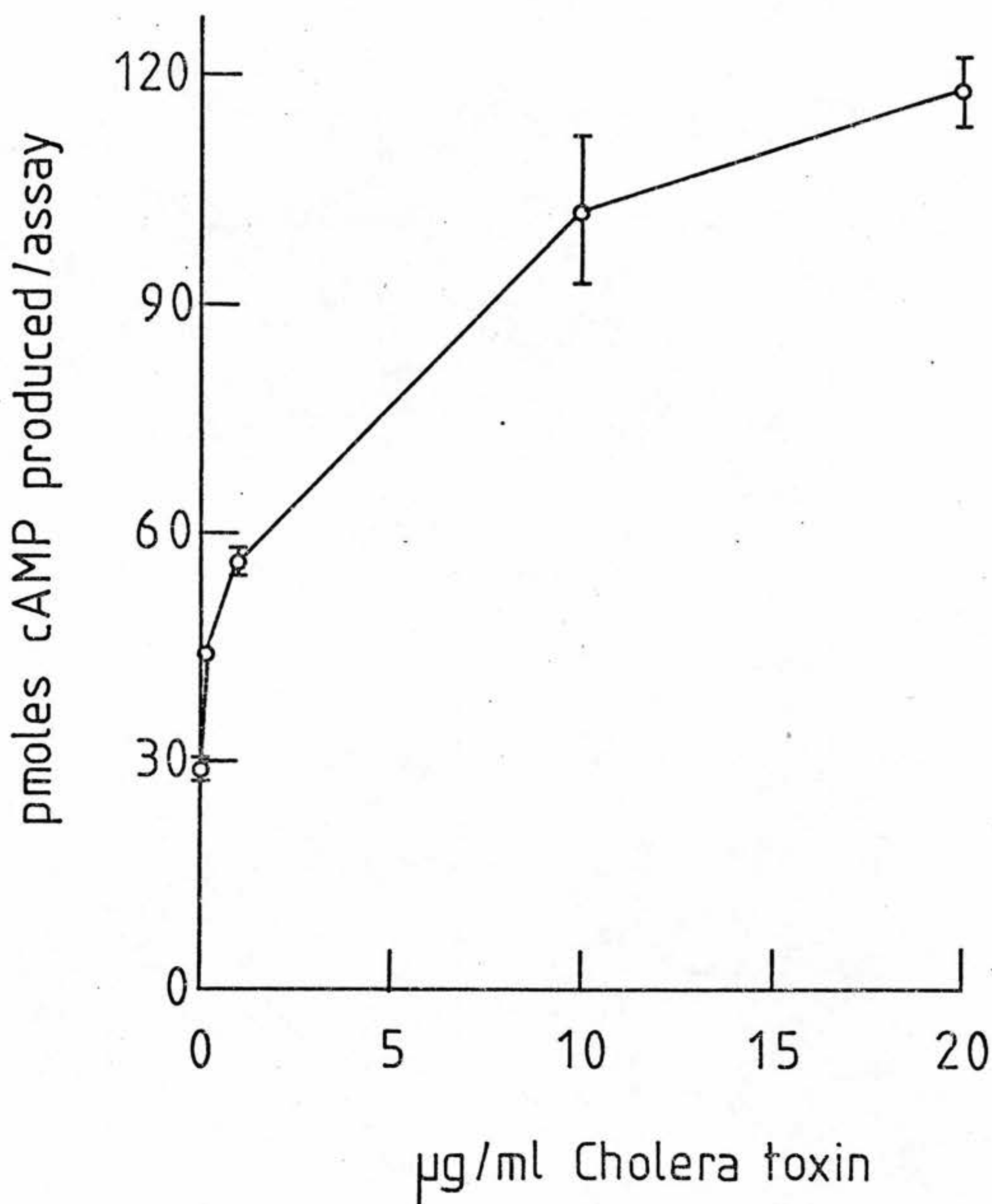


Fig.5.33. Enhanced activity of cholera toxin in the presence of nicotinamide and thymidine.

Crude rat liver membranes were prepared in Buffer System D as described in Ch.2.5. and 10µl aliquots were incubated in a total volume of 25µl in assay medium II (Ch.3.3.2.3) supplemented with 1mM-nicotinamide, 20mM-thymidine, and the indicated concentrations of preactivated cholera toxin. After 10min at 25°C, 5µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{3}H]cAMP. Error bars indicate the range of duplicate determinations.

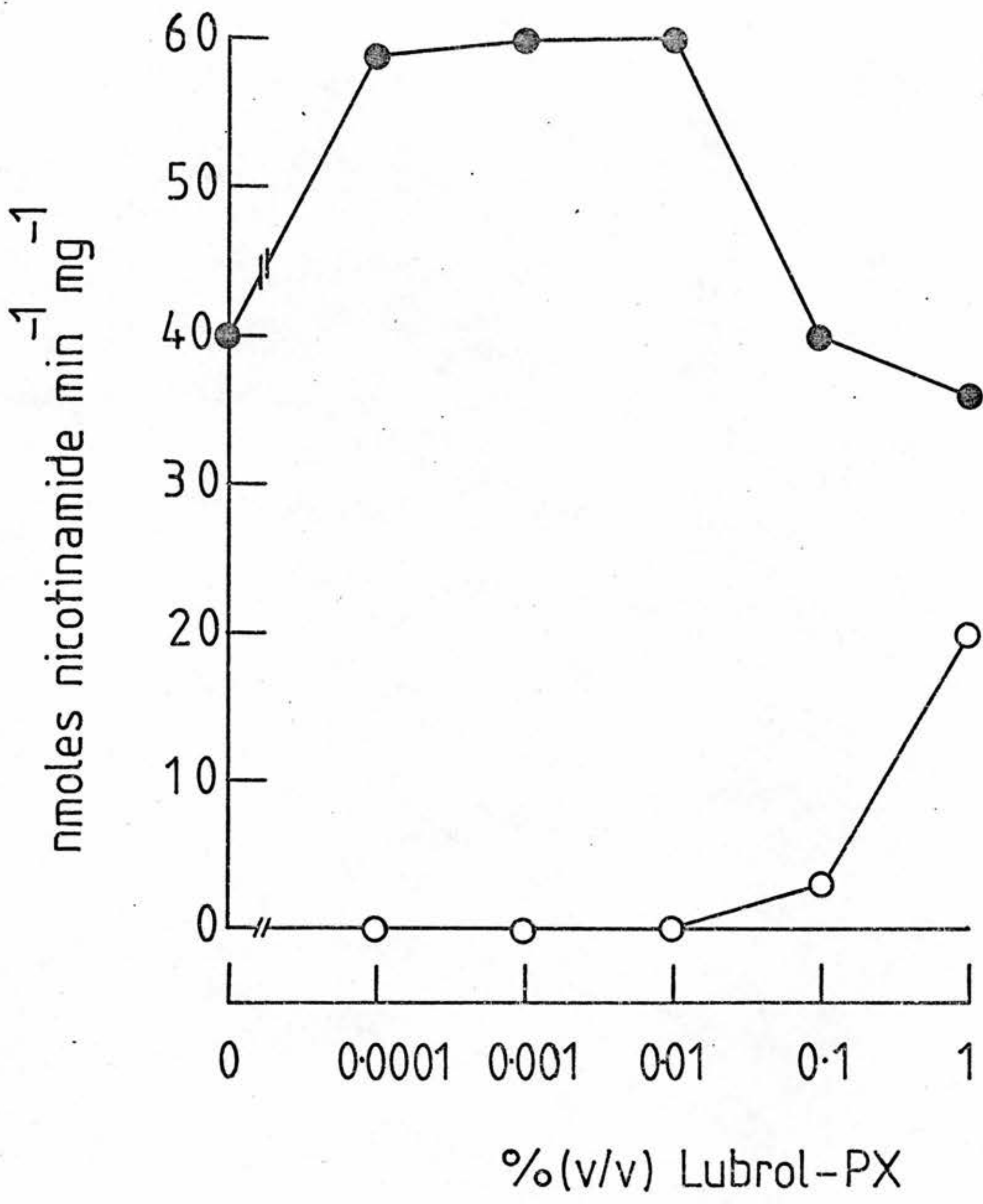
5.5. <u>The action of cholera toxin on solubilised adenylate cyclase</u>

As described in Ch. 5.3.2. it was not possible to inhibit endogenous rat liver NADases to a sufficient extent to enable investigation of toxin action in the absence of interfering activities. However a method was devised to separate adenylate cyclase from these activities based on the relative ease of solubilisation of the former with the non-ionic detergent Lubrol-PX, which is effective in the range 0.01 to 0.1% (v/v). In contrast, endogenous NADase activity was found to be extremely resistant to solubilisation with this detergent (Fig. 5.34.) NADase activity first appeared in the supernatant after solubilisation with 0.1% Lubrol-PX but this represented only about 5% of the total activity present in the membranes. Even with 1% Lubrol-PX the majority of NADase activity remained membrane-bound. It is interesting to note that on addition of very low concentrations of detergent (0.001%), there was a significant increase in the specific activity of membrane-bound NADases. This could be due to a direct stimulatory effect of Lubrol=PX on the enzymes or to an 'unmasking' of previously buried active sites on the membrane surfaces.

An adenylate cyclase preparation solubilised with 0.25% Lubrol-PX catalysed NAD^+ hydrolysis with a specific activity of about 3 nmoles nicotinamide $min.^{-1} mg^{-1}$ (Fig.5.35) which was about 5% of the activity in the parent membrane preparation. However, solubilised adenylate cyclase presented several new problems in itself, not least of which was the instability of the enzyme after activation by cholera toxin (Fig. 5.36.). Cholera toxin caused a 2-fold stimulation of activity which was maximal after a 2 min.

Fig.5.34. Solubilisation of endogenous rat liver NADases with Lubrol-PX.

Crude rat liver membranes were prepared in Buffer System B as described in Ch.2.5. and 7ml aliquots were mixed with an equal volume of Lubrol-PX in the same buffer to give the indicated final concentrations. After mixing and centrifugation at 100,000g x 30min, the supernatants were removed and the pellets resuspended in an equal volume of buffer. 30μl aliquots of the supernatant (○—○) and particulate fractions (●—●) were incubated in a total volume of 50μl with 5mM-NAD^+ and 50nCi of [carbonyl-^{14}C]NAD^+. After 5min (particulate preparations) or 15min (supernatants) at 25°C, 50μl of 5%-TCA were added, precipitated protein was removed by centrifugation, and 10μl of each supernatant taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.



nmoles nicotinamide min^{-1} mg^{-1}
60
50
40
30
20
10
0
0
0·0001
0·001
0·01
0·1
1
%(v/v) Lubrol-PX

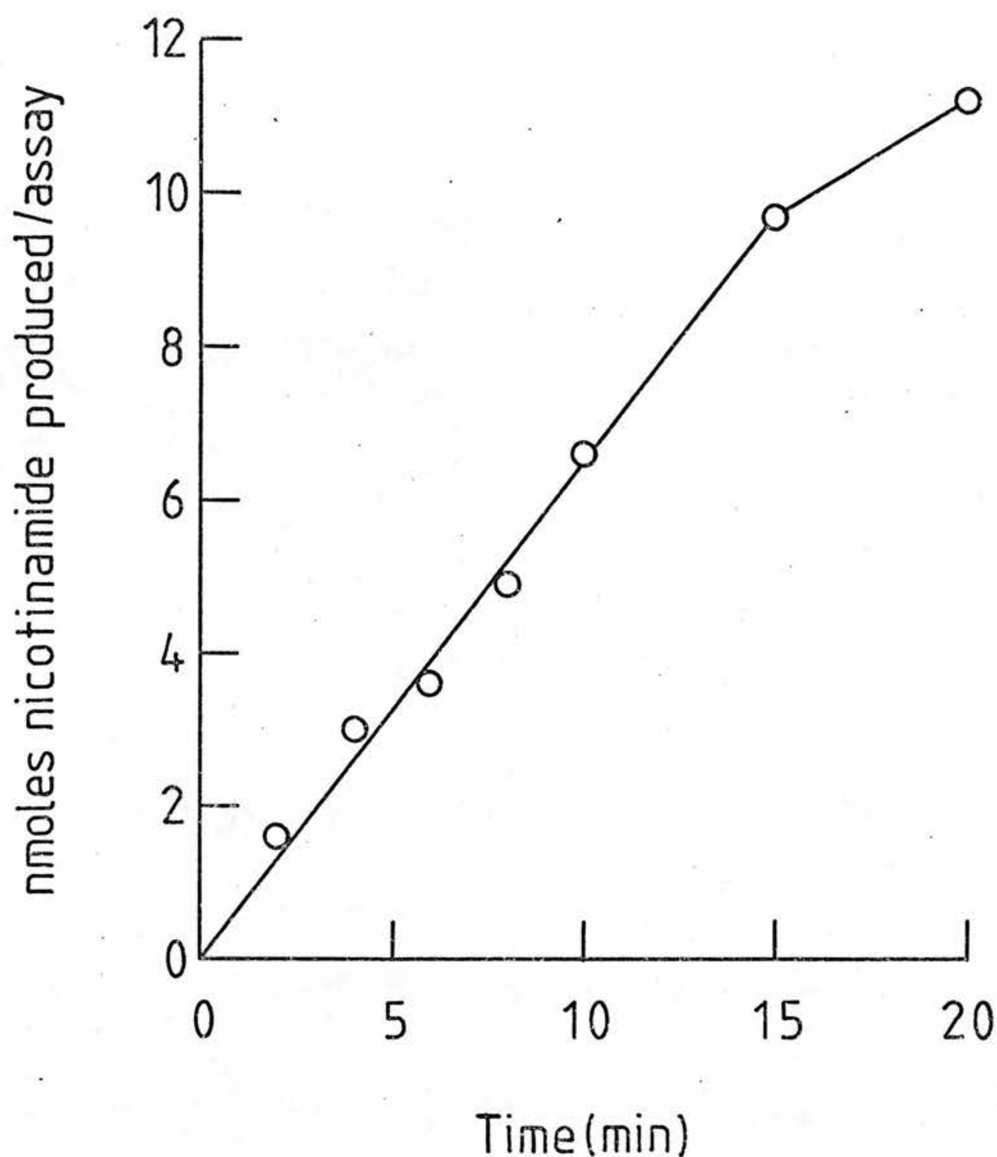


Fig.5.35. Hydrolysis of NAD^+ in rat liver homogenates solubilised with 0.25%-Lubrol-PX.

A rat liver homogenate was prepared in Buffer System B and solubilised with 0.25%-Lubrol-PX as described in Ch.2.5. 30µl aliquots were incubated in a total volume of 50µl with 2mM-NAD^+ and 25nCi of [carbonyl-^{14}C]NAD^+. After the indicated times at 25°C, 50µl of 5%-TCA were added, precipitated protein was removed by centrifugation, and 10µl of each supernatant taken for estimation of [^{14}C]nicotinamide. All values were single determinations only.

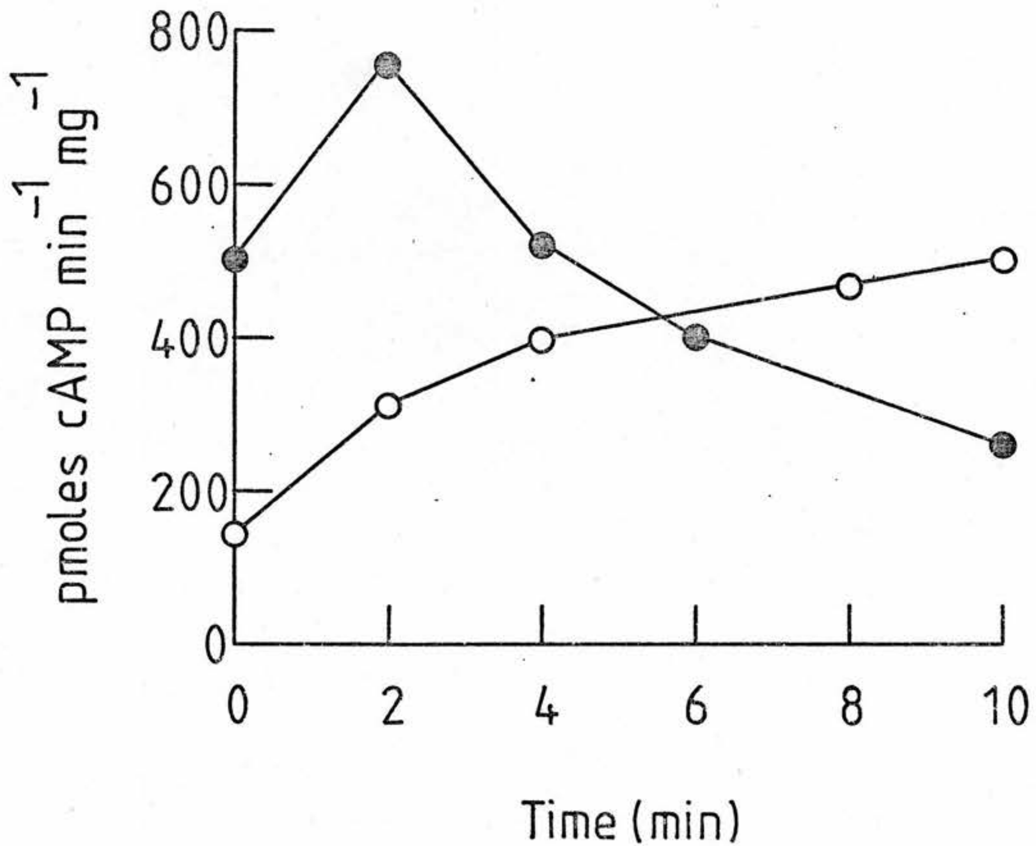


Fig.5.36. Activation of Lubrol-solubilised adenylate cyclase by cholera toxin.

A rat liver was prepared in Buffer System B and solubilised with 0.25%-Lubrol-PX as described in Ch.2.5. 150µl aliquots were incubated in a total volume of 200µl with 1mM-ATP, 1mM-DTT, 1mM-NAD^+, and either 10µg/ml cholera toxin (●—●) or buffer for controls (○—○). After the indicated times at 25°C, 15µl aliquots were transferred to tubes containing 10µl of assay medium I (Ch.3.3.2.3) to give a final ATP concentration of 1mM. After a further incubation at 25°C for 5min, 10µl of 25%-TCA were added, precipitated protein was removed by centrifugation, and 5µl of each supernatant taken for estimation of [^{3}H]cAMP. All values were single determinations only.

preincubation time but on longer incubations with toxin, the stimulated activity decayed rapidly and within 6 min. had fallen below the activity of the unstimulated, control enzyme. Similar results have been reported previously (van Heyningen, 1976 b). Short incubation times (2 min.) were therefore employed in all future work on this system.

Activation of solubilised adenylate cyclase by cholera toxin was completely analogous to activation of the membrane-bound enzyme. 0.01 μg/ml toxin caused some activation, activation was maximal with 1μg/ml, and addition of more toxin up to a concentration of 50μg/ml had no further effect (Fig. 5.37.) Maximal activation was about 3-fold over basal levels. However estimations of ADP-ribose incorporation in an identical series of incubations produced the surprising result that addition of cholera toxin caused marked inhibition of ADP-ribose incorporation. In the presence of 10μg/ml toxin, when adenylate cyclase was already maximally activated, ADP-ribose incorporation was less than 10% of that in the absence of toxin. 50μg/ml toxin caused only a further slight reduction in ADP-ribose incorporation.

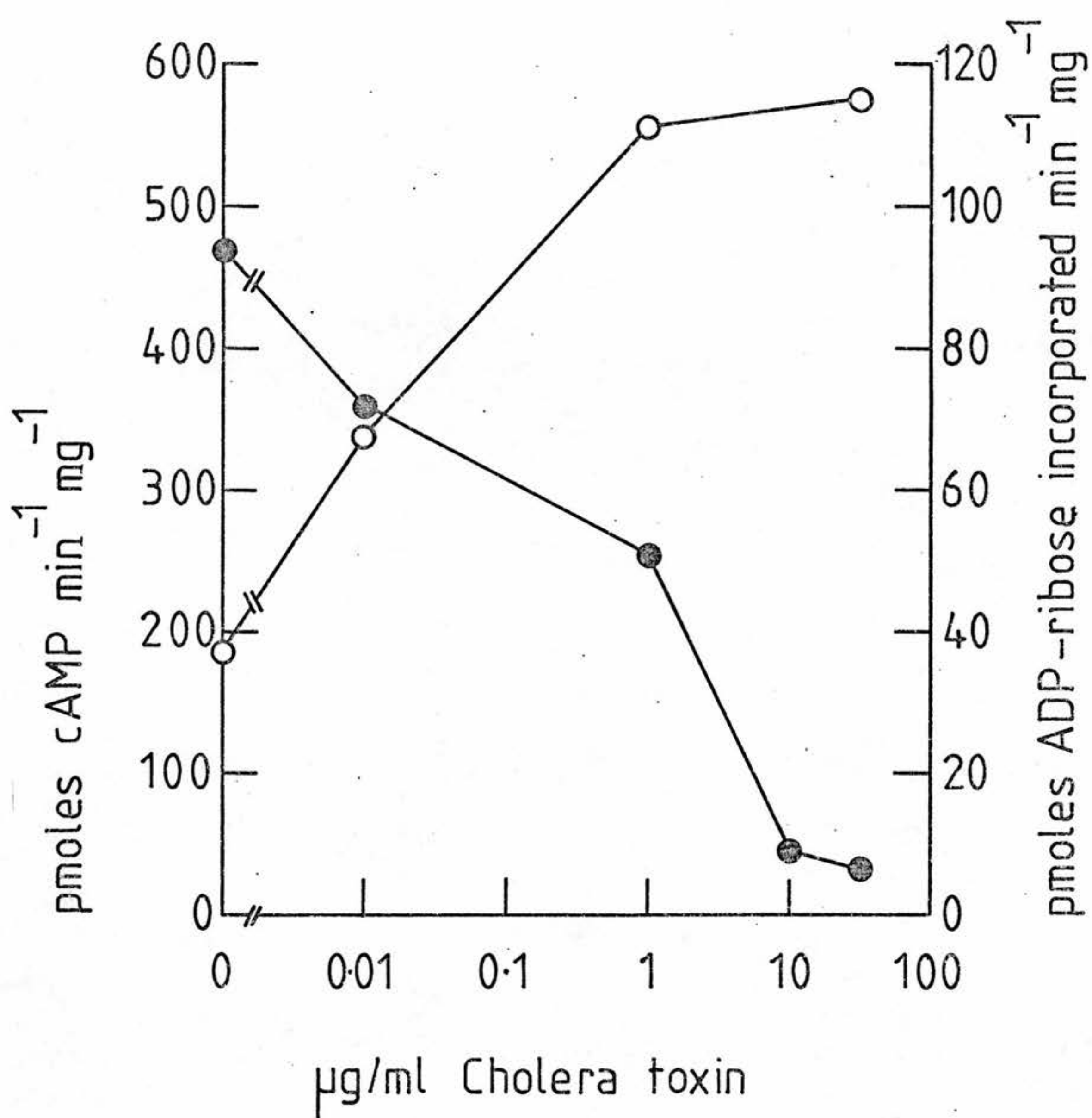
The action of cholera toxin as an inhibitor of endogenous ADP-ribosyltransferases is clearly illustrated in Fig. 5.38. which shows time courses of ADP-ribose incorporation in the presence of several different concentrations of cholera toxin. At all points on the time courses, the rate of ADP-ribose incorporation decreased with increasing toxin concentration with 50% inhibition occurring at about 0.1μg/ml toxin. Although cyclase activation was maximal after 2 min. of incubation with toxin, the toxin-induced inhibition of ADP-ribosyltransferases was maintained for at least 10 min.

Fig.5.37. Activation of adenylate cyclase and inhibition of ADP-ribose incorporation by cholera toxin in solubilised rat liver homogenate.

A rat liver homogenate was prepared in Buffer System B and solubilised with 0.25%-Lubrol-PX as described in Ch.2.5. 50µl aliquots were incubated in a total volume of 100µl with 5mM-ATP, 5mM-DTT, 200µM-NAD^+, 50nCi of [adenine U-^{14}C]NAD^+, and the indicated concentrations of cholera toxin. After 2min at 25°C, 15µl aliquots were transferred to tubes containing 10µl of assay medium II (Ch.3.3.2.3) to give a final ATP concentration of 5mM, and adenylate cyclase activities were determined after a 5min assay at 25°C. 50µl of 5%-TCA were added to the remainders of the preincubation mixtures and 100µl of each suspension were taken for estimation of [^{14}C]ADP-ribose incorporation. All values were single determinations only.

(○—○): Adenylate cyclase activities.

(●—●): ADP-ribose incorporation.



pmoles cAMP min^{-1} mg^{-1}
600
500
400
300
200
100
0
pmoles ADP-ribose incorporated min^{-1} mg^{-1}
120
100
80
60
40
20
0
0
0·01
0·1
1
10
100
µg/ml Cholera toxin

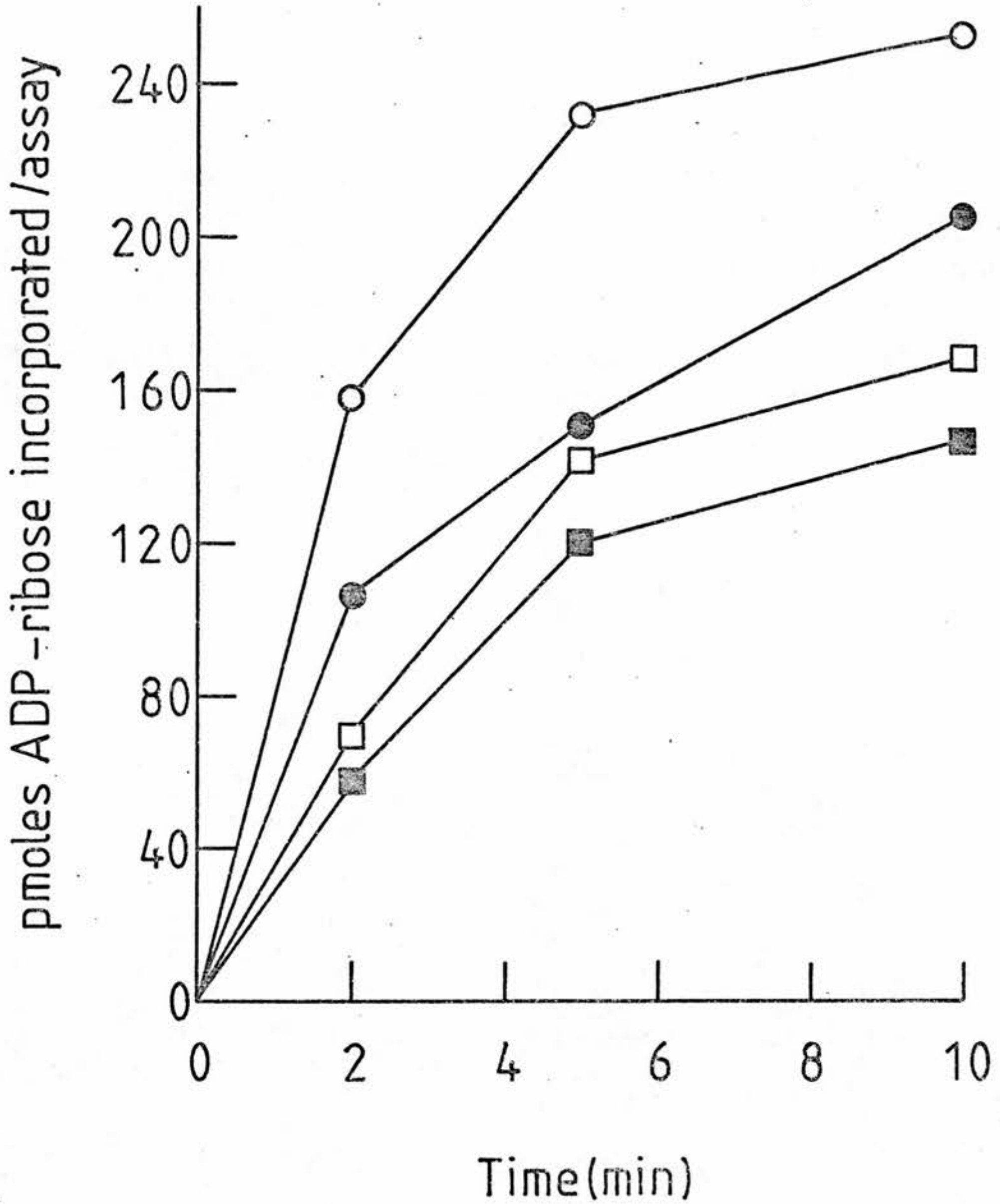


Fig.5.38. <u>Effect of cholera toxin on ADP-ribosylation in solubilised rat liver homogenate</u>.

A rat liver homogenate was prepared in Buffer System B and solubilised with 0.25%-Lubrol-PX as described in Ch.2.5. 50µl aliquots were incubated in a total volume of 100µl with 2mM-ATP, 5mM-DTT, 200µM-NAD^+, 100µM-GTP, 50nCi of [adenine U-^{14}C]NAD^+, and cholera toxin at final concentrations of 0 (○—○), 0.01 (●—●), 0.1 (□—□), and 1 (■—■) µg/ml. After the indicated times at 25°C, 10µl of 25%-TCA were added and 80µl of each suspension taken for estimation of [^{14}C]ADP-riboee incorporation. All values were single determinations only.

Chapter 6. Discussion

6.1. The NADase activity of cholera toxin.

6.1.1. NAD^+ hydrolysis by purified preparations of cholera toxin.

As outlined in Ch. 1.10, NAD^+ was initially implicated in the mechanism of action of cholera toxin by Gill (1975) who showed that pigeon erythrocyte lysates, when depleted of endogenous NAD^+, became refractory to cholera toxin and that on readdition of exogenous NAD^+, toxin sensitivity was restored. On the basis of these observations Gill suggested that one possible mechanism of toxin action could be the transfer of the ADP-ribose moiety of NAD^+ to an intracellular acceptor protein, possibly a part of the adenylate cyclase complex itself. Such a mechanism would be analogous to the already established action of diptheria toxin. Once inside a cell, diphtheria toxin uses endogenous supplies of NAD^+ as a substrate for the ADP-ribosylation of elongation factor-2 (EF-2), an event which inhibits the translocation of nascent polypeptide chains from the acceptor to the donor sites on ribosomes, thereby inhibiting protein synthesis (see Collier, 1975).

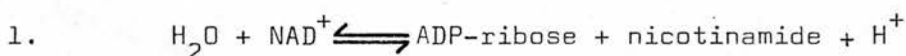
Possible analogies with diphtheria toxin can also be suggested from the similarities in the structures of the two toxins despite the fact that, superficially, there appear to be few points of comparison. Unlike cholera toxin, diphtheria toxin does not possess a complicated subunit structure and consists only of one 62,000 dalton polypeptide. However the toxin is readily cleaved proteolytically to produce a species known as 'nicked' toxin which consists of two distinct

polypeptide chains held together covalently by one disulphide bridge. On reduction with thiol reagents the two fragments, termed A and B (of molecular weights 24,000 daltons and 38,000 daltons respectively), are released. Fragment A alone has been shown to be the enzymically active species (Gill and Dinius, 1971; Gill and Pappenheimer, 1971; Collier and Traugh, 1969), and since this fragment is not toxic towards intact cells, fragment B is presumed to be responsible for the binding of toxin to cell surfaces and possibly also for assisting fragment A in its passage through the cell membrane to its intracellular site of action. Since the enzymic activity of diphtheria toxin is only manifested after the proteolytic and reductive generation of fragment A (Collier and Traugh, 1969), this process presumably occurs _in vivo_ and could perhaps be catalysed by host cell proteases at some stage after the initial toxin binding event. Thus the two functional domains of diphtheria toxin are entirely analogous to the A and B subunits of cholera toxin, although in the case of diphtheria toxin, much less is known about the specific cell surface receptor recognised by the B fragment of the toxin.

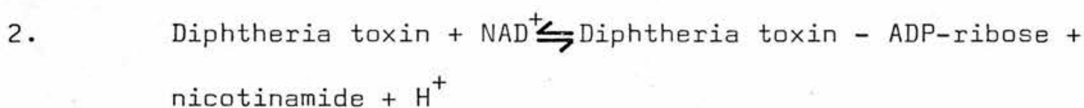
The possibility that subunit A of cholera toxin is also an enzyme possessing an ADP-ribosyltransferase activity was supported by the observations of Moss _et al_ (1976b) who found that cholera toxin was capable of hydrolysing NAD^+ to nicotinamide and ADP-ribose. The results presented in Fig. 5.6. confirm this observation and, also in agreement with these workers, high concentrations of cholera toxin and long incubation times were necessary before a detectable NADase activity could be demonstrated. Pigeon erythrocyte cytosol had no effect on the NADase activity of cholera toxin (Fig. 5.7.) thereby ruling out

the possibility that the cytosolic factors implicated in the mechanism of toxin action (Gill, 1975; 1976b) were not involved in the enhancement of an enzymic activity of cholera toxin towards NAD^+. As shown in Fig. 5.5. a requirement for cytosolic factors in toxin action was not, in any case, observed in the rat liver system, a result which is in agreement with those of Martin et al (1977) who demonstrated activation of adenylate cyclase by cholera toxin in purified rat liver plasma membranes. However some of the results discussed later suggest the existence of cytosolic factors which, although not essential requirements for toxin action, may at least assist the toxin in adenylate cyclase activation.

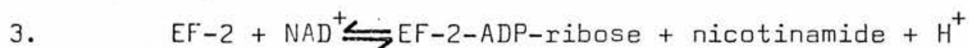
The discrepancy between the concentrations of cholera toxin required for cyclase activation and those required to demonstrate an NADase activity does not necessarily argue against an enzymic mechanism of toxin action involving NAD^+ as a substrate. Diphtheria toxin is known to catalyse three different reactions:



1. $H_2O + NAD^+ \rightleftharpoons$ ADP-ribose + nicotinamide + H^+



2. Diphtheria toxin + $NAD^+ \rightleftharpoons$ Diphtheria toxin - ADP-ribose + nicotinamide + H^+



3. EF-2 + $NAD^+ \rightleftharpoons$ EF-2-ADP-ribose + nicotinamide + H^+

Only reaction 3, the ADP-ribosylation of EF-2, is considered to be of physiological importance, the other two reactions (NAD^+ hydrolysis and self-ADP-ribosylation) being merely unphysiological

manifestations of the true activity which are catalysed at a much slower rate. NAD^+ hydrolysis by diphtheria toxin has been calculated to proceed at a rate which is about 2×10^6 times less than that of EF-2 ADP-ribosylation under conditions of saturating NAD^+ (Kandel et al, 1974). However it is essential, when using high concentrations of toxins, to establish that any observed effect is a true property of the toxin under study and that it is not due to small amounts of contaminating proteins, the activities of which would not normally be detectable at physiological concentrations of the toxin. The problems of purity in toxin preparations and the possibilities of contaminating activities, even in apparently highly purified preparations, have been discussed recently by Wadstrom (1978) and it is interesting to note the case of at least one other bacterial toxin to which an enzymic activity was wrongly assigned. The streptolysin O from Streptococcus pyogenes was initially thought to possess an intrinsic NADase activity but subsequent studies demonstrated that this activity was in fact due to a contaminant and could be separated from the toxin by iso-electric focussing(Alouf and Raynaud, 1970; 1973; Shany et al,1973; Smyth and Fehrenbach, 1974).

In the case of diphtheria toxin there is good evidence to suggest that NADase activity is a genuine property of the toxin and indeed this activity, by allowing kinetic and inhibitor studies to be conducted in simple, well defined buffer systems (Kandel et al, 1974), has been useful in elucidating the detailed mechanism of the toxin's action. In a similar way, the demonstration of a cholera toxin-associated NADase activity provided opportunities for studying the action of cholera toxin in a simplified system.

However some of the results presented in Ch. 5.2. were not consistent with the supposition that NADase activity was a genuine reflection of the toxin's true activity and emphasised the need for caution in extrapolating from results obtained in studies of this activity to the mechanism of toxin action on adenylate cyclase. Before discussing these results, it is important to consider some aspects of the stability of NAD^+ in different buffer systems.

6.1.2. Effects of ionic strength on the stability of NAD^+

Stimulation of the NADase activity of cholera toxin in phosphate buffers as reported by Moss et al (1976b) and Moss and Vaughan (1977), was not confirmed in this work. The results in Fig. 5.8. indicate that the increased rate of NAD^+ hydrolysis by cholera toxin with increasing potassium phosphate concentrations is not due to a direct stimulatory effect of phosphate on the toxin's activity, but to a non-specific effect on the stability of NAD^+. Also in contrast to the results of Moss and coworkers, the NADase activity of the toxin used in this study was independent of buffer composition and activities were comparable, after appropriate blank subtraction, in 50mM-Tris/HCl, 200mM-sodium acetate, or various concentrations of potassium phosphate. As Fig. 5.9. shows, the non-specific effect on NAD^+ hydrolysis was not unique to phosphate, and NaCl in high concentrations also resulted in a high degree of non-specific NAD^+ hydrolysis.

These results are in agreement with previous reports on the stability of NAD^+ in various buffer systems at different pH values. Colowick et al (1951) noted that with phosphate buffers, the cleavage rate of NAD^+ at 100^oC increased dramatically at pH values of 6 and above and when several buffers were tested, all at 0.1M concentration and pH 7.5, only phosphate and citrate increased the rate of NAD^+ destruction. Lowry et al (1961) confirmed these observations and described 3.5-fold and 7-fold increases in the cleavage rate of NAD^+ at pH 6.2 and 6.8 respectively by 0.1M-phosphate, whether at 25 , 38 or 60^oC. These workers also described enhanced cleavage of NAD^+ in the presence of NaCl and several other salts.

It should be noted that the buffer systems employed by Moss and coworkers routinely contained 0.2M to 0.4M-potassium phosphate at pH 7.0, under which conditions the non-specific cleavage rate of NAD^+ is high. Although no references were made to the problems of high blank values in this system, the use of 0.4M-phosphate in the work described here resulted in blank values which were only slightly lower, and often greater, than the values obtained in the presence of cholera toxin. Since the non-specific effects of NaCl on the stability of NAD^+ were even more marked (in Fig. 5.9. for example, almost 25% of all available NAD^+ was destroyed by 1M-NaCl in the absence of cholera toxin), the apparent ten-fold increase in the activity of toxin on increasing the NaCl concentration from zero to 1M, was not considered to be of any real significance.

6.1.3. NAD^+ hydrolysis by culture filtrates of *V. cholerae*

The fact that cholera toxin appeared to be capable of hydrolysing NAD^+ raised the possibility of monitoring toxin purification enzymically, thereby improving significantly on the qualitative techniques of Ouchterlony immunodiffusion and SDS gel electrophoresis used previously. However, as shown in Fig. 5.10. crude culture filtrates of *V. cholerae* possessed an NADase activity which was almost 200 times greater than that of the purified toxin. Furthermore, NADase activity purified separately from cholera toxin and the non-toxin NADase eluted from DEAE-cellulose was again about 200 times more active than the toxin finally obtained in this purification. It should be emphasised that all preparations of cholera toxin purified during the course of this work (which all appeared to be homogeneous on polyacrylamide gel electrophoresis), displayed different specific activities of NADase, depending presumably on the efficiency of removal of the non-toxin NADase in different purifications. However since all preparations displayed at least some degree of NADase activity, the possibility of a toxin-associated NADase was not ruled out by this observation alone. Nevertheless the results do emphasise the importance of purity in toxin preparations when using high concentrations of the toxin. From the results in Fig. 5.11. it can be calculated that all of the NADase activity observed in the purified toxin (0.75µg of protein per assay), could be accounted for by only 4.7 ng of non-toxin NADase contaminant. 50µg of the same preparation could therefore contain 0.3µg of non-toxin NADase and thus, even with large loadings on SDS-polyacrylamide gels, the proposed contaminant would not be visible.

6.1.4. Arguments against the significance of NADase activity in relation to the action of cholera toxin on adenylate cyclase.

If NAD^+ hydrolysis by purified preparations of cholera toxin was a useful indication of the activity of toxin with respect to adenylate cyclase activation, treatments which alter cyclase activation by the toxin would be expected to alter the NADase activity in the same way. However this was not found to be the case. Preincubation of cholera toxin in a dilute solution of SDS and DTT enhances the subsequent ability of the toxin to activate adenylate cyclase in broken cell preparations (Gill, 1976b; van Heyningen, 1977b). This is presumed to be due to the release of active peptide A_1 from the intact toxin molecule during the SDS-DTT treatment. Such a treatment might therefore be expected to enhance the toxin's NADase activity but on the contrary, preincubation with SDS and DTT markedly inhibited the NADase reaction catalysed both by purified cholera toxin and by the non-toxin NADase (Table 5.5.) Similarly, preincubation with antitoxin, a procedure which prevents toxin action on subsequent addition to an adenylate cyclase preparation, had no effect on the NADase activity of either culture filtrates of V. cholerae or of purified cholera toxin (Table 5.6.).

Direct evidence that the NADase activity of purified cholera toxin was not a genuine property of the toxin was derived from the results presented in Fig. 5.12. In this experiment cholera toxin, in both crude and purified preparations, comigrated on polyacrylamide gels with an expected peak of adenylate cyclase activating activity

but in the purified material this did not coincide with any detectable NADase activity. It appears therefore that the cholera toxin purified in this work possesses an NADase activity which can be wholly accounted for by a contaminating enzyme present in small amounts. Although it remains possible that a more sensitive assay method may have detected a toxin-associated NADase activity, this alone cannot account for the differences between these results and those of Moss et al (1976b) who, using similar assay procedures, demonstrated NADase activity in one slice from a polyacrylamide gel of purified cholera toxin in a comigrating position with the stained protein band. The mobility of their toxin on the gel was similar to that observed here, but no other peaks of NADase activity were detected. Adenylate cyclase activating activities of the gel slice fractions were not reported.

It is also possible that a specific toxin-associated NADase activity may have been detected had the assays been conducted in the presence of agents which have been reported to enhance the NADase activity of the toxin. Moss and Vaughan (1977) have described marked enhancement of this activity in the presence of arginine, a phenomenon which was explained by suggesting that arginine, by acting as a more efficient acceptor of ADP-ribose than water, encouraged the cleavage of NAD^+ by cholera toxin. Direct evidence for this suggestion was also presented when a compound with the characteristics of an ADP-ribose-arginine adduct was isolated from incubation mixtures containing cholera toxin, NAD^+ and arginine. However as shown in Tables 5.1. and 5.2., the NADase activity of cholera toxin purified in this laboratory, as well as a sample of purified toxin given by Dr. R. Rappaport, was consistently unaffected by arginine. At present, no satisfactory

explanation for this major discrepancy with the results of Moss and coworkers can be put forward.

It is interesting to note that more than one species of adenylate cyclase activating material was observed in both crude and purified toxin preparations (Fig. 5.12.) The crude toxin (culture filtrate) displayed one major peak distinct from the toxin itself at mobility 0.45. It seems probable that this represents free subunit A of the toxin since a similar gel of the purified subunit, stained for protein, displayed one band in this position (results not shown). After purification much of this material was removed but was replaced with several other smaller peaks of activity and in fact, the purified toxin was more heterogeneous with respect to adenylate cyclase activating species than was the crude material. The most likely explanation for this phenomenon is the proteolytic generation of fragments of active subunit A during purification. The problems of proteases in some batches of crude toxin have already been referred to in Ch. 4. and a recent report by Gill and Rappaport (1979) has implicated the possible importance of extracellular V. cholerae proteases in the production of the 'nicked' A_1 and A_2 peptides of cholera toxin. In this report also, similar experiments to the one described above were reported in which active cyclase stimulating material was only observed in association with intact subunit A or with peptide A_1 on SDS-polyacrylamide gels. No other peaks of activity analogous to those in Fig. 5.12.B. were detected. This may simply reflect a greater purity of the toxin preparation used by these workers but since their experiments were conducted on SDS gels, the presence of fragments differing by only a few hundred daltons cannot be excluded.

Such fragments would migrate in essentially the same position as intact subunit A on SDS gels but the loss of only one or a few amino acids may result in markedly different migration characteristics on native polyacrylamide gels where overall charge, as well as size and shape, contributes to the observed mobility. Thus the various peaks of activity present in Fig. 5.12.B do not necessarily represent very small fragments of subunit A but may differ from this, and from each other, in only several amino acids. On the other hand, significantly smaller fragments are not excluded either. Matuo et al (1976) reported the generation of active fragments of cholera toxin by proteases present in Sarcoma 180 cell membranes, and the smallest fragment which still retained activity was reported to have a molecular weight of only 1,400 daltons i.e. about ten amino acids in length. Although it is difficult to reconcile an enzymic mechanism of toxin action with the retention of activity by such a small fragment, it is interesting to note that recent studies on the amino acid sequence of peptide A_1 of cholera toxin have revealed a small area of sequence homology with the α subunit of thyrotropin stimulating hormone (TSH), and that this same sequence is remarkably similar to the nonapeptide neurohypophyseal hormones oxytocin and vasopressin (see Kohn et al, 1978). It will be of obvious interest to determine the amino acid sequence of the active 1,400 dalton fragment since this may not activate adenylate cyclase in the same way as intact peptide A_1. The selective advantages of a toxin which could still activate pathogenic processes (perhaps by a different non-enzymic mechanism) even if severely degraded by intracellular proteases, are obvious. Furthermore, if small fragments of cholera toxin generated intracellularly were capable of binding directly to adenylate cyclase, some of the results of Cuatrecasas and coworkers described in Ch.1.10. could also be more readily explained.

The major conclusion to be drawn from the above experiment is that the adenylate cyclase activating activity of the cholera toxin purified in this laboratory was not associated with an NADase activity and that the small amount of the latter activity in these preparations was due to a contaminant enzyme. The possible role of a highly active NADase secreted extracellularly by V. cholerae is obscure and to the author's knowledge, the enzyme has not been described previously. NADases are widely distributed in nature and have been described in several microorganisms. In general, procaryotic NADases are soluble enzymes which are resistant to inhibition by nicotinamide and by other pyridine compounds (including INH) and which are often accompanied by heat-labile inhibitor proteins (Everse and Kaplan, 1968). The major NADase of V. cholerae was also resistant to inhibition by INH (Table 5.4.) and thus resembles most other bacterial NADases, but since the enzyme was active without a requirement for heat treatment, it apparently is not accompanied by a heat labile inhibitor protein. In a recent report (Fernandes et al, 1979), membrane-bound NADases have been described in the inner and outer membranes of V. cholerae. In the inner membrane most of the NADase activity was associated with a 29,000 dalton protein while in the outer membrane the major NADase had a molecular weight of 22,000. The inner membrane NADase was also capable of ADP-ribosylating a 44,000 dalton protein also in the inner membrane and antibodies against subunit A of purified cholera toxin inhibited this ADP-ribosylation. The authors suggested that these activities may represent the membrane-bound precursors of cholera toxin and that ADP-ribosylation of the 44,000 dalton protein in the inner membrane may be of importance in cellular regulation. It is interesting to note that although

antibodies against subunit A of purified toxin inhibited the ADP-ribosyltransferase activity of the putative inner membrane-bound precursor of toxin, it had no effect on the NADase activity. In the same way, activation of adenylate cyclase by cholera toxin, which may involve an ADP-ribosyltransferase activity, is inhibited by cholera antitoxin, but NADase activity was unaffected by antitoxin treatment (Table 5.6.). These results lend support to the hypothesis that the inner and outer membrane NADases of V. cholerae are associated with the precursors of cholera toxin and suggest that NADase activity, if a genuine property of the purified toxin, occurs independently of the putative ADP-ribosyltransferase activity and may be catalysed at a different site. Since the NADases described in these studies were membrane-bound, they are probably unrelated to the major NADase described in this work which was a soluble enzyme, secreted extracellularly by V. cholerae.

Kinetic studies with different preparations of purified cholera toxin did not fully support the conclusion drawn from the results in Fig. 5.12. that cholera toxin is devoid of an NADase activity. As shown in Figs. 5.13. and 5.14., the NADase activity in culture filtrates of V. cholerae and in the toxin purified therefrom, exhibited the same kinetic affinity for NAD^+ (0.2mM), confirming that both activities are due to the same enzyme. However kinetic analyses of the reactions catalysed by purified cholera toxin given by Dr. R. Rappaport and Dr. R.A. Finkelstein both displayed ten-fold greater K_m values for NAD^+ (Figs. 5.15. and 5.16.). Even this latter value of 2mM is considerably less than that of 3.8 mM initially reported by Moss et al (1976b), although

the use of low concentrations of phosphate (10mM) and analysis of the data by the statistically more accurate Direct Linear method in the work described here, may account for this difference. Despite the wide variations in the kinetic parameters of the NADase reactions catalysed by different toxin preparations, the two most widely differing samples (the toxin purified in this laboratory and the toxin from Dr. R.A. Finkelstein) produced virtually identical effects on rat liver adenylate cyclase when added in equal concentrations (Fig. 5.17.).

The above results indicate that the study of the NADase activity of cholera toxin is of questionable value in investigating the mechanism of toxin action. Although it cannot be concluded with certainty that cholera toxin does not possess an NADase activity, these results and those of Fernandes et al (1979) suggest that this activity is at least independent of the activity responsible for adenylate cyclase activation. Thus the only artifical system which may be expected to yield valuable information on the mechanism of cholera toxin action would be one which is directed specifically towards the ADP-ribosyltransferase activity of the toxin. The system initially reported by Moss and Vaughan (1977) in which the toxin-catalysed ADP-ribosylation of arginine was described, has recently been modified by Mekalanos et al(1979a,b) who have studied the detailed molecular mechanisms involved in the transfer of the ADP-ribose moiety of NAD^+ to guanidine groups on artifical acceptor molecules catalysed by cholera toxin.

Studies with this artificial system are likely to provide useful information on the mechanism of cholera toxin action.

In this work however, it was decided to study the role of NAD^+ as a putative substrate for toxin-catalysed ADP-ribosylation under conditions in which adenylate cyclase activation is observed i.e. in crude cellular systems with physiologically meaningful concentrations of toxin and of reactants. In particular, attempts were made to demonstrate toxin-specific ADP-ribosylation of rat liver membrane proteins as was reported in pigeon erythrocyte membranes concurrently with the execution of this work (Gill and Meren, 1978; Cassel and Pfeuffer, 1978). In this way it was hoped to establish that the mechanism of toxin action in complex membranes was at least similar to its action on the relatively simple pigeon erythrocyte system.

6.2. The role of NAD^+ in the activation of adenylate cyclase by cholera toxin.

6.2.1. Requirement for NAD^+ in toxin action and interference by endogenous rat liver enzymes.

The NAD^+ requirement for cyclase activation by cholera toxin first reported by Gill (1975) in pigeon erythrocyte lysates, and later verified in many tissues including rat liver plasma membranes (Martin et al, 1977), was also confirmed here (Fig. 5.18.) However, while Martin and coworkers noted a continually increasing extent of cyclase activation with increasing NAD^+ concentrations up to at least 5mM, Fig. 5.18. indicates that maximum toxin action in crude rat liver membranes was observed with 1mM-NAD^+, and 5mM-NAD^+ was less effective. These results are similar to those of Flores et al (1976) who also observed a maximum effect of cholera toxin in rat liver homogenates with 1mM-NAD^+. These differences may reflect some property of the crude systems used by the latter authors and in this work, as opposed to the purified plasma membranes employed by Martin and coworkers. As shown in Fig. 5.18., the extent of cyclase activation was not directly proportional to the NAD^+ concentration as might be expected if NAD^+ was the substrate for a toxin-catalysed reaction, but this is undoubtedly related to the high levels of contaminating NADases in the crude membrane preparation (Fig. 5.19.). Since cholera toxin action was not supported at low NAD^+ concentrations, it may be postulated that under these conditions the toxin is unable to compete effectively for substrate with the endogenous NADase activities and that the latter enzymes may exhibit a higher affinity

for NAD^+ than does the toxin. Mammalian NADases in general tend to possess high affinities for NAD^+ and K_m values of 16μM for bovine erythrocyte NADase (Pekala and Anderson, 1978), 56μM for calf-spleen NADase (Schuber and Travo, 1976), and 100μM for soluble seminal fluid NADase (Yuan and Anderson, 1971) have been reported. Although these values are certainly much lower than that for the NADase activity of cholera toxin, for the reasons discussed in the previous section it is not possible to make any meaningful comparisons until the K_m value for NAD^+ in the toxin-associated ADP-ribosyltransferase activity has been determined.

It should be emphasised that the endogenous rat liver activities referred to collectively here as 'NADase', probably represent a variety of enzymes which catalyse the release of nicotinamide from NAD^+ and in crude rat liver membranes there are likely to be two major activities contributing to total NAD^+ destruction:

(i) poly (ADP-ribose) polymerase

(ii) microsomal NADase

Poly (ADP-ribose) is a polymer of repeating ADP-ribose residues linked ribosyl (1 → 2) ribose with adenosine at the growing end which has been found covalently attached to histones (Nishizuka et al, 1968; Otake et al, 1969), and which has been implicated in the control of DNA synthesis and gene expression (Burzio and Koide, 1970; 1971; Lehmann et al, 1974; Rickwood et al, 1977). However the exact function of this curious polymer remains obscure. The enzyme responsible for its synthesis, poly (ADP-ribose) polymerase, has been reported to exhibit a K_m value for NAD^+ of 0.25mM both in nuclei (Nakazawa et al, 1968) and in soluble extracts of tissue homogenates (Gill, 1972). Since the crude rat liver membrane preparations

employed in the work described here undoubtedly contained nuclear material, poly (ADP-ribose) polymerase is likely to be an important competitor for NAD^+ in this system. However, since purified plasma membranes (which should be devoid of this enzyme) still displayed high levels of NAD^+ - utilising activities (Table 5.7.), the membrane-bound microsomal NADases probably represent the major pathway for NAD^+ destruction. NADases have been reported previously to co-purify along with plasma membranes as well as with microsomal fractions (Bock et al, 1971). Since the endogenous NAD^+-utilising activities could therefore not be separated from adenylate cyclase simply by purifying the plasma membrane fraction, several attempts were made to selectively inhibit these activities leaving the action of cholera toxin unaffected.

NADases from different species exhibit curious differences in their susceptibility to inhibition by INH. For example the enzymes from homogenates of beef spleen and brain are inhibited by more than 50% with 0.75mM-INH, while in the corresponding tissues from rat and mouse, NADases are relatively unaffected by 25mM-INH. In general, NADases from ruminants and birds are 'INH-sensitive' while those from all other species are 'INH-insensitive' (Zatman et al, 1954). The results in Fig. 5.20. indicate that INH in high concentrations did have an inhibitory effect on rat liver NADases and maximal inhibition (about 60%) was observed with 100mM-INH. Although this appears to be a significant inhibition, the remaining activity was still at a high level and continued to interfere with the action of cholera toxin. Similarly, other inhibitory techniques tested were either totally ineffective (preincubation with nicotinamide) or only partially

effective (preincubation with NAD^+; treatment with methylxanthines) as inhibitors of the endogenous rat liver NADases.

It is evident therefore that the major problem encountered by Gill and Meren (1978) in attempting to demonstrate cholera toxin-specific ADP-ribosylation in pigeon erythrocyte membranes was exactly reversed in the rat liver membrane system. In the former case, endogenous NAD^+ in the pigeon erythrocyte lysate diluted high specific radioactivity $[^{32}P]$ NAD^+ added exogenously, thereby rendering the small amount of ADP-ribose incorporated by the toxin undetectable. In rat liver membranes it was not possible to add low concentrations of highly radioactive NAD^+ since such low concentrations (10μM) were insufficient to support cholera toxin action, being metabolised preferentially through the endogenous NAD^+-utilising pathways. Although it proved possible to effectively separate adenylate cyclase from these activities, based on the selective solubilisation of the former with Lubrol-PX, new difficulties arose from the use of solubilised preparations, the major problem being the instability of adenylate cyclase after solubilisation. Nevertheless, some interesting results were obtained with this system and these are discussed later (Ch. 6.3.), but because of the problems of cyclase stability, studies were continued mainly with the particulate crude membrane preparation.

6.2.2. Incorporation of ADP-ribose into rat liver proteins.

When adenylate cyclase activity in rat liver homogenate was determined after various times of incubation with or without cholera toxin, in conjunction with estimations of ADP-ribose incorporation, the results shown in Fig. 5.24. were obtained. 1 mM-NAD^+ was employed in this experiment which was therefore sufficient to support maximum cholera toxin action and after 15 min. of incubation with toxin, the cyclase was activated maximally by about two-fold. Despite this clear activation, no toxin-specific incorporation of ADP-ribose was evident and indeed, at later time points, ADP-ribose incorporation in the presence of toxin was significantly inhibited. Time courses of ADP-ribose incorporation in crude extracts of mammalian tissues characteristically show the pattern illustrated in this figure, namely an initial rapid burst of incorporation, followed by a decrease in the rate of incorporation, and then an overall decline in the net amount of ADP-ribose incorporated. This probably reflects alterations in the relative rates of synthesis and degradation of protein-ADP-ribose complexes. In particular, mammalian nuclei contain two enzymes which are responsible for the degradation of poly (ADP-ribose) polymers; a nucleolar phosphodiesterase which hydrolyses pyrophosphate bonds at the terminal poly (ADP-ribose) residue and which may also hydrolyse NAD^+ (Futai and Mizuno, 1967; Futai et al, 1968), and a soluble poly(ADP-ribose) glycohydrolase which catalyses hydrolysis of the ribosyl-ribose bonds (Ueda et al, 1972; Miwa and Sugimura, 1971). After the first ten to fifteen minutes of incubation in the above experiment, NAD^+ levels will be severely depleted as a result of the action of endogenous microsomal NADase activities (Fig.5.19.)

and since substrate concentration will therefore become limiting, the rate of ADP-ribose incorporation will decrease and eventually cease altogether, leaving visible only the rate of degradation of protein -ADP-ribose complexes.

A comparison of Figs. 5.24.B. and 5.19. indicate that the majority ofthe NAD^+-utilising activities in rat liver homogenates are probably of the NADase type. Total NAD^+ destruction after 10 min. in Fig. 5.19. amounted to about 70 nmoles of nicotinamide released per assay after the initial addition of 2mM-NAD^+, while in the presence of 1mM-NAD^+ in the above experiment, only about 250 pmoles of ADP-ribose were incorporated into protein after the same time. Although these two experiments were conducted under slightly different conditions and at different incubation temperatures, it is clear that ADP-ribosyltransferase activites constitute only a small fraction of the total NAD^+ - utilising activities in rat liver homogenates.

The apparent inhibition of ADP-ribose incorporation by cholera toxin at all time points, and especially after 10 min. of incubation, is discussed later when the same effect was observed, but to a greater extent, in solubilised rat liver homogenates. It is evident at this stage that if cholera toxin catalyses an ADP-ribosylation, then the techniques employed in the above experiment were not sufficiently sensitive to detect this process.

6.2.3. <u>Effects of nicotinamide and thymidine on the action of cholera toxin.</u>

Nicotinamide, an effective inhibitor of NADases and ADP-ribosyltransferases from most eucaryotic species (Zatman et al, 1953; 1954; Gill, 1972; Pekala and Anderson, 1978), was found to exhibit selective effects between the activities of endogenous NAD^+-utilising enzymes and of cholera toxin in rat liver membranes. As Fig. 5.26. shows, a preliminary experiment indicated that nicotinamide could inhibit the action of cholera toxin but only when present in high concentrations.At lower concentrations (up to 1mM) the compound appeared to result in enhancement of adenylate cyclase activation by the toxin. A more detailed study of the effects of nicotinamide confirmed this finding and three major conclusions can be drawn from the results summarised in Figs. 5.27 to 5.29.

(i) the action of cholera toxin on adenylate cyclase in crude rat liver membranes can be supported with low concentrations of NAD^+ provided that nicotinamide is also present, and indeed with a combination of 100μM-NAD^+ and 1mM-nicotinamide, toxin action is greater than with 1mM-NAD^+ alone. At concentrations of nicotinamide greater than 1mM, inhibition of toxin action begins to occur. In contrast, when the NAD^+ concentration is high, low concentrations of nicotinamide do not enhance the action of cholera toxin and a slow progressive inhibition occurs on the addition of this compound.

(ii) High concentrations of nicotinamide, although reducing the maximal extent of cyclase activation observed after a 10 min. incubation time, result in an initial rapid burst of adenylate cyclase activation

after 2 min. when an effect of cholera toxin is not normally evident. This effect is seen with both low (100μM) and high (1mM) concentrations of NAD^+.

(iii) Even with high concentrations of nicotinamide (10 mM to 25mM), cholera toxin action is not completely inhibited and significant cyclase activation still occurs. This result is not in agreement with those of Fig. 5.26. where 10mM-nicotinamide was found to completely inhibit toxin action. No definite explanation can be offered for this discrepancy but since the above results were reproducible in several different experiments, they are probably more reliable than the preliminary results of Fig. 5.26.

ADP-ribose incorporation assays conducted under exactly the same conditions as the above adenylate cyclase assays (Figs. 5.30. and 5.31.) showed nicotinamide to be an effective inhibitor of endogenous ADP-ribosyltransferase enzymes but again, even with maximal inhibition (about 90% at 10mM to 25mM-nicotinamide) there was no detectable toxin specific ADP-ribosylation. This was also true for the conditions (100μM-NAD^+ and 1mM-nicotinamide) under which cholera toxin action was maximal.

Thus the use of nicotinamide alone did not enable the observation of the putative cholera toxin-catalysed ADP-ribosylation possibly because the effect of the toxin, which is likely to be very small even under optimal conditions, would be even smaller when partially inhibited by high concentrations of nicotinamide. However when thymidine (20mM) was included in assays under optimal cyclase activating conditions of 100μM-NAD^+ and 1mM-nicotinamide, the background

rate of ADP-ribose incorporation was almost completely abolished. Since thymidine is an effective inhibitor of poly (ADP-ribose) polymerase (Tanuma et al,1978; 1979; Clark et al, 1971; Preiss et al, 1971) it seems likely that the majority of endogenous ADP-ribosylation is due to this enzyme. However, thymidine had no effect on the action of cholera toxin (Fig. 5.33.) and therefore these conditions supported maximum toxin action with the minimum of interference from endogenous NAD^+-utilising enzymes. High concentrations of nicotinamide alone can also reduce endogenous ADP-ribosylation to comparable levels (see Figs. 5.30. and 5.31.) but only at the expense of a loss in toxin activity and although 20mM-thymidine alone would probably be sufficient to completely inhibit poly (ADP-ribose) polymerase, it is unlikely to have an extensive inhibitory effect on the microsomal NADases which constitute the greatest proportion of endogenous NAD^+-utilising activities. Thus optimal conditions for cholera toxin action can only be obtained with a combination of an optimal NAD:nicotinamide ratio and sufficient thymidine to inhibit poly (ADP-ribose) polymerase by almost 100%. Under these conditions a cholera toxin-specific incorporation of ADP-ribose into rat liver membrane proteins was observed (Fig. 5.32.)

With cholera toxin at a concentration of 2 µg/ml (the concentration employed in all of the experiments discussed above), only about 0.3 pmoles of ADP-ribose were incorporated by the toxin after a 5 min. incubation time and it is therefore not surprising that all previous attempts to demonstrate this activity of the toxin (against a background of tens or hundreds of pmoles of ADP-ribose being incorporated), were unsuccessful. However by increasing the toxin

concentration up to 9µg/ml the amount of ADP-ribose incorporated by the toxin was increased significantly. Since maximum adenylate cyclase activation has previously been shown to occur with only 1µg/ml cholera toxin (Fig. 5.4.), this would not be expected if all ADP-ribosylations were relevant to the process of cyclase stimulation. However in the presence of 100µM-NAD^+, 1mM-nicotinamide, and 20mM-thymidine, maximum cyclase activation was no longer observed with 1µg/ml cholera toxin but continued to increase dramatically with toxin concentrations up to 20µg/ml (Fig. 5.33.). Thus under these conditions cholera toxin was a considerably more effective stimulator of adenylate cyclase and it therefore seems likely that at least some of the ADP-ribosylations observed with high toxin concentrations are of relevance to cyclase activation.

However there was still no direct proportionality between the amount of ADP-ribose incorporated and the extent of adenylate cyclase activation. While 1µg/ml cholera toxin catalysed the incorporation of 0.2pmoles of ADP-ribose with a two-fold increase in cyclase activity, 9µg/ml toxin catalysed the incorporation of almost 4 pmoles of ADP-ribose with only an approximate 3.5-fold stimulation of adenylate cyclase. Other results by Gill and Meren (1978) and Gill (1979) have indicated a similar situation in pigeon erythrocyte lysates where more than one protein was specifically ADP-ribosylated by the toxin. As yet, the possible significance of these 'extra' ADP-ribosylations is not understood.

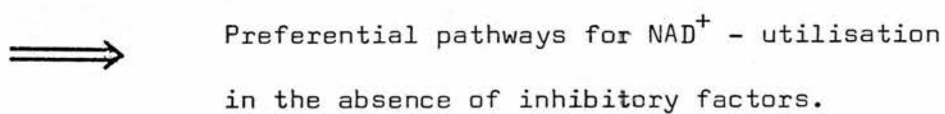
6.2.4. Model of cholera toxin action in rat liver membranes.

The results discussed above can be integrated into the scheme outlined in Fig. 6.1. which summarises the factors involved in the action of cholera toxin in crude rat liver membranes. A similar situation may be found to exist in most complex tissues which contain endogenous NAD^+ - utilising activities, although it should be remembered that in intact cells, compartmentation of cellular functions may eliminate or modulate some of these processes.

In the crude membrane preparation with no additions, endogenous NAD^+-utilising activities are uninhibited and since they are likely to be present in a large excess over cholera toxin, and since they may exhibit a higher affinity for NAD^+ then does the toxin, small amounts of NAD^+ added exogenously are metabolised preferentially by these enzymes. Only when high concentrations of NAD^+ are employed (1mM), when the endogenous enzymes are presumably approaching saturation point, is there sufficient NAD^+ available to support the activation of adenylate cyclase by cholera toxin. 1mM-nicotinamide inhibits endogenous ADP-ribosyltransferase activities by about 75% and although the effect of nicotinamide on the microsomal NADase activities was not studied in detail, preliminary experiments indicated that these enzymes were almost completely inhibited under these conditions. Thus in the presence of 1mM-nicotinamide, low concentrations of NAD^+ are effective in supporting cholera toxin action, and the inhibition of toxin action at high nicotinamide concentrations may reflect end product inhibition of a toxin-catalysed ADP-ribosylation. However, since cholera toxin is relatively resistant to inhibition by nicotinamide, the immediate almost 100% inhibition of all endogenous NAD^+-utilising enzymes, which

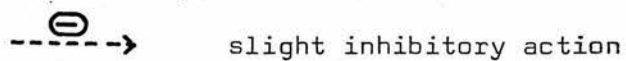
Figure 6.1. Competition for NAD^+ between cholera toxin and endogenous enzymes in rat liver membrane fractions.

NIC : Nicotinamide

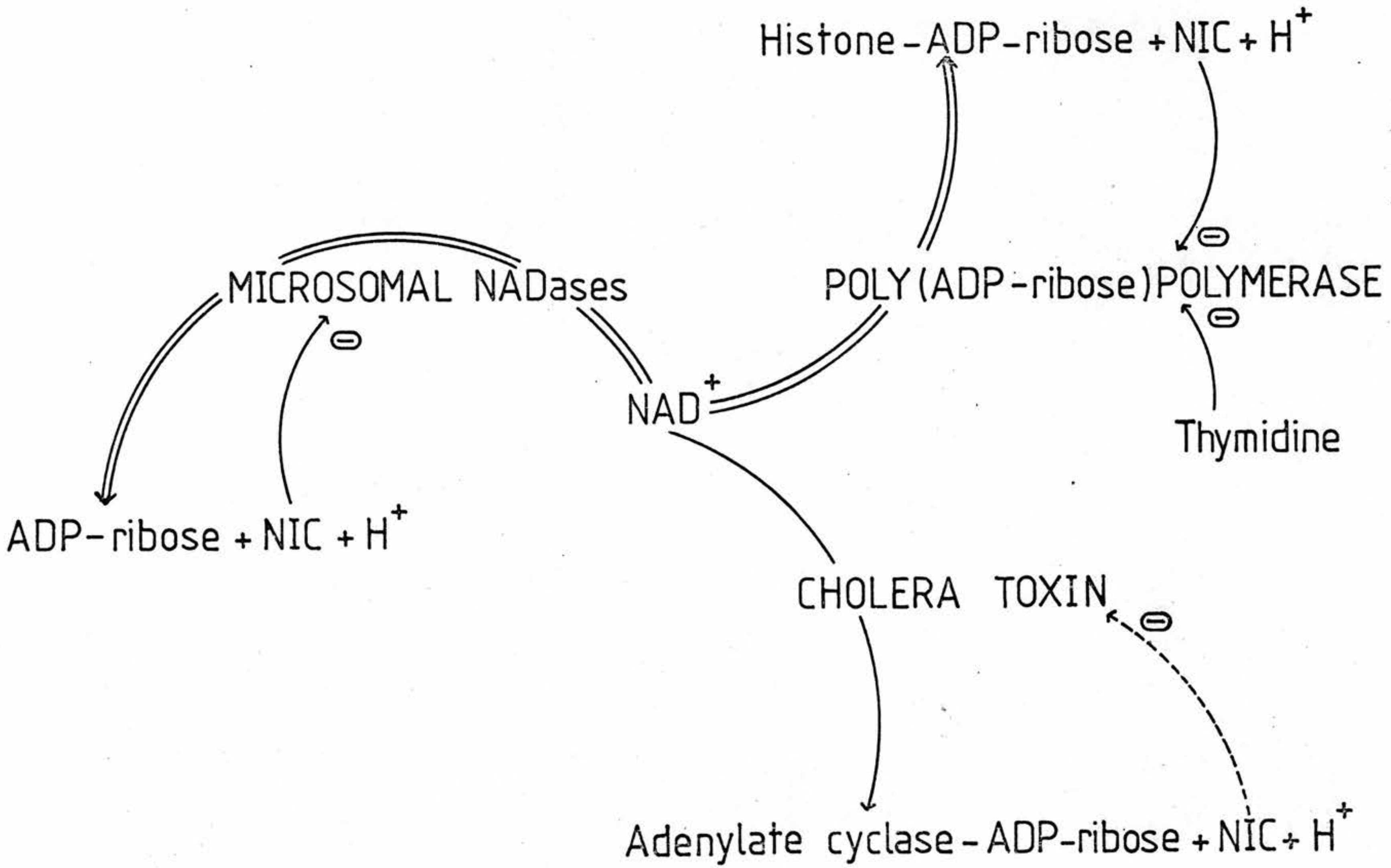


Preferential pathways for NAD^+ - utilisation in the absence of inhibitory factors.

strong inhibitory action



slight inhibitory action



Histone-ADP-ribose + NIC + H+
MICROSOMAL NADases
POLY(ADP-ribose)POLYMERASE
NAD+
Thymidine
ADP-ribose + NIC + H+
CHOLERA TOXIN
Adenylate cyclase-ADP-ribose + NIC + H+

probably occurs on addition of high nicotinamide concentrations, may be responsible for the initial rapid burst of toxin action observed under these conditions. When optimal adenylate cyclase activating conditions are employed, with nicotinamide in a ten-fold molar excess over NAD^+, addition of 20mM-thymidine causes almost 100% inhibition of the remaining poly (ADP-ribose) polymerase activity, and thus cholera toxin becomes one of the few NAD^+-utilising enzymes in the preparation which is not inhibited, the result being abnormally high levels of cyclase activation in conjunction with a detectable toxin-specific ADP-ribosyltransferase activity.

The results in Fig. 5.18. suggested that high concentrations of NAD^+ in this system (e.g. 5mM) inhibit the action of cholera toxin. Despite reports that NAD^+ itself at this concentration inhibits basal adenylate cyclase activity by about 50% (Martin et al, 1977), corrections for this phenomenon would not fully account for the extent of inhibition observed and it seems possible that this result may represent substrate inhibition of cholera toxin. This suggestion is supported by the observation that with 100µM-NAD^+, addition of 1mM-nicotinamide produced enhancement of toxin action, presumably by making NAD^+ available to the toxin through inhibition of endogenous activities (Fig. 5.27). With 1mM-NAD^+ however, the same addition of nicotinamide did not result in enhancement of toxin action but caused significant inhibition (Fig. 5.28). Although it is possible that cholera toxin may be more susceptible to inhibition by nicotinamide in the presence of high NAD^+ concentrations, it is perhaps more likely that the inhibition of endogenous activities by 1mM-nicotinamide in the presence of 1mM-NAD^+ makes such a large excess of NAD^+ available for the toxin

that the result is the same as that seen with 5mM-NAD^+ alone, namely substrate inhibition.

6.2.5. Nature of the substrate for cholera toxin-catalysed ADP-ribosylation.

Having demonstrated that specific ADP-ribosylation by cholera toxin could be observed in crude rat liver membranes, several attempts were made to identify the substrate(s) for toxin action in order to investigate the possibility that the same 42,000 dalton acceptor protein labelled by cholera toxin and [^{32}P] NAD^+ in pigeon erythrocytes (Gill and Meren, 1978; Cassel and Pfeuffer, 1978), was also the target for toxin action in rat liver membranes. These experiments involved incubations with cholera toxin and [adenine U - ^{14}C]-NAD^+ in crude membrane preparations followed by solubilisation of the membranes and application to SDS or native polyacrylamide gels, which were sliced and counted for radioactivity as described in Ch. 2.3.4. However these attempts were unsuccessful owing to the fact that so few radioactive counts were incorporated into protein by the toxin. For example, from about 1μCi of [adenine U-^{14}C] NAD^+ added to an assay, only several hundred counts were specifically incorporated as ADP-ribose by cholera toxin, and although this was reproducible in the assays described in the previous section, it was too little to be detected with certainty after application to and elution from slices of polyacrylamide gels. The use of [^{32}P]NAD^+, as described by other workers, should enable these experiments to be performed more readily.

However it seems likely that the major target protein of 42,000 daltons may be the cholera toxin substrate in rat liver also since this has been a common feature of toxin action in all systems studied to date, namely pigeon erythrocytes (Gill and Meren, 1978; Cassel and Pfeuffer, 1978; Gill, 1979), fat cell ghosts (Malbon and Gill, 1979), mouse S49 lymphoma cells, turkey erythrocytes, and human erythrocytes (Kaslow _et al_, 1979). The latter authors, in attempting to demonstrate cholera toxin-specific ADP-ribosylation in turkey erythrocyte membranes, also encountered the difficulties of endogenous NADases discussed above, a problem which they overcame by partially purifying solubilised fractions of the membranes before incubation with cholera toxin and $[^{32}P]$ NAD^+. This possibility had previously been attempted in this work when solubilised rat liver membranes were subjected to ion-exchange chromatography on DEAE-cellulose or gel filtration on Sepharose-6B, and each fraction assayed for ADP-ribose accepting activity in the presence and absence of cholera toxin. Again however the observed effects with [adenine U-^{14}C] NAD^+ as substrate were too small to allow any definite conclusions to be made.

6.3. Other activities of cholera toxin.

Several observations on the mechanism of action of cholera toxin suggest that ADP-ribosylation of an adenylate cyclase-associated GTP-binding protein may not be the only action of the toxin, or at least that the consequent inhibition of the GTPase activity of this protein may not be the only manifestation of the toxin-catalysed ADP-ribosylation. For example, fluoride stimulation of adenylate cyclase, a process which is modulated by cholera toxin (see Ch.1.12.), is not itself dependent on guanylnucleotides (Ross et al, 1977). The fact that cholera toxin appears to ADP-ribosylate more than one protein (Gill and Meren, 1978; Gill, 1979) also suggests other, as yet unknown, actions of the toxin, although it remains possible that the majority of these ADP-ribosylations are of no physiological significance and simply reflect the occurrence of several intracellular proteins with similar sequences to the true toxin substrate.

However some of the results presented in this report indicate the existence of at least one other effect of cholera toxin which can be directly related to the proposed mechanism of the toxin's action. This activity came to light as a result of studies on solubilised preparations of rat liver homogenates. As shown in Fig. 5.34. endogenous NADase activities in rat liver homogenates were extremely resistant to solubilisation with Lubrol-PX, a property which is shared by other mammalian NADases (Pekala and Anderson, 1978). Adenylate cyclase on the other hand could be readily solubilised

with 0.25% Lubrol-PX and the resultant preparation, which was relatively free of NADase activity (compare Figs. 5.19. and 5.35.), appeared to be suitable for studying cholera toxin action in the absence of interfering activities. However, although the solubilised adenylate cyclase was capable of being activated by cholera toxin (Fig. 5.36.), the stimulated enzyme was unstable and rapidly decayed to levels below that of the unstimulated control cyclase. When this preparation was incubated with increasing concentrations of cholera toxin for the optimal time of 2 min., and estimations were made of adenylate cyclase activity and of ADP-ribose incorporation, the surprising result shown in Fig. 5.37. was obtained. While adenylate cyclase was stimulated to a maximum of about three-fold over basal levels, the amount of ADP-ribose incorporation decreased markedly with increasing toxin concentrations. It therefore appears that cholera toxin has inhibited, to a large extent, the activity of endogenous ADP-ribosyltransferases. Confirmation of this conclusion is illustrated in Fig. 5.38. However it is important to note that ADP-ribose incorporation was not completely abolished even at 50µg/ml cholera toxin, and the interesting possibility is therefore raised that the residual rate of ADP-ribosylation which cannot be inhibited by cholera toxin may in fact be due to the toxin itself.

The above results can be compared with those in Fig. 5.24.B. where an inhibition of background ADP-ribosylation by cholera toxin was also seen in particulate homogenates although in this case, the effect was not marked until after 10 min. of incubation with toxin. It is interesting to relate these results to the earlier observation (Fig. 5.25.) that the endogenous ADP-ribosyltransferases were located almost exclusively in the membrane fraction. Cytosol contained neither

the enzymes nor the substrates but on addition of cytosol to the membranes there was a significant decrease in the rate of endogenous ADP-ribosylation, suggesting that cytosol may contain some ADP-ribosyltransferase-inhibiting factor. Furthermore, although cholera toxin had little effect on the rate of ADP-ribosylation either in the membranes or in the membranes plus cytosol, there may have been a further small decrease in the amount of ADP-ribose incorporated in the latter condition on addition of cholera toxin. Although the difference here is too small to be assigned any real significance, when combined with the above observations, the results suggest that cytosol contains an ADP-ribosyltransferase-inhibiting factor which is more effective in the presence of cholera toxin. Alternatively, cytosol may contain enzymes (such as the poly (ADP-ribose) glycohydrolase referred to earlier) which degrade protein-ADP-ribose complexes and cholera toxin may exert a direct stimulatory effect on these enzymes.

All of the solubilised preparations used in the above experiments were derived from homogenates i.e. membranes solubilised in the presence of cytosol and it would be of obvious interest to repeat these experiments on membranes solubilised in the absence of cytosol. As shown in Figs. 5.30 and 5.31. cholera toxin had no effect on the rate of background ADP-ribosylation in crude rat liver membranes, again suggesting the importance of cytosolic factors in mediating the inhibitory effect of cholera toxin on endogenous ADP-ribosyltransferases. Since the effects of cholera toxin on these enzymes were greatly enhanced in the solubilised preparations (compare Figs. 5.25.B. and 5.37.), it is possible that solubilisation renders the ADP-ribosyltransferases more accessible for inhibition by the unknown factor after toxin treatment.

Although there was insufficient time to pursue these results further, the advantages of an ADP-ribosyltransferase-inhibiting action of cholera toxin are obvious. In nature it seems likely that only a few molecules of cholera toxin subunit A will penetrate cell membranes and reach their intracellular site of action, and the probability of such few molecules initiating the pathogenic processes of cholera would be greatly increased if the toxin was capable of inhibiting at least some of the endogenous NAD^+-utilising activities. However, it should be emphasised that the solubilised preparations used in the above experiments represent an unphysiological situation and thus extensive inhibition of endogenous ADP-ribosyltransferases may not occur in vivo. The true situation may be more closely related to the results shown in Fig. 5.24.B. for rat liver homogenates, where the toxin had a smaller effect on the endogenous activities after a longer time period. Because of the time taken for a significant inhibitory effect to occur in this experiment, the possibility that ADP-ribosyltransferase inhibition is an indirect secondary effect as a result of adenylate cyclase activation and elevation of cAMP levels, is not ruled out. Further work in this area is necessary to establish the true nature of the inhibitory effect and of its possible significance in the mechanism of cholera toxin action.

Chapter 7. Conclusions and future prospects

The mechanism of action of cholera toxin has now been defined with some certainty. Recent studies have provided strong evidence in favour of Gill's original suggestion (Gill, 1975) that the toxin was an enzyme catalysing the NAD^+-dependent ADP-ribosylation of some component of the adenylate cyclase complex. In several different tissues the toxin has been shown to ADP-ribosylate a 42,000 dalton GTP-binding protein which is involved in adenylate cyclase regulation, and the consequent inhibition of this protein's GTPase activity is probably responsible for the stimulation of adenylate cyclase (Cassel and Selinger, 1978; Cassel and Pfeuffer, 1978; Gill and Meren, 1978; Kaslow et al, 1979; Malbon and Gill, 1979, Farfel et al, 1979) Although the action of cholera toxin in rat liver membranes was not defined in such detail in this work, all results were consistent with the above hypothesis and under conditions in which interference from endogenous NAD^+ - utilising activities was minimised, it was possible to demonstrate an ADP-ribosyltransferase activity of cholera toxin.

It is now possible to note some striking similarities between the action of cholera toxin and that of diphtheria toxin. Some similarities in the structures of these two toxins have already been discussed (Ch. 6.1.) and both are now also known to catalyse the ADP-ribosylation of an intracellular target protein. Most striking however, are the similarities between these target proteins. Like the GTPase component of adenylate cyclase which is attacked by cholera toxin, EF-2, the diphtheria toxin substrate, is also a GTP-binding protein which acquires a GTPase

activity when in association with another macro-molecular complex (mRNA, ribosome, and aminoacyl tRNA) (Skogerson and Moldave, 1968; Raeburn et al, 1968). ADP-ribosylation of EF-2 by diphtheria toxin specifically inhibits this factor's ribosome-dependent GTPase activity (Raeburn et al, 1968; Honjo et al, 1969), and therefore both toxins attack an intracellular GTPase, the activity of which is inhibited upon ADP-ribosylation.

In this respect it is interesting to note an early observation of Bennett et al (1975b) who, while investigating the effect of protein synthesis inhibitors on the action of cholera toxin, employed diphtheria toxin to eliminate protein synthesis before treatment with cholera toxin. Under these conditions, the authors noted a small, but consistent, activation of adenylate cyclase after incubation with diphtheria toxin alone. The observation received no further attention but in retrospect, it seems possible that diphtheria toxin, as well as ADP-ribosylating EF-2, may also have had some limited effects on the cyclase-associated GTPase, causing a slight stimulation of adenylate cyclase activity. It seems likely therefore that the substrates for the two toxins probably exhibit some similarities in amino acid sequence around the GTPase active site.

In the light of these observations, it is possible to envisage for the first time, an effective treatment for cholera (and possibly also for closely related diseases such as the diarrhoea induced by E. coli heat-labile enterotoxin) based on detailed biochemical considerations.

Any treatment aimed at lowering the levels of cyclase activity elicited by cholera toxin would probably be ineffective since the possibilities of interfering with the normal hormonal responses of adenylate cyclase would be significant. However the substrate for ADP-ribosylation by cholera toxin is now known specifically and furthermore, the toxin will also catalyse the ADP-ribosylation of several artificial acceptor molecules such as arginine (Moss and Vaughan, 1977) and related compounds (Mekalanos et al, 1979 a,b). The possibility of a unique sequence in GTPase enzymes which is recognised by ADP-ribosylating toxins, makes it feasible to define the target amino acid, and the surrounding sequence, for toxin-catalysed ADP-ribosylation and thus to manufacture an artificial substrate which is at least as effective, and possibly more effective, as an acceptor for ADP-ribose than the natural substrate. Such a compound would be a potent competitive inhibitor of cholera toxin action.

Several other aspects of cholera toxin action are not well understood and are worthy of further research. For example, the process of membrane penetration by subunit A of the toxin remains obscure. Although Gill (1976a) has suggested several possible mechanisms for this process, in which the B subunits, bound to Gm_1 gangliosides, form a 'tunnel' in the membrane through which subunit A is extruded, direct evidence for any such mechanism is still lacking. Similarly, the role of cytoplasmic factors required for toxin action in pigeon erythrocytes (Gill 1975; 1976b) has not been studied in detail. Since these factors were not required for toxin action in rat liver (Ch. 5.1.), their involvement in avian erythrocytes may indicate some fundamental evolutionary differences in adenylate cyclase systems between birds and mammals.

Toxin production and secretion by V. cholerae are also poorly understood phenomena and in particular, the recent report by Fernandez, et al (1979) describing membrane-bound NADases of V. cholerae which may represent cholera toxin precursors, is especially interesting. Their observation that the inner membrane NADase was also capable of ADP-ribosylating a 44,000 dalton protein, also in the inner membrane, raises the possibility that the ADP-ribosyltransferase activity of cholera toxin may also be involved at some stage in toxin production and secretion. Studies on the nature of the 44,000 dalton protein ADP-ribosylated in this system should be undertaken in order to investigate any possible similarities between this and the toxin substrate in eucaryotic cells, and to study the possible role of this protein in the cellular control mechanisms of V. cholerae.

APPENDIX I Materials

Material	Supplier
Acrylamide	BDH
Adenylate kinase	Boehringer
ATP	Sigma
cAMP	Boehringer
Cellulose microcrystalline	Merck
Coomassie Brilliant Blue-G	Sigma
Coomassie Brilliant Blue-R250	Sigma
DEAE-cellulose (DE-52)	Whatman
DEAE-paper (DE-81)	Whatman
Dimethyl-POPOP	Koch-Light
DTT	Sigma
Ethylenegycolmonoethylether	Koch-Light
GF/C filtres	Whatman
GTP	Sigma
Hepes	BDH
Isobutylmethylxanthine	Koch-Light
Isoniazid	BDH
Lubrol-PX	Sigma
2 -Mercaptoethanol	BDH
N,N'-(Methylenebis) acrylamide	BDH

NAD^+	Boehringer
Naphthalene	BDH
NCS Tissue solubiliser	Amersham
Nicotinamide	BDH
PEI	BDH
PEP	Boehringer
PMSF	Sigma
PPO	Koch-Light
Pyruvate Kinase	Boehringer
SDS	Sigma
TEMED	BDH
Thymidine	Sigma
Ultrogel AcA-44	LKB
Xylene (Scintillation grade)	BDH

Radioactive chemicals

$[8-^3H]$cAMP, $[$adenine $U-^{14}C]$ cAMP, $[8-^3H]$ ATP, $[$carbonyl-$^{14}C]$-NAD^+, and $[$adenine $U-^{14}C]$ NAD^+ were obtained from Amersham.

Cholera toxin

Crude culture filtrates of V. cholerae were kindly supplied by Dr. R.O. Thompson of the Wellcome Research Laboratories, Langley Court, Beckenham, Kent.

Purified cholera toxin prepared by the method of Finkelstein and LoSpalluto (1970) was a gift from the National Institutes of Health, Bethesda, M.D. U.S.A., for whom it wasy prepared under contract.

A sample of purified cholera toxin received from Dr. R. Rappaport is also gratefully acknowledged.

APPENDIX II Determination of kinetic rate parameters by the Direct Linear Method.

Kinetic rate parameters were determined using the Direct Linear Plot as described by Eisenthal and Cornish-Bowden (1974). In this method, each experimental observation of substrate concentration and velocity (s and v) are marked off on the -x and y axes respectively and a line is drawn through the two points and extended into the first quadrant. For a theoretically ideal experiment, all lines will intersect at a common point in the first quadrant whose co-ordinates provide values for V_{max} and K_m.

In real situations which are subject to error, lines will not intersect at a common point and in these cases V_{max} and K_m values are taken as the median of all points of intersection. When there are an even number of observations the median is taken as the mean of the middle two estimates, and where more than two lines intersect at a common point, the total number of intersections taken into account in calculating the median value is $\frac{1}{2}n\ (n-1)$, where n = the number of intersecting lines.

The theory behind the Direct Linear method for calculation of rate parameters is discussed in detail by Eisenthal and Cornish-Bowden (1974) and the same authors have also presented theoretical arguments which indicate that the method is statistically more accurate than other existing techniques (Cornish-Bowden and Eisenthal, 1974).

For some of the results presented in this report (Figs. 5.15. and 5.16.), where K_m values were high (and therefore intersection points were far along the x - axis), data were more conveniently presented by a later modification to the Direct Linear Plot (Cornish-Bowden and Eisenthal, 1978), in which values corresponding to s/v and 1/v for each observation are marked off on the x and y axes respectively and joined by a line. As above, the median of all intersection points has co-ordinates equivalent to $1/V_{max}$ and K_m / V_{max}.

As described by the authors, this modification also improves the statistical accuracy of the method by eliminating the bias given by intersections in the third quadrant which sometimes occurs when using the original method. Such intersections yield negative values for both K_m and V_{max} and lead to inaccuracies in the final estimate if taken at face value. In the modified method, intersections which do not occur in the first quadrant require no special treatment (Cornish-Bowden and Eisenthal, 1978).

APPENDIX III List of Abbreviations

A	ADENINE
ACTH	ADRENOCORTICOTROPHIC HORMONE
Ad	ADENOSINE
ADP	ADENOSINE 5'-DIPHOSPHATE
ADP-RIBOSE	ADENOSINE 5'-DIPHOSPHATE RIBOSE
AMP	ADENOSINE 5'-MONOPHOSPHATE
ARg-OMe	ARGININE METHYL ESTER
BIS-ACRYLAMIDE	N,N'- (METHYLENEBIS) ACRYLAMIDE
BMX	ISOBUTYLMETHYLXANTHINE
BSA	BOVINE SERUM ALBUMIN
cAMP	ADENOSINE 3', 5'-MONOPHOSPHATE
cGMP	GUANOSINE 3', 5'-MONOPHOSPHATE
DEAE	DIETHYLAMINOETHYL
DIMETHYL-POPOP	DIMETHYL 1, 4-BIS- [2-(PHENYLOXAZOLYL)] BENZENE
DNP-LYSINE	2,4-DINITROPHENYL LYSINE
DTT	DITHIOTHREITOL
EF-2	ELONGATION FACTOR-2
G	GUANINE
GDP	GUANOSINE 5'-DIPHOSPHATE
Gd_{1a}	N-ACETYLNEURAMINYL-GALACTOSYL-N-ACETYLGALACTOSAMINYL-[N-ACETYLNEURAMINYL] - GALACTOSYLGLUCOSYLCERAMIDE
Gm_1	GALACTOSYL-N-ACETYLGALACTOSAMINYL- [N-ACETYLNEURAMINYL] - GALACTOSYLGLUCOSYLCERAMIDE

Gm_2	N-ACETYLGALACTOSAMINYL - [N-ACETYLNEURAMINYL] - GALACTOSYLGLUCOSYLCERAMIDE
Gm_3	N-ACETYLNEURAMINYL-GALACTOSYLGLUCOSYL CERAMIDE
GMP	GUANOSINE 5'-MONOPHOSPHATE
$Gpp(CH_2)p$	5' - GUANYLYL METHYLENE DIPHOSPHONATE
Gpp (NH)p	5'-GUANYLYLIMIDODIPHOSPHATE
GTP	GUANOSINE 5'-TRIPHOSPHATE
$GTP_{\gamma}S$	GUANOSINE 5'-O- (3-THIOTRIPHOSPHATE).
Gu	GUANOSINE
Hepes	4-(2-HYDROXYETHYL)-1-PIPERAZINE-ETHANESULPHONIC ACID
HX	HYPOXANTHINE
IDP	INOSINE 5'-DIPHOSPHATE
IMP	INOSINE 5'-MONOPHOSPHATE
In	INOSINE
INH	ISO-NICOTINIC ACID HYDRAZINE (ISONIAZID)
ITP	INOSINE 5'-TRIPHOSPHATE
NAD^+	NICOTINAMIDE ADENINE DINUCLEOTIDE
NADase	NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE
PEI	POLYETHYLENEIMINE
PEP	PHOSPHOENOLPYRUVATE
PMSF	PHENYLMETHYLSULPHONYLFLUORIDE
PPi	PYROPHOSPHATE
PPO	2,5-DIPHENYLOXAZOLE
SDS	SODIUM DODECYL SULPHATE
TEMED	N,N,N',N'-TETRAMETHYLETHYLENE DIAMINE
TLC	THIN LAYER CHROMATOGRAPHY
TRIS	TRIS (HYDROXYMETHYL) AMINOMETHANE
TSH	THYROTROPIN STIMULATING HORMONE

VIP	VASOACTIVE INTESTINAL POLYPEPTIDE
X	XANTHINE
Xa	XANTHOSINE
XMP	XANTHOSINE 5'-MONOPHOSPHATE

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The Adenylate Cyclase-Activating Activity of Cholera Toxin is not Associated with a Nicotinamide–Adenine Dinucleotide Glycohydrolase Activity

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The NAD^+ glycohydrolase activity of cholera-toxin samples can be separated from their adenylate cyclase-activating activity by polyacrylamide-gel electrophoresis and is inhibited by sodium dodecyl sulphate (which does not inhibit the action of toxin on cells), but not by antibodies to pure toxin. It is therefore probably not a true property of the toxin.

Cholera toxin is a potent activator of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in a wide variety of eukaryotic cell membrane systems (for general review, see van Heyningen, 1977*a*). The activation requires NAD^+, ATP, a thiol reducing agent, and an unidentified factor from the cell sap, which is probably a protein (Gill, 1976). Recent evidence also indicates a requirement for GTP (Cassel & Selinger, 1977). There have been several reports that purified cholera toxin has an NAD^+ glycohydrolase activity (Moss *et al.*, 1976, 1977) and evidence that the toxin can catalyse an ADP-ribosylation with arginine as acceptor has been presented (Moss & Vaughan, 1977). There is also evidence for self ADP-ribosylation of cholera toxin (Trepel *et al.*, 1977). It has therefore been suggested that cholera toxin, in an analogous way to diphtheria toxin, may act in catalysing an ADP-ribosylation perhaps of adenylate cyclase itself, or at least of some component of the cyclase complex, thus leading to the activation of this enzyme.

This paper provides evidence that the simple NAD^+ glycohydrolase activity is not associated with the activation of adenylate cyclase, and so may well be due to contaminating protein.

Experimental

Purification of cholera toxin

Cholera toxin prepared from crude culture filtrates of *Vibrio cholerae*, kindly given by Dr. R. O. Thomson of the Wellcome Laboratories, Beckenham, U.K. (batch number VT 2214E), was purified as follows. All operations were carried out at 4°C, and all buffers were made 0.1 mM in phenylmethanesulphonyl fluoride immediately before use. The crude solution was made up to 30% of saturation (measured at 25°C) in $(NH_4)_2SO_4$, the precipitate discarded, and the supernatant made up to 60% of saturation in $(NH_4)_2SO_4$. The precipitate was dissolved in water, dialysed against 50mM-Tris/HCl, pH 7.4, and applied to a column (2cm diameter × 26cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with the same buffer. Material reacting with cholera antitoxin (van Heyningen, 1976*a*) was eluted with 200ml of a linear gradient of 0 to 0.1M-NaCl in the buffer, freeze-dried, dissolved in water, dialysed against 0.1M-Tris/HCl/0.1M-NaCl, pH 7.4, and applied to a column (2cm diameter × 92cm) of Ultrogel AcA-44 (LKB, South Croydon, Surrey, U.K.). The antigenic product was homogeneous as judged by the visible bands present on polyacrylamide gels in the presence and absence of sodium dodecyl sulphate and migrated with the same mobility as a sample of toxin purified by the method of Finkelstein & LoSpalluto (1970) given to us by the National Institutes of Health, Bethesda, MD, U.S.A., for whom it was prepared under contract.

Polyacrylamide gels

Polyacrylamide gels at pH 8.3 were run by the method of Davis (1964), without a stacking or sample gel. Crude cholera toxin (150 μg) and purified toxin (50 μg) were applied to gels in duplicate. One of the duplicate gels was stained for protein with Coomassie Brilliant Blue R250, and the other was sliced into 2mm segments which were eluted for 24h with shaking at 25°C in about 0.4ml of water. The gel slices were removed, the eluates were freeze-dried, and the residues were taken up in 30 μl of water. Then 5 μl of each eluate was taken for NAD^+ glycohydrolase assay, 10 μl for adenylate cyclase-activating assay, and 5 μl for immunodiffusion against rabbit antitoxin (van Heyningen, 1976*a*).

NAD$^+$ glycohydrolase assay

NAD$^+$ glycohydrolase activities were determined by the addition of 5μl of each gel-slice eluate to 10μl of an assay medium containing 0.2M-sodium acetate/0.02M-dithiothreitol/2mM-NAD$^+$ and 10nCi of [*carbonyl*-^{14}C]NAD$^+$ of specific radioactivity 53Ci/mol (The Radiochemical Centre, Amersham, Bucks., U.K.), pH6.2. Incubations were conducted at 37°C for 60min, after which time 10μl samples from each assay were spotted on 20cm×2cm strips of DE-81 chromatographic paper (Whatman, Maidstone, Kent, U.K.), and [^{14}C]nicotinamide was separated as previously described (Apps & Nairn, 1977), for the separation of NAD$^+$ and NADP. Under these conditions, nicotinamide migrated with the solvent front, and NAD$^+$ had an R_F of about 0.4.

Adenylate cyclase assay

The source of adenylate cyclase was a crude rat liver homogenate prepared by the method of van Heyningen (1976*b*). The crude particulate adenylate cyclase preparation was then made 2.5mM in NAD$^+$, ATP, dithiothreitol and isonicotinic acid hydrazide, an inhibitor of endogenous rat liver NAD$^+$ glycohydrolase that had no effect on the NAD$^+$ glycohydrolases of *V. cholerae*.

A 10μl sample of each gel-slice eluate was added to 15μl of crude particulate adenylate cyclase and incubated at 37°C. After 20min, 15μl of each preincubation mixture was transferred to tubes containing 10μl of assay medium. Each assay (final volume, 25μl) contained 20mM-Tris/HCl/5mM-$MgCl_2$/5mM-KCl/5mM-phosphoenolpyruvate/1mM-dithiothreitol/0.5mM-cyclic AMP/0.5mM-isobutylmethylxanthine/0.58mM-ATP/80μg of pyruvate kinase/ml/80μg of adenylate kinase/ml and 0.6μCi of [8-^{3}H]ATP (The Radiochemical Centre; specific radioactivity 23Ci/mmol). The assay medium was adjusted to pH8.0 with 1M-NaOH by using Phenol Red as internal indicator, before use.

Incubations were conducted at 37°C for 20min and reactions were terminated by the addition of 10μl of 25% trichloroacetic acid. Precipitated protein was removed by centrifugation and 10μl samples of each clear supernatant were spotted on 20cm×50cm glass plates spread to a layer thickness of 0.3mm with cellulose microcrystalline (Merck; obtained from BDH Chemicals, Poole, Dorset, U.K.), impregnated with a 1% solution of poly(ethyleneimine) (BDH Chemicals), and cyclic [^{3}H]AMP was separated by the method of Keirns *et al.* (1974) Cyclic [*adenine*-U-^{14}C]-AMP (5nCi) (The Radiochemical Centre; specific radioactivity 287Ci/mol) was spotted along with each sample to act as a check on individual plate recoveries. Cellulose scrapings were placed in 10ml of a Triton X-100/xylene-based scintillation cocktail (Fricke, 1975), 2ml of 25mM-$MgSO_4$ was added to solubilize the cyclic AMP from the cellulose particles, and the samples were counted for radioactivity in a Searle MK III liquid-scintillation spectrometer.

Results and Discussion

During our routine purification of cholera toxin from concentrated culture filtrates of *V. cholerae*, NAD$^+$ glycohydrolase activity was consistently found to decrease in fractions containing toxin and increase in fractions not containing toxin after each step in the purification procedure. In particular those fractions eluted from a DEAE-cellulose column containing no material precipitating with antibodies to pure cholera toxin were found to have about 90–99% of all the NAD$^+$ glycohydrolase activity eluted from the column. This immediately suggested that the NAD$^+$ glycohydrolase was a property of proteins other than the toxin. To test this hypothesis several experiments were done.

Treatment of purified cholera toxin with sodium dodecyl sulphate, a procedure known to increase the activation of adenylate cyclase by toxin (Gill, 1976; van Heyningen, 1977*b*), brought about a loss of most of the NAD$^+$ glycohydrolase activity in several different experiments. An identical treatment of the NAD$^+$ glycohydrolase eluted from DEAE-cellulose as described above caused a loss of nearly all the activity (Table 1).

Antibody prepared in rabbits against a different sample of cholera toxin purified by the method of Finkelstein & LoSpalluto (1970) (see van Heyningen, 1976*a*) inhibits the adenylate cyclase-activating activity of crude and purified toxin in cells. However, it had no effect on the NAD$^+$ glycohydrolase activity (Table 1).

Table 1. *Effect of sodium dodecyl sulphate and cholera antitoxin on the NAD$^+$ glycohydrolase activity of toxin and non-toxin preparations*

The results are the means of three experiments.

Preparation	NAD$^+$ glycohydrolase activity (nmol of NAD$^+$ hydrolysed/min per mg of protein)
Purified toxin	8.9
Purified toxin preincubated with rabbit antitoxin	9.5
Purified toxin preincubated with 0.1% sodium dodecyl sulphate for 10min at 37°C	2.7
Non-toxin protein eluted from DEAE-cellulose	5400
Same non-toxin protein preincubated with 0.1% sodium dodecyl sulphate for 10min at 37°C	47

It is also possible to separate the two activities by polyacrylamide-gel electrophoresis. Figs. 1(*a*) and 1(*b*) show the activity of NAD^+ glycohydrolase and adenylate cyclase-activating activities eluted from slices of gels of purified and crude toxin respectively. Activities are plotted against the mobility of the mid-point of each gel slice. In the pure-toxin gel, cholera toxin was detected by Ouchterlony immunodiffusion with mobilities 0.02–0.18, and in the crude-toxin gel (where cholera toxin constitutes only about 2% of the total protein) the toxin was detected with mobilities 0.09–0.20. In both gels at these mobilities there is, as expected, a strong peak of adenylate cyclase-activating activity. However, also in both cases, the NAD^+ glycohydrolase activity at these mobilities is virtually zero.

The very powerful NAD^+ glycohydrolase activity in crude culture filtrates of *V. cholerae* runs at mobility 0.5–0.6. The corresponding band on a gel stained for protein was very faint. Specific enzyme activities could not be calculated owing to the small

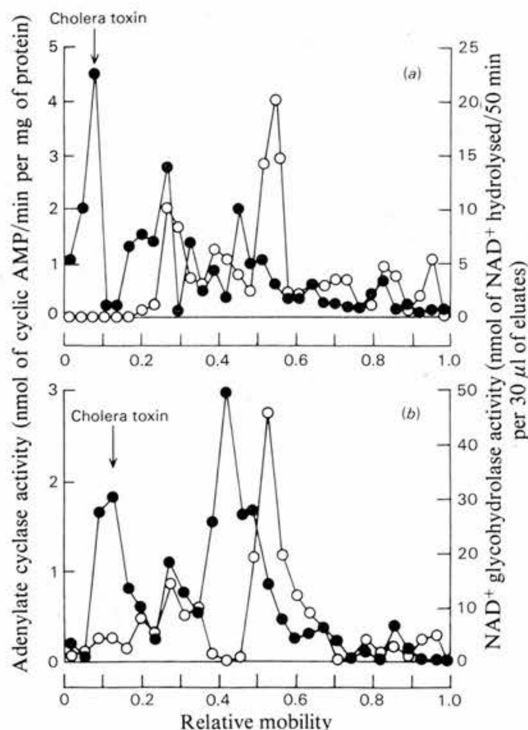

Fig. 1. *Activity of material eluted from polyacrylamide gels*
Purified (*a*) or crude (*b*) cholera toxin was subjected to polyacrylamide-gel electrophoresis at pH 8.3. Slices from each gel were immersed in water and the eluted protein was assayed for NAD^+ glycohydrolase activity (○) and for activation of adenylate cyclase in rat liver membranes (●) as described in the text.

amounts of material in each gel-slice eluate, but in a separate experiment the $V_{max.}$ of this NAD^+ glycohydrolase (partially purified) was found to be in the region of 3000–4000 nmol of NAD^+/min per mg of protein. In the pure-toxin gel in which only one band was visible on protein staining, that of cholera toxin, some of this NAD^+ glycohydrolase activity has persisted (mobility 0.5–0.6 in Fig. 1*a*). Thus although the purified material gives only one band on protein staining with a characteristic mobility for cholera toxin, the toxin is evidently not pure, and contains a significant amount of contaminating NAD^+ glycohydrolase activity.

A similar gel of cholera toxin, prepared by the method of Finkelstein & LoSpalluto (1970), gave similar results showing more than one band of adenylate cyclase-activating activity, but in this material the NAD^+ glycohydrolase activity was lower.

The peak of adenylate cyclase-activating activity at mobility about 0.4 in both gels may be due to free subunit A of the toxin. A similar gel of subunit A alone (purified by the method of van Heyningen, 1974) gave one band running in this position. Evidently the majority of this material has been removed from the toxin during purification. The other minor peaks of adenylate cyclase-activating activity, particularly on the gel of the purified toxin, may be due to small proteolytic fragments of cholera toxin, which are still capable of activating adenylate cyclase. Such fragments have been observed previously (Matuo *et al.*, 1976).

In neither of the two gels does the position of cholera toxin, nor the putative position of free subunit A, coincide with any appreciable NAD^+ glycohydrolase activity. Rather, this latter is present only as a contaminant clearly separable from the peaks of adenylate cyclase-activating activity.

One possible reason for differences between our results and those of other workers (Moss *et al.*, 1976, 1977; Moss & Vaughan, 1977), may be that their experiments were done in 0.2–0.4 M-phosphate buffer at pH 7.0. At this pH, even small concentrations of phosphate are known to catalyse the non-specific breakdown of NAD^+ to ADP-ribose and nicotinamide (Colowick *et al.*, 1951). In our experiments the use of such phosphate buffers resulted in as much NAD^+ hydrolysis whether cholera toxin was present in the assay mixtures or not. Acetate buffer at pH 6.2, which does not lead to extensive NAD^+ breakdown, was therefore used in the experiments described here.

Although NAD^+ is a strict requirement for adenylate cyclase activation by cholera toxin, the results presented in this paper do not support the hypothesis that cholera toxin (and specifically subunit A) acts as an enzyme directly catalysing the transfer of ADP-ribose at least to water. It remains possible that

the ADP-ribosylation of some other acceptor is part of the normal mechanism of the toxin.

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