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Recognition

Series B Volume 1

The Specificity and Action of Animal, Bacterial and Plant Toxins

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Receptors and Recognition

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About the series

Cellular recognition – the process by which cells interact with, and respond to, molecular signals in their environment – plays a crucial role in virtually all important biological functions. These encompass fertilization, infectious interactions, embryonic development, the activity of the nervous system, the regulation of growth and metabolism by hormones and the immune response to foreign antigens. Although our knowledge of these systems has grown rapidly in recent years, it is clear that a full understanding of cellular recognition phenomena will require an integrated and multidisciplinary approach.

This series aims to expedite such an understanding by bringing together accounts by leading researchers of all biochemical, cellular and evolutionary aspects of recognition systems. The series will contain volumes of two types. Firstly, there will be volumes containing about five reviews from different areas of the general subject written at a level suitable for all biologically oriented scientists (Receptors and Recognition, series A). Secondly, there will be more specialized volumes, (Receptors and Recognition, series B), each of which will be devoted to just one particularly important area.

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Preface

Although throughout the history of medicine toxic substances have been of great concern to scientists and society, it has only been in recent years that the primary scientific emphasis toward naturally occurring toxins has shifted from the concern for health, pathology and clinical management to their use as powerful tools for the study of complex biological phenomena. This volume deals with recent advances in our understanding of the mechanism of action of various classes of extraordinarily potent 'toxic' substances which exist in nature. The primary orientation of this volume and the studies it describes is not toward understanding the traditionally 'toxic' nature of these substances, in the sense of causing disease, but rather toward the use of these substances to probe and elucidate fundamental processes in biology. Thus, although the substances described are indeed powerful 'toxins', it is not their 'toxic' properties *per se* which are of primary concern here.

A powerful 'poison' is by definition a substance which has potent biological activity in miniscule quantities. It follows therefore that its action must be highly *selective* and of *high affinity*, and that it must involve crucial and essential functions of cells and tissues. These features predict the potential value of toxins in probing receptors and important biological interactions and regulatory functions of cells. The topics described in this volume illustrate vividly and with certainty the extraordinary investigational utility of several such toxins. We are now seeing the realization of Claude Bernard's vision, expressed in 1875 in his classic work, *Experimental Science*, when he said that to the physiologist ' . . . the poison becomes an instrument which dissociates and analyzes the most delicate phenomenon of living structures, and by attending carefully to their mechanism in causing death, he can learn indirectly much about the physiological processes of life.'

Rather than trying to cover exhaustively (but, necessarily, superficially) the wide and enormous spectrum of naturally occurring toxic substances, this volume presents highly detailed and critical analyses of a relatively small number of representative classes of the better studied and understood toxins. In all cases presented, substantial information exists concerning the chemical structure of the toxin as well as the pharmacologic, physiologic and biochemical bases of action, and understanding of the latter has already shed new insights into normal control mechanisms. The protein toxin elaborated by *Vibrio cholerae* (choleragen) stimulates ubiquitously the adenylate cyclase activity of eukaryotic plasma cell membranes by mechanisms relevant to the normal processes by which natural hormones and regulatory substances modulate the activity of

this important enzyme system. The extraordinary selectivity of its action is initiated by interaction with a highly specific, chemically defined receptor known to be a cell membrane glycolipid (ganglioside). Other protein toxins described (diptheria, *Pseudomonas,* abrin and ricin) present some interesting and provocative analogies with choleragen in that the molecular region of the toxin molecule involved in recognition and binding to cell surface receptors is structurally distinct (i.e., different subunits) from that portion which endows the molecule with the ability to subsequently exert specific biological effects. Despite these general similarities, important and illuminating differences exist. The specific receptors for all these toxins are different and unique, and their precise loci of action are different.

Although the primary action of several of the toxins (diptheria, *Pseudomonas,* ricin, abrin and colicin E_3) described appears to be inhibition of protein synthesis, different mechanisms of achieving this exist. In all cases, however, special mechanisms exist for translocating the toxin molecule, or a portion thereof, to the interior of the cell where protein synthesis is interrupted by catalytic, toxin-directed mechanisms. Not only have these studies shed light on important features of the regulation of protein synthesis, but they also have important implications for the structure and permeability of cell membranes and the possible mechanisms by which certain macromolecules may normally traverse these seemingly impervious cell barriers.

The target cell specificity of some potent toxins is also illustrated by the exquisite selectivity of tetanus toxin for the central nervous system, where it affects certain types of inhibitory synapses. The mechanism by which this toxin ascends from the nerve terminals up the spinal cord, and by which it subsequently interacts with receptors and interrupts function, will no doubt provide important new insights into fundamental functions of the central nervous system. Botulinum toxin and β-bungarotoxin (from snake venom) are also proteins with selectivity for nervous tissues, but in their case the principal action is at peripheral cholinergic nerve endings where neuromuscular transmission is affected. Both of these toxins act presynapticly (but by different mechanisms) to interfere with the normal release of acetylcholine, whereas α-bungarotoxin blocks neuromuscular transmission by binding to and thus inactivating the acetylcholine receptor of the post-synaptic membrane. The action of α-bungarotoxin will be considered separately and in depth in a chapter dealing with acetylcholine receptors in a forthcoming volume of this series, Receptors and Recognition, (M.E. Eldefrawi and A.T. Eldefrawi in Series A, Vol. 4)

The only non-protein toxic substances considered in this volume are the animal steroidal alkaloids, which demonstrate highly selective modulation of ion transport mechanisms and have thus been of invaluable assistance in the study of the structure-function relationships of electrogenic membranes.

An important class of protein toxins, the cytolytic bacterial toxins, exert their primary effect by physical or chemical dissolution of eukaryote and

prokaryote cell membranes. This fascinating but heterogeneous group of toxin molecules disrupts membrane function by acting as surfactants, enzymes (i.e., hydrolysis of lipids or proteins) or by binding to specialized membrane molecules. These toxins have served as excellent probes for elucidating biomembrane organization and function, and for studying factors that control membrane integrity, permeability and function.

June, 1976 *Pedro Cuatrecasas*

Contents

1 Cholera Toxin: Membrane Gangliosides and Activation of Adenylate Cyclase

V. BENNETT *and* P. CUATRECASAS

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1. Abbreviations used: cyclic AMP: 3′, 5′ adenosine monophosphate; ACTH: adrenocorticotropic hormone; gangliosides are referred to by the nomenclature of Svennerholm (1963).
2. Estimates of molecular weights of the toxin and its component subunits vary depending on the laboratory. The values used here represent average of those quoted in the literature.

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1.1 INTRODUCTION

Asiatic cholera is a disease caused by the local action of a specific bacterial exotoxin on the small intestine and is characterized by a profuse outpouring of fluid at rates up to 20 liters per day (Pierce *et al.* 1971). The victims rapidly exhibit symptoms of hypovolemic shock, and if unassisted, may succomb within 8 hours of the onset of diarrhea. One of the earliest descriptions of cholera by a European records epidemics near Goa in 1503 and 1545:

> . . . so grievous was the throe and of so bad an aspect that the very worst poison seemed there to take effect as proved by vomiting and cramps that fixed the sinew to the joints . . . the eyes dimmed to sense . . . and the nailes of the hands and feet black and arched. (Pollitzer, 1959).

Cholera was confined primarily to Asia until the nineteenth century when the disease erupted in a series of pandemics which involved most of the countries in Europe as well as the United States and several South American nations (Pollitzer, 1959; Rosenberg, 1969). The disease lessened or disappeared by 1923, and did not spread beyond Asia again until 1947 when a localized epidemic broke out in Egypt. In 1959, however, an outbreak of cholera began in Thailand and expanded into a major pandemic involving 42 countries by 1971 (Finkelstein, 1973). The present international spread of cholera is continuing, and it is anticipated that South America may soon become infected (Finkelstein, 1973; Goodgame and Greenough, 1975).

The recent epidemics have been credited with renewing the scientific interest in cholera (Finkelstein, 1973), and may be indirectly responsible for the significant advances in the understanding of the molecular basis of this disease in the last few years. The purification of a protein exotoxin which completely reproduced the cholera syndrome (Finkelstein and LoSpalluto, 1969; Richardson and Noftle, 1970), and the exciting discovery of the role of cyclic AMP[1] and activation of adenylate cyclase in the pathogenesis of cholera (Field, 1971; Sharp and Hynie, 1971; Kimberg *et al.*, 1971), provided a sound basis for the work reviewed in this chapter. Direct measurements of the interaction of cholera toxin with cell surfaces have since led to the elucidation of the chemical nature of the membrane receptor for the toxin, GM_1 monosialoganglioside, a molecule which is present in the plasma membrane of all eukaryotic cells and which may be involved in very distinct processes such as contact inhibition and viral transformation in tissue culture systems (see below).

The biological effects of cholera toxin are not restricted to stimulation of secretion in the intestine, but have been observed in diverse tissues, and in all cases

Table 1.1 Biological effects of choleragen

Effects	Reference
↑ intestinal secretion in many species	Sharp (1973)
↑ lipolysis in rat fat cells	Vaughan *et al.* (1970)
↓ insulin-stimulated glucose oxidation in rat fat cells	Hollenberg and Cuatrecasa (1975)
↑ pancreatic electrolyte secretion in cats and rats	Case and Smith (1975); Kempen *et al.* (1975)
↑ glycogenolysis in liver and platelets	Zieve *et al.* (1970)
↑ hormone release from cultured rat pituitary cells	Rappaport and Grant (1974)
↑ steroid secretion in cultured mouse adrenal cells, rat testis, and rat adrenal cells	Donta *et al.* (1973); Wolff *et al.* (1973); Sato *et al.* (1975); Haksar *et al.* (1975)
↓ fluid absorption in rabbit gall bladder	Mertens *et al.* (1974)
↑ alkaline phosphatase activity in rat liver	Baker *et al.* (1973)
mimics dopamine in CNS of rats	Miller and Kelly (1975)
↓ IgE-mediated release of histamine from human leukocytes and ↓ cytolytic activation of mouse lymphocytes	Lichtenstein *et al.* 1973)
↓ DNA and RNA biosynthesis in human fibroblasts, mouse spleen lymphocytes, human peripheral lymphocytes, mouse thymocytes and transformed mouse epithelial cells	Hollenberg and Cuatrecasas (1973); Sultzer and Craig (1973); DeRobertis *et al.* (1974); Holmgren *et al.* (1974); Hollenberg *et al.* (1974)
↓ expression of F_c receptors in mouse lymphocytes	Zuckerman and Douglas (1975)
↑ tyrosinase activity in cultured mouse melanocytes	O'Keefe and Cuatrecasas (1974)
modulation of *in vivo* immune response in mice	Henney *et al.* (1973): Northrup and Fauci (1972); Chisari *et al.* (1974); Kately *et al.* (1975)
modulation of *in vitro* early anamnestic antibody response in rabbits	Cook *et al.* (1975)
alters T-cell helper function in mice	Kately and Friedman (1975)
↓ lymphocytes, ↑ corticosterone levels, ↑ serum calcium, ↑ serum glucose, ↓ liver glycogen following intravenous injection	Morse *et al.* (1975); Chisari and Northrup (1974); Hynie *et al.* (1974)
↑ serotonin N-acetyltransferase and cyclic AMP phosphodiesterase activities in cultured pineal glands of rats	Minneman and Iversen (1976)

they mimic the action of cyclic AMP (Table 1.1). Cholera toxin also activates adenylate cyclase ubiquitously, and recent studies of the mechanism of stimulation indicate important similarities to the regulation of cyclase by naturally occurring hormones. Thus, cholera toxin, at one time of interest to a selected group of clinicians and microbiologists, now appears to be a potent new tool for investigation of membrane structure and function.

1.1.1 Pathogenesis of cholera and purification of cholera toxin

Vibrio cholerae, the bacterium responsible for the symptoms of clinical cholera, was isolated by Pacini in 1854 (Hugh, 1964) and by Koch in 1884, and was found to be a motile, comma-shaped, gram negative rod. Koch proposed (1884) that the vibrios cause disease by secretion of a soluble exotoxin in analogy to the formation of tetanus and botulinum toxins. Koch speculated further that the cholera toxin necrotized the intestinal epithelium and induced a systemic intoxication.

Unfortunately, the erroneous concepts were accepted that the pathogenesis of cholera requires damage to the intestinal epithelium and that extraintestinal tissues play an important role in the disease, while Koch's suggestion of an exotoxin was abandoned until 1960. Various attempts were made between 1885 and 1950 to isolate a toxin, which was felt to be derived from the bacterial cell wall or cytoplasmic membrane (Pollitzer, 1959). These investigations were severely hampered, however, by the use of animal models which were designed on the assumption that cholera symptoms would be elicited by the systemic application of bacterial products. De and Chatterje (1953) reintroduced the rabbit ileal loop model (Viole and Crendiropoulo, 1915), in which the ileum was ligated at intervals and cholera vibrios were injected, with a resulting outpouring of fluid similar to that observed in the disease. Dutta and Habu (1955) revived the suckling rabbit system of Issaeff and Kolle (1894) which involved administration of bacteria to young rabbits which subsequently developed diarrhea. These were simple and valid bioassay systems which permitted a rational assessment of the factors involved in the disease. De (1959) and Dutta *et al.* (1959) reported that bacteria-free culture filtrates were enterotoxic, thus implicating some soluble factor. The leading candidates for a toxin material included a mucinase activity detected in culture filtrates (Burnet and Stone, 1947), a hemolytic activity (Koch, 1887; Shottmuller, 1904), and a poorly defined endotoxin (Pollitzer, 1959). De *et al.* reported in a seminal paper (1960) that the symptoms of cholera could be elicited with a heat-labile factor, distinct from mucinase or hemolytic activities, which was clearly not an endotoxin. Also, at this time, the misconception that the disease involved gross anatomical damage to the intestinal epithelium, which had been refuted in an overlooked study in 1923 (Goodpasture), was re-evaluated by Gangarosa and co-workers. No detectable intestinal epithelial mucosal cell lesions were observed in ileal biopsies from cholera victims (Gangarosa *et al.*, 1960), a finding subsequently confirmed (Formal *et al.*, 1961; Fresh *et al.*, 1964). Furthermore, Gordon (1962) found that intravenously administered

^{131}I-polyvinylpyrrolidone was retained during cholera, and thus extended the concept of an intact eipthelium to include the vasculature as well. These findings also strongly suggest that the hemolytic and mucinase activities were not significant in production of diarrhea, and provided a rational basis for the purification of the exotoxin postulated by De *et al.* (1960).

The impetus for the isolation of the toxin was provided by Finkelstein, who developed a simple growth medium for production of a 'choleragen' (Finkelstein *et al.*, 1964) which produced clinical cholera in humans (Benyajati, 1966). Enterotoxic activity was subsequently separated from endotoxin by gel filtration (Finkelstein *et al.*, 1966a, b) and the toxin was eventually isolated in a highly purified form (Finkelstein and LoSpalluto, 1969). An immunologically identical protein was also purified which was biologically inactive, and termed choleragenoid. Subsequent studies demonstrated that choleragenoid was derived from choleragen (Finkelstein *et al.*, 1971). Pure choleragen was described as a protein of about 84 000 MW which contained no detectable lipid or carbohydrate (LoSpalluto and Finkelstein, 1972) and did not exhibit the following enzymatic activities: neuraminidase, protease, elastase, lipase, lecithinase, hyaluronidase, chondroitin sulphatase, DNase, RNase, mucinase, or staphylolytic enzymes (unpublished data cited by Finkelstein, 1973). Technical aspects of the purification were improved (Finkelstein and LoSpalluto, 1970) and choleragen and choleragenoid have since been crystallized (Finkelstein and LoSpalluto, 1972). Significant contributions to the techniques of large-scale purification have been made by other workers (Richardson and Noftle, 1970; Spyrides and Feeley, 1970; Rappaport *et al.*, 1974). Affinity adsorbents have been developed which quantitatively adsorb choleragen and choleragenoid, and may provide a single-step method of purification (Cuatrecasas *et al.*, 1973).

1.1.2 Discovery of activation of adenylate cyclase by choleragen

The availability of pure preparations of choleragen made possible unambiguous studies on its mechanism of action at the molecular level. Field and co-workers (1968a, b) reported that addition of cyclic AMP to the basal surface of isolated ileal mucosa rapidly induced a large increase in the short circuit current which then gradually declined. After 30 to 60 min the net sodium flux in the absence of glucose fell to zero, and the direction of chloride transport was reversed such that Cl^- was actively secreted from the mucosal side of the preparation. These effects could quite conceivably cause a passive eflux of water, and thus diarrhea. The potential significance of these findings was quickly realized, and it was soon reported that dialysed filtrates of *Vibrio cholerae* cultures could mimic exactly the effects of cyclic AMP (Field *et al.*, 1969). Further evidence for a possible role of cyclic AMP in the pathogenesis of cholera was provided by the demonstration that injection of prostaglandins or theophylline into the superior mesenteric artery of dogs caused diarrhea (Pierce *et al.*, 1970).

It was concluded that active secretion of chloride ion into the intestinal lumen

could explain the loss of fluid during cholera, and that choleragen acted via some mechanism related to cyclic AMP (Field, 1971). It was subsequently discovered that choleragen was also a potent activator of lipolysis in isolated rat fat cells (Vaughan *et al.*, 1970; Greenough *et al.*, 1970), and also that it stimulated glycogenolysis in liver and platelets (Zieve *et al.*, 1971). Choleragen has since been found to mimic the biological effects of cyclic AMP in every tissue so far examined.

Choleragen was subsequently demonstrated to increase the tissue content of cyclic AMP (Shafer *et al.*, 1970), and to stimulate adenylate cyclase activity in intestinal mucosa with no demonstrable effect on cyclic AMP phosphodiesterase activity (Sharp and Hynie, 1971; Kimberg *et al.*, 1971). Choleragen was unusual in that it was effective only if incubated with intact cells, and activation occurred after a latency phase of 15 to 60 min. Stimulation of adenylate cyclase by choleragen, once obtained, persisted in washed membrane preparations. Choleragen was interpreted as acting on the existing adenylate cyclase molecules rather than stimulating *de novo* synthesis of the enzyme since fluoride ion activated the toxin-strimulated cyclase activity to a greatly reduced extent compared to that of the control preparation (Sharp and Hynie, 1971; Kimberg *et al.*, 1971). Measurements of adenylate cyclase activity in biopsy tissue from humans during the diarrheal phase of cholera revealed a similar activation as obtained in animal models, which decreased in the convalescent period (Chen *et al.*, 1971, 1972). Guerrant *et al.*, (1972) analyzed carefully the relationships between the net water and sodium fluxes and the stimulation of adenylate cyclase by choleragen in the small bowel, and obtained a good correlation between the time course and extent of cyclase activation and the changes in secretion.

Considerable evidence has thus accumulated that the clinical symptoms of cholera could be explained solely by stimulation of the adenylate cyclase activity of the intestinal mucosa by choleragen (reviewed by Sharp, 1973). Activation of adenylate cyclase by choleragen has also been demonstrated in such diverse tissues as rat (Beckman *et al.*, 1974; Bennett *et al.*, 1975a) and mouse (Gorman and Bitensky, 1971), liver, human neutrophils (Bourne *et al.*, 1973), cultured mouse adrenal cells (Wolfe *et al.*, 1973; Donta *et al.*, 1973), and melanoma cells (O'Keefe and Cuatrecasas, 1974), erythrocytes of turkeys (Field, 1974; Bennett and Cuatrecasas, 1975b), pigeons (Gill and King, 1975), rats (Bennett and Cuatrecasas, 1975a), and toads (Bennett and Cuatrecasas, 1975b), as well as many others (Table 1.2). As a universal activator of adenylate cyclase, choleragen assumes general importance as a means of elucidating the diverse biological effects of cyclic AMP, and as a potential tool in deciphering the mechanism of regulation of this enzyme.

1.1.3 Subunit structure of choleragen

The first study on the subunit structure of choleragen (LoSpalluto and Finkelstein, 1972) reported that both toxin and its biologically inactive derivative, choleragenoid, dissociated reversibly to 15 000 MW subunits in the presence of mild acid, as