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The Cell Envelope of Gram-Negative Bacteria: New Aspects of Its Function in Transport and Chemotaxis

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1 Introduction

1.1 Evolutionary Significance of the Cell Envelope

The appearance of protein synthesis controlled by nucleic acids was one of the crucial events in prebiotic evolution. Such systems, able to perform metabolism and replication of macromolecules, existed long before the first cells. We sometimes overlook the evolutionary significance of the inception of membranes. The evolution of nucleic acids coding for the primitive enzymes was extremely slow in prebiotic times before development of membranes. If a variant of nucleic acid (RNA) arose that made a superior type of enzyme, the new enzyme could not selectively contribute to the replication of the new RNA in its competition with the neighboring old RNA copies. Sequestering of macromolecules within a cell envelope greatly accelerated evolution because it linked selective propagation of this new, advantageous RNA much more tightly to the function of the superior gene product (EIGEN et al. 1981).

All present-day cells are surrounded by membranes composed of phospholipids and proteins. There are no fossil records that trace the origins of the first cell. Important evolutionary steps towards optimized cell envelopes and towards optimized cellular metabolism are still conserved in present day organisms. Fascinating aspects of microbial evolution were reviewed by WOESE (1981). About 20 years ago almost no information was available on the natural relationship among bacteria. Today, as reviewed by SCHLEIFER and STACKEBRANDT (1983), the fundamentals of a phylogenetic tree have been established by comparative nucleic acid sequencing, reassociation experiments, and immunological methods.

1.2 Regulatory Significance on the Cell Envelope

The cell envelope of microorganisms functions as a barrier between the outer environment and the cytoplasm. In contrast to the cellular environment in multicellular organisms, the environment of microorganisms changes rapidly and contains the necessary metabolites and ions in very low concentrations (10^{-6} *M* and below). The microbial cell envelope controls the cellular concentrations of the essential metabolites and ions via specific transport systems. Transport of exogenous solutes is the first step in regulation of the cellular catabolic and anabolic pathways. Therefore transport is sometimes referred to as the pacemaker of metabolic pathways.

Distinct mechanisms for regulation of uptake in bacteria are known (DILLS et al. 1980). The expression of most transport systems is regulated at the level of transcription of the genes encoding the system. Induction or derepression is observed only in situations in which the presence or absence of a solute demands a new transport activity. Induction of the maltose transport system by maltose (see Sect. 5.5) and derepression of the *pho* regulon-dependent *ugp* transport system (for *sn*-glycerol-3-phosphate uptake) upon phosphate limitation (see Sect. 3.5) are good examples of this principle.

If two substrates are present at the same time, the bacterial cell has a strict order in which the substrates are used. Thus, if glucose is present, expression of other systems is repressed via the decrease of the cellular concentration of the second messenger cAMP (see Sect. 5.5). Even the activity of existing transport systems is inhibited. This inhibition (inducer exclusion) is probably due to an action of a protein involved in the transport of glucose (the enzyme III^{glc}, product of the gene *crr*) on a variety of specific transport components.

Intracellular sugar concentrations are modulated by substrate exit mechanisms. Recent evidence suggests that a regulatory protein of the bacterial phosphotransferase system (PTS) catalyzes the ATP-dependent phosphorylation of the low molecular weight, heat stable protein HPr, thereby controlling sugar accumulation by exclusion and/or expulsion processes (REIZER et al. 1985).

1.3 Structure of the Cell Envelope in Gram-Negative Bacteria

The cell envelope of gram-negative bacteria is composed of three layers or compartments: (a) an outer membrane, (b) the periplasmic space containing soluble proteins and the peptidoglycan layer, and (c) the inner or cytoplasmic membrane. A schematic diagram shows the organization of the cell envelope of gram-negtive bacteria (Fig. 1, adapted from a recent review of LUGTENBERG and VAN ALPHEN 1983).

In enteric gram-negative bacteria, which live in the intestinal tract of animals, the outer membrane has developed into a very effective barrier, protecting cells from the detergent action of bile salts and degradation by digestive enzymes (reviewed by NIKAIDO and VAARA (1985). Besides its protective function, the outer membrane provides the cell with an additional compartment, the so-called

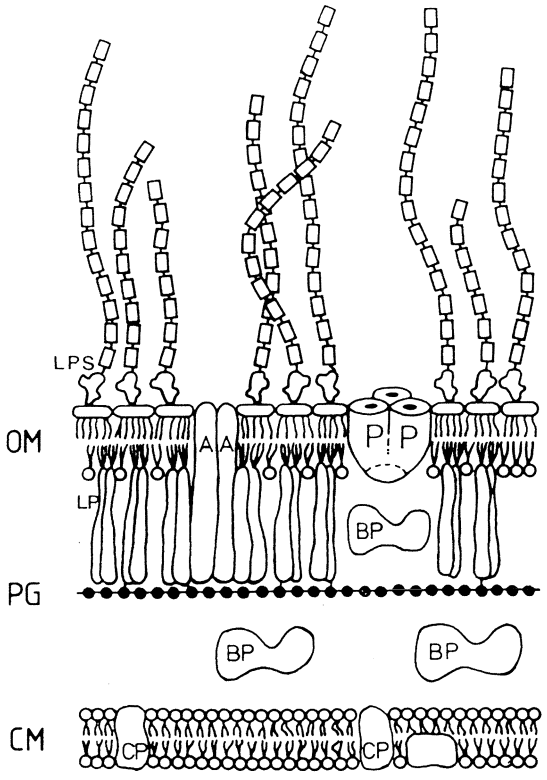

Fig. 1. Organization of the cell envelope of *Escherichia coli*. The cytoplasmic membrane (*CM*) containing carrier proteins (*CP*) involved in active transport, the rigid peptidoglycan layer (*PG*) and the outer membrane (*OM*) are indicated. The asymmetric composition of the *OM* (lipopolysaccharide (*LPS*) in the outer leaflet, phospholipid predominantly in the inner leaflet) is shown. Divalent cations (not indicated) are supposed to play important roles in LPS interaction, and thus in permeability barrier functions of the outer membrane. Three types of OM proteins are shown: OmpA protein (*A*), lipoprotein (*LP*), and porin protein (*PP*), the last being composed of three subunits with a triplet inlet converging to a single outlet (DORSET et al. 1984). The periplasm, the space between *OM* and *CM*, contains a variety of soluble binding proteins (*BP*) which are involved in transport of nutrients. Some binding proteins, such as the maltose binding protein (MBP), also function as chemoreceptors for these nutrients. MBP and other binding proteins which have been studied in detail show a pronounced two-domain protein structure (QUIOCHO et al. 1979). For further details see text. (Adapted from LUGTENBERG and VAN ALPHEN 1983)

periplasmic space between inner and outer membrane. This membrane-bounded compartment harbors a variety of soluble proteins and the rigid peptidoglycan layer. Peptidoglycan is responsible for the maintenance of cell shape and for the ability of the cell to withstand the very high internal osmotic pressure in dilute environments. Morphology is dependent on the proper interplay of outer membrane and growing peptidoglycan.

The cytoplasmic membrane is the osmotic barrier of the cell. Integrated into this membrane are most of the carrier proteins which are responsible for accumulation of substrates in the cytoplasm. In addition, the cytoplasmic membrane contains some of the proteins necessary for transduction of chemotactic

signals. It also contains the bacterial respiratory chain as well as most of the enzymes necessary for phospholipid and peptidoglycan synthesis.

2 Molecular Architecture of the Outer Membrane

2.1 Lipid Components

The outer membrane is about 75 Å thick (GLAUERT and THORNLEY 1969) and appears to be a planar lipid bilayer. The lipid constituents of the outer membrane are asymmetrically localized (Fig. 1). Lipopolysaccharide (LPS) is found exclusively in the outer leaftlet of this membrane. Phospholipids [90% phosphatidylethanolamine (LUGTENBERG and PETERS 1976) and small amounts of phosphatidylglycerol and diphosphatidylglycerol] are found only in the inner leaflet of the membrane. Interestingly, this asymmetry is dependent on the presence of an intact peptidoglycan layer in the periplasm, since degradation of peptidoglycan with lysozyme results in a redistribution of LPS within both leaflets (TAKEUCHI and NIKAIDO 1981). Phospholipids and LPS isolated from *Escherichia coli* do not mix and persist in separate domains.

LPS from different gram-negative bacteria have attracted interest because of their role in assembly and maintenance of the outer membrane permeability barrier (LEIVE 1965; reviewed in NIKAIDO and VAARA 1985), their suggested role in correct localization of outer membrane proteins during secretion (DATTA et al. 1977; see however, Sect. 5.7), and their importance for the biological function of most outer membrane proteins (reviewed in NIKAIDO and VAARA 1985), as well as their function as phage receptors (reviewed in BRAUN and HANTKE 1981), and their endotoxic effects (WESTPHAL and LÜDERITZ 1954).

LPS isolated from various enterobacterial species consist, as the name implies, of a polysaccharide and a lipid A moiety. The hydrophobic lipid A is a phosphorylated and extensively acylated glucosamine disaccharide (see Fig. 2). Most of the fatty acids in lipid A are short (14–16 carbon atoms) and saturated. Some are branched and carry hydroxyl groups (RIETSCHEL et al. 1972). The hydrophilic polysaccharide chain of *E. coli* K12 is shown in Fig. 3. Lipid A and the proximal sugar residues of the polysaccharide chain (the 3-deoxy-D-manno-octulosomic acid residues) carry a large number of predominantly negatively charged residues which are responsible for binding of divalent cations. High affinity (μM range) and a low affinity (mM range) binding site for Mg^{2+} or Ca^{2+} have been characterized by fluorescence quenching of dansylated LPS (SCHINDLER and OSBORN 1979). Recent work (STRAIN et al. 1983) attributes the high affinity calcium binding site to the glycoside diphosphate moiety of lipid A (Fig. 2). Binding of divalent cations to LPS neutralizes repulsion between the anionic groups and permits a tightly folded conformation of lipid A and the core saccharides. The importance of metal ion crossbridges beween LPS molecules is demonstrated by the loss of LPS and decrease in permeability barrier function of the outer membrane after treatment of cells with ethylenediaminetetraacetate (EDTA) (LEIVE 1965; see Sect. 6.1). Mutants with shortened polysaccharide side chains (rough mutants; see Fig. 3) show an increased permeability of the outer membrane to hydrophobic compounds (see Sect. 2.3).

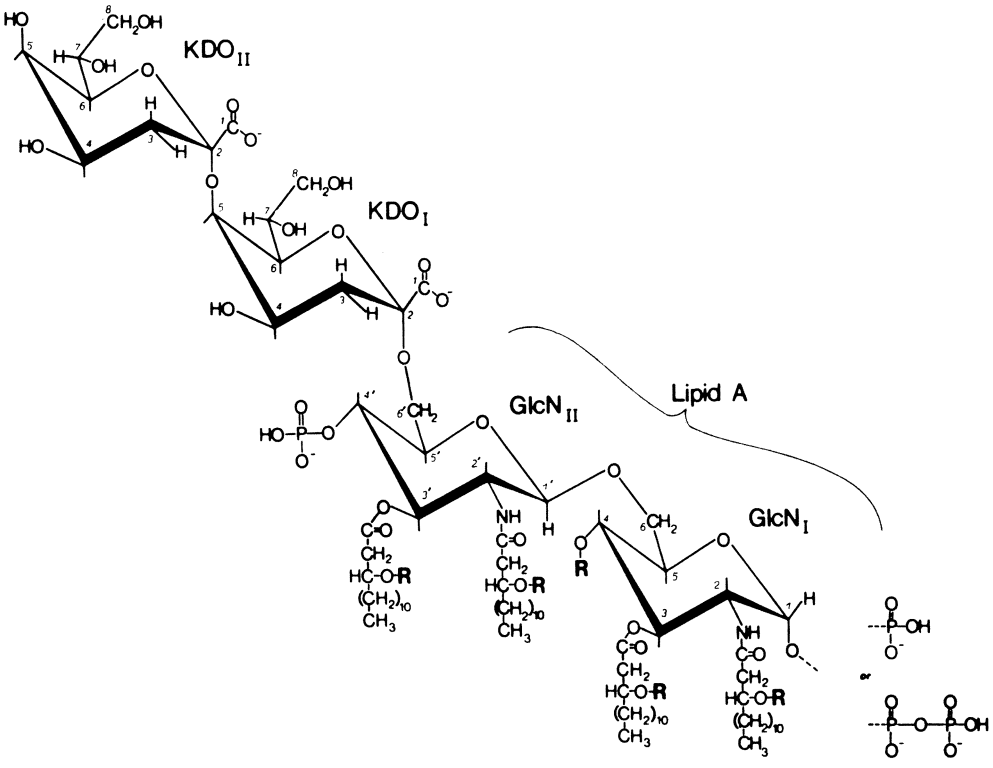

Fig. 2. Lipopolysaccharide of *E. coli*. Structure of lipid A and the negatively charged inner core KDO sugars. *GlcN*$_{I}$ and $_{II}$, glucosamine; *R* and *H*, lauroyl and myristoyl or D-3-hydroxymyristoyl; *KDO*$_{I}$ and $_{II}$, 2-keto-3-deoxyoctulosomic acid. (Reprinted with permission of STRAIN et al. 1983)

2.2 Outer Membrane Proteins

At first glance, the protein composition of the outer membrane appears relatively simple compared to the complex inner membrane. It is dominated by the presence of four to six very prominent proteins, which are expressed at high copy numbers (about $1–2 \times 10^5$/cell). After separation of outer membrane proteins of *E. coli* K12 according to their apparent molecular weight in urea-containing polyacrylamide (PUGSLEY and SCHNAITMAN 1978), four prominent outer membrane proteins can be easily identified by the use of specific mutants lacking one or more of these proteins (Fig. 4). If sensitive methods for detection are used (antibodies, phages, colicins, etc.) many more outer membrane proteins can be detected. These are expressed at lower copy number (about $1 \times 10^3–1 \times 10^4$/cell). Table 1 lists the functions and the regulatory pattern of some of the outer membrane components. Regulation of expression is observed in both high and low copy number proteins. The outer membrane is poor in enzymatic activities. The presence of different lipases, proteases, and a few other enzymes was, however, reported (reviewed in LUGTENBERG and VAN ALPHEN 1983).

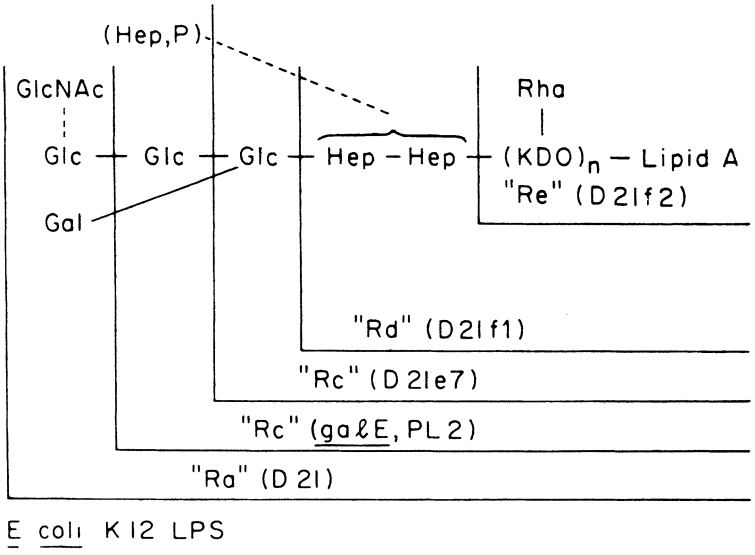

Fig. 3. Oligosaccharide chains of wild-type *E. coli* K12 LPS and LPS mutants. *GlcNAc,* N-acetyl-D-glucosamine; *Glc,* glycose; *Gal,* D-galactose; *Hep,* L-glycero-D-manno-heptose; *EtN* ethanolamine; *P,* phosphate; *KDO,* 2-keto-3-deoxyoculosomic acid. *Ra-Re* refer to the chemotype of the mutant LPS. (Reprinted with permission of the review of NIKAIDO and VAARA 1985)

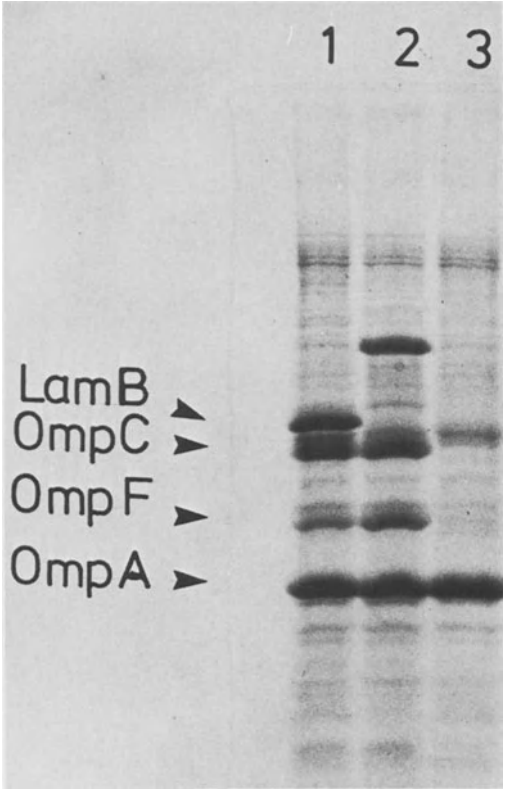

Fig. 4. The major outer membrane proteins in wild-type and porin mutants of *E. coli.* Washed cell envelopes from 2×10^8 broken cells (containing inner and outer membranes) were separated on urea containing polyacrylamide gels (for details see BRASS et al. 1984). The positions of the major outer membrane proteins *OmpA, OmpC, OmpF* and *λ-receptor* (maltoporin) are indicated. *Lane 1,* wild-type LA5612 (*malT^c-1* λ receptor constitutively expressed); *lane 2,* JB42 (*lamB102 malT^c-1*; λ receptor missing) (band above λ-receptor probably flagellin); *lane 3,* JB107 (*ompR lamB102 malT^c-1*; λ receptor and OmpF and OmpC porins missing). (Modified from BRASS et al. 1985)

Table 1. Functions and expression of outer membrane components of *E. coli* K12. (Adapted from LUGTENBERG 1981)

Outer membrane constituent	Apparent mol wt	Number of molecules per cell	Structural gene	Conditions for induction or derepression	Part of receptor for phage/colicin	Function
Lipopolysaccharide	4500	3.4×10^6	–	Constitutive	T3, T7, C21, P1	Stabilization of outer membrane structure; protection against bile salts; required for biological activity of several proteins; specific role in acceptor cell in stabilization of mating aggregates in I-pilus-mediated conjugation
Lipoprotein	7200	Bound form 2.5×10^5; free form 5×10^5	*lpp*, min 36.3	Constitutive		Anchoring outer membrane to peptidoglycan; influence on cell shape; stabilization of outer membrane structure
OmpA protein	35159 (boiled)	10^5	*ompA*, min 21.5	Constitutive	K3, TuII*	Role in acceptor cell in stabilization of mating aggregates in F-pilus mediated conjugation; influence on cell shape; requires LPS for biological activity
OmpC porin	36000	Up to 10^5	*ompC*, min 47.1	Preferentially expressed in media of high osmolarity	TuIb, Mel, T4, PA-2 434	General pore for hydrophilic solutes with a mol wt up to about 700; requires LPS for biological activity
OmpF porin	37205	Up to 10^5	*ompF*, min 20.7	Preferentially expressed in media of low osmolarity	TuIa, T2, TP1	General pore for hydrophilic solutes with a mol wt up to about 700; requires LPS for biological activity
PhoE porin	40000	$1–2 \times 10^5$	*phoE*, min 5.9	Phosphate limitation	TC23, TC45	General pore for hydrophilic solutes; preference for anionic solutes; recognized by polyphosphate; probably required for uptake of polyphosphate
Maltoporin	50000	$1–2 \times 10^5$	*lamB*, min 91.0	Presence of maltose	λ, K10, TP1	Pore for small hydrophilic solutes; recognized by maltose and maltodextrins, required for uptake of higher maltodextrins
Tsx protein	28000	$\sim 10^4$	*tsx*, min 9.2		T6, ColK	Pore for nucleosides
BtuB protein	60000		*btuB*, min 89.0	Vitamin B_{12} limitation	BF23, E. colicins	Uptake vitamin B_{12}
Cir protein	74000		*cir*, min 44	Fe^{3+} limitation	ColI, ColV	Uptake complexed Fe^{3+}
TonA protein	78000		*tonA*, min 3.4	Fe^{3+} limitation	T1, T5, $\phi 80$, ColM	Uptake ferrichrome
Fec protein	80500		*fec*, min 7	Fe^{3+} limitation		Uptake Fe^{3+} citrate
FepA protein	81000		*fep*, min 13.0	Fe^{3+} limitation	ColB	Uptake Fe^{3+} enterochelin
Protein a	40000	Up to $\sim 4 \times 10^4$	*lep*, min 12.5	constitutive	LP81	Protease

The most abundant protein of the outer membrane, lipoprotein (mol.wt. 7200; not seen in Fig. 4), is in part (30%) covalently bound, via the ε-NH_2 group of the C-terminal lysine, to aminopimelic acid residues of the underlying peptidoglycan network (BRAUN and REHN 1969; BRAUN et al. 1976). The NH_2-terminal amino acid of lipoprotein, cysteine, is modified in a unique manner: its NH_2-group is substituted with a fatty acid (amide linkage) and its sulfhydryl group is substituted with a diglyceride (BRAUN 1975). Lipoprotein is bound to the outer membrane via hydrophobic intractions of its three fatty acids. It is apparently not, exposed at the outer surface of the cells, since, unlike all other outer membrane proteins, it does not function as a receptor for phages. Lipoprotein is to a large degree α-helical (BRAUN et al. 1976), which is unique among outer membrane proteins (see below). Like all other outer membrane proteins (and also the periplasmic proteins; see Sect. 5.7), lipoprotein is synthesized as a precursor containing a 20-amino acid signal-sequence, which is split off from the 58 amino acids of the mature protein during secretion and translocation of the protein to the outer membrane.

Lipoprotein-deficient mutants (*lpo*) show a significant loss of outer membrane in the form of vesicles (WEIGAND et al. 1976; DIX and BRASS unpublished), indicating that lipoprotein plays an important role in the structure and stability of the outer membrane. Under laboratory conditions, however, lipoprotein seems to be dispensable for growth. Very similar lipoproteins are found in most of the Enterobacteriaceae (INOUYE 1982).

The OmpA protein, which is also present in high copy number in the outer membrane (see Fig. 4), seems to play a role in the structure of the outer membrane. Mutants lacking an OmpA protein produce unstable outer membranes. *lpo ompA* double mutants show a defective morphology (egg-shaped) and are extremely sensitive to osmotic stress or EDTA (SONNTAG et al. 1978). OmpA seems to be associated with lipoprotein and interacts with murein via ionic bonds. The protein apparently consists of two domains, which can be separated by cleavage with proteases. The NH_2-terminal domain is inserted in the outer membrane, while the COOH-terminal domain is exposed in the periplasmic space (CHEN et al. 1980). In contrast to lipoprotein, the OmpA protein of *E. coli* has, similar to the porin proteins OmpC and OmpF (see below), a high β-sheet content (CHEN et al. 1980). OmpA is exposed at the outer surface of the cell since it acts as receptor for phages (DATTA et al. 1977). Despite the fact that OmpA exhibits a certain amount of sequence homology with the porin proteins OmpC and OmpF (NIKAIDO and WU 1984), OmpA does not increase the permeability of the outer membrane for solutes. The observed homology might rather indicate common sequences essential for outer membrane localization during secretion (see Sect. 5.7) or interaction with LPS.

OmpA protein and LPS are involved in the stabilization of cell-cell contacts during F-factor mediated conjugation (DAVIS and REEVES 1975). The first contacts, mediated by F-pili, are OmpA independent. Solubilized OmpA protein, after reconstitution with LPS, competitively inhibits conjugation (VAN ALPHEN et al. 1977).

The OmpA protein has still another interesting function: cells deficient in OmpA are tolerant to colicin K. Colicins are toxic proteins which are produced

by some strains of *E. coli* and which kill sensitive strains of *E. coli*. Colicin K (as well as E_1, A, Ia and Ib) functions by the formation of ion channels in the inner membrane (KAYALAR et al. 1984). It is not well understood how colicins reach their target. Colicin K first binds to its receptor, the Tsx porin. Tsx-deficient strains are resistant to colicin K. Further internalization of colicin K apparently requires a functional interaction of Tsx with OmpA and LPS. OmpA-deficient strains bind colicin K but are tolerant against it. Two interesting explanations were proposed (HINZ 1983): OmpA and Tsx might interact to create a local disturbance of the membrane to facilitate permeation of colicin K through the rigid outer membrane. Alternatively, the penetration of colicin K might depend on the close contact of outer and inner membrane, in which case OmpA protein might be involved in the stabilization of positions where inner and outer membranes are in close contact.

Many of the other outer membrane proteins are involved in solute transport through the permeability barrier of the outer membrane. Entry of the necessary substrates and minerals through the outer membrane into the periplasm of gram-negative cells is accomplished in two ways: (a) passive entry of low molecular weight solutes present at relatively high concentrations (around 10^{-6} *M*) proceeds through water-filled pore-forming proteins, so-called porins (NAKAE 1975); (b) entry of essential solutes which are too large to pass through the porins, and which are present at very low concentrations (down to 10^{-20} *M*), proceeds after binding of substrate or a substrate-chelator complex to outer membrane receptor proteins. Examples of receptor-mediated uptake processes are the BtuB-mediated uptake of vitamine B_{12}, and the Cir-, TonA-, Fec-, and FepA-mediated uptake of ferric ion chelator complexes (see Table 1 and Sect. 4.6).

Seven different porins have been described for *E. coli*: OmpC, OmpF, PhoE, maltoporin, Tsx, protein K, and NmpC. The gene coding for NmpC is silent in wild-type *E. coli* K12 (HINDAL et al. 1984). Some of the porins, such as OmpC and OmpF of *E. coli* K12 or *S. typhimurium,* do not seem to belong to a particular transport system and are therefore called general porins. The OmpF and OmpC porins have approximately the same size specificity for hydrophilic solutes (optimal permeability for solutes of molecular weight below 200; exclusion of solutes above 650). Some of the porins, such as maltoporin, PhoE, or Tsx, are specifically suited for diffusion of bulky substrates; they belong to specific transport systems. Maltoporin and PhoE also function as efficient general porins for other solutes (BAVOIL et al. 1977; HENNING et al. 1977; BRASS et al. 1985). Tsx is not restricted to the uptake of nucleosides through the outer membrane but also acts as porin for amino acids (HEUZENROEDER and REEVES 1981). A more detailed consideration of pore specificity is given in Chap. 3.

Porins function in vivo and in vitro as trimers with identical subunits with molecular weights between 35000 and 45000 d (NAKAE et al. 1979; ICHIHARA and MIZUSHIMA 1979). Porin trimers exhibit the remarkable ability to maintain their oligomeric association even in the presence of sodium dodecyl sulfate (SDS) (1%) at moderate temperatue (below 60° C). Porin proteins span the outer membrane and interact via ionic bonds or indirectly via lipoprotein or OmpA with the peptidoglycan layer. The amino acid composition of OmpF

and OmpC is not particularly hydrophobic (reviewed in BENZ 1985). The longest hydrophobic sequence of OmpF comprises about 10 amino acids, which is enough β-sheet structure to span the membrane. The large number of charged amino acids is presumably used to stabilize the tertiary and quaternary structure of the porin trimers which contain a hydrophilic channel and a hydrophobic exterior. Studies of negatively stained porin crystals revealed one triplet per unit cell. The three-dimensional structure of the triplets was reconstituted from tilted projections recorded in the electron microscope. From these data it was concluded that porins have a triplet inlet converging into a single outlet at the periplasmic site of the membrane (see Fig. 1; DORSET et al. 1984).

2.3 The Hydrophobic Barrier of the Outer Membrane

In contrast to most biological membranes the outer membrane does not allow passage of hydrophobic and amphipathic molecules. This can easily be concluded from the remarkable resistance to detergents and hydrophobic antimicrobial agents like actinomycin (NIKAIDO and NAKAE 1979; NIKAIDO and VAARA 1985). The asymmetry in the lipid composition of the outer membrane of Enterobacteriaceae protects these bacteria against an appreciable number of detergents (cholate) and free fatty acids present in their natural environment (the colon). The outer leaflet of the outer membrane contains almost exclusively LPS as lipid and also is not dissolved by detergents, since the LPS molecules are held together not only by hydrophobic interactions but also by strong ionic interactions. In addition, the saturated LPS hydrocarbons are much less mobile than phospholipid hydrocarbon chains (NIKAIDO et al. 1977). Hydrophobic compounds therefore have great difficulty making a hole between the associated hydrocarbon chains (GALEY et al. 1973).

Among LPS mutants of S. typhimurium, only in deep rugh mutants (Rd-mutants; see Fig. 3), with a strongly reduced outer membrane protein content, is the hydrophobic barrier function affected. In E. coli K12 already mutants with less severe LPS-defects (lacking galactose and glucose) are affected. These mutants tend to fill empty positions not only with LPS but also with phospholipids (SMIT et al. 1975; SCHWEIZER et al. 1976). They become sensitive to hydrophobic antibiotics, detergents, and exogenous phospholipase (NIKAIDO 1976; VAN ALPHEN et al. 1976, 1977; AGABIAN and UNGER 1978. Diffusion of hydrophobic molecules in Rd-mutants is extremely dependent on temperature ($Q_{10}=10$) and there is no clearcut size limit for hydrophobic solutes. It is not clear at the moment if the sensitivity of Rd-mutants is due to the presence of phospholipid molecules in the outer leaflet of the outer membrane or to decreased interaction of Rd-LPS molecules (reviewed in NIKAIDO and VAARA 1985). Rd-mutants show normal, or even reduced, sensitivity to hydrophilic antibiotics like penicillin (ROANTREE et al. 1977). In contrast, however, to the Enterobacteriaceae, gram-negative bacteria such as *Neisseria gonorrhoeae,* which live in an environment free of bile salts and free fatty acids, are quite sensitive to hydrophobic antibiotics and detergents (MANESS and SPARLING 1973).

Porins exclude hydrophobic molecules (see Sect. 3.4). Insertion of sex (F) plasmid- and R plasmid-coded pili into the cell envelope weakens the remarkable barrier function of the outer membrane of wild-type *E. coli* against hydrophobic molecules. Selection of F-episome free strains, a procedure quite useful for bacterial genetics, takes advantage of the higher detergent resistance of F-episome free cells grown in 5% SDS (TOMEDA et al. 1968).

3 Permeability Properties of the Outer Membrane Porins

What is the history of the discovery of porins? What was the first indication for a pore-forming protein in the outer membrane and what is known today about the specificity of the different porins present in *E. coli*?

Taking into account the low partition coefficient of hydrophilic solutes in biological membranes, Nikaido postulated the existence of a mechanism in the outer membrane to facilitate the slow diffusion of solutes through this membrane. He and his group studied this postulated mechanism first by measuring the penetration of solutes into the enlarged periplasm of plasmolyzed cells. They used oligosaccharides of different size, like raffinose (galactosyl-sucrose) or stachyose (galactosyl-galactosyl-sucrose) neither of which penetrated through or were actively transported across the cytoplasmic membrane (DECAD and NIKAIDO 1976). They found a clearcut size limit for penetration of these oligosaccharides into the periplasm: sucrose (342 daltons) and raffinose (504 daltons) entered the accessible space (50% of the cell volume) extremely rapidly even at 0° C, whereas stachyose (666 daltons) or larger oligosaccharides showed only very slow entry. Interestingly, these results were consistent with earlier data of others who found that peptides larger than 500–600 daltons could not enter nonplasmolyzed *E. coli* cells (PAYNE and GILVARG 1968). The possibility still existed that the cell wall, the rigid peptidoglycan layer underlying the outer membrane, was acting as the size-limiting molecular sieve. This possibility was excluded by the observation that lysozyme-treated, plasmolyzed cells with degraded peptidoglycan showed the same permeability characteristics as intact cells (NAKAE and NIKAIDO 1975).

The hypothesis that outer membrane proteins play an essential role in the outer membrane permeability was finally proven by reconstitution experiments with liposomes doped with extracted outer membrane protein fractions (NAKAE 1975) or purified proteins (NAKAE 1976). The pores produced in phospholipid vesicles by addition of outer membrane proteins and LPS allowed free diffusion of sucrose and raffinose, but excluded larger oligosaccharides. Addition of LPS alone was ineffective. In these early reconstitution experiments permeability or exclusion of a molecule was assayed by gel filtration of vesicles preloaded during the vesicle formation with impermeable ^{3}H-dextran and ^{14}C-labeled molecules to be tested. Excluded molecules comigrated with the vesicles while permeable solutes were separated from the much faster migrating vesicles after exit from the vesicle interior. Porins integrated in these vesicles facilitated diffusion of a variety of other small hydrophilic molecules such as amino acids, sugars,

and nucleosides smaller than 600 daltons. The rate of porin mediated diffusion could not be resolved with this approach.

Further proof for the involvement of outer membrane proteins in outer membrane permeability and the first indication of the great importance of these porin proteins for transport and growth rates of bacteria stemmed from a genetic approach. In 1971, a specific class of *E. coli B/r* mutants was described; they required increased (20–500-fold) nutrient and ion concentrations for half maximal growth rates and showed a concomitant pleiotropic decrease in the apparent affinities of different transport systems for these nutrients and ions (VON MEYENBURG 1971). The phenotype of these mutants was at first wrongly interpreted as cell envelope modification which weakens the attachment of various specific binding proteins to the inner membrane. It was assumed that this could produce a general increase in the K_m value of the different transport systems (*kmt* mutation). More than 5 years later the right interpretation was found. It was shown that the decreased apparent affinity for the uptake of most nutrients was due to a lack of the major outer membrane protein, previously shown to produce transmembrane diffusion channels in vesicle reconstitution experiments in vitro (BAVOIL et al. 1977). The *kmt* mutation was mapped at 73.7 min by P1-transduction. We know now that this region of the chromosome contains a regulatory gene (*ompR*) which is essential for expression of porin protein OmpC in *E. coli B/r* and OmpC and OmpF in *E. coli* K12 (HALL and SILHAVY 1981).

The pleiotropic transport deficiency of *kmt* (*ompR*) mutants can now easily be explained. The porin deficiency of *kmt* strains results in a strongly reduced flow of solutes into the periplasm. The rate of entry is dependent on the external solute concentration. At high external substrate concentrations the residual flow of substrate through other minor porins is still sufficient to compensate for the number of molecules which are taken up from the periplasm into the cytoplasm by the inner membrane transport components. At low external substrate concentrations, however, permeation through the outer membrane is not sufficient. The periplasmic solute concentration drops far below the external concentration and the inner membrane transport components are no longer saturated. This produces a phenotype showing decreased apparent substrate affinity in many transport systems.

A similar K_m-shift in the apparent affinity of the maltose transport system for maltose (from 10^{-6} *M* in wild-type cells to 10^{-4} *M*; unchanged V_{max}) is observed in *lamB* mutants defective in the specific maltoporin (SZMELCMAN et al. 1976). The necessary periplasmic maltose concentration for half-maximal saturation of the periplasmic maltose binding protein (10^{-6} *M*) is only achieved at 10^{-4} *M* external concentration of maltose in a *lamB* mutant. The efficiency of permeation of maltose through the OmpF and OmpC general porins in vivo is 100-fold lower than that through maltoporin (see Sect. 3.2).

Interestingly, suppressor mutations could be selected from the porin deficient *kmt* mutants which regained their ability to grow on low concentrations of lactose, e.g. Cells carrying such suppressor mutations always contained new outer membrane proteins. We know today that these newly synthesized proteins were indeed specific porins (maltoporin and PhoE porin) which are expressed in wild-type cells only after growth on maltose, or derepressed by growth at

limiting phosphate concentrations. Constitutive expression of these porins in *kmt*-suppressor strains provides a considerable selective advantage since these proteins not only function as specific, but also as general porins, allowing entry of a great variety of solutes into the periplasm.

3.1 Solute Size Specificity of General Porins

In a single bacterium we find multiple species of porins. The expression of these porins is controlled at the transcriptional level by the growth conditions (see Table 1) (NIKAIDO and NAKAE 1979). Growth at high and low salt concentrations favors production of OmpC and OmpF porins, respectively (KAWAJI et al. 1979). Growth at low P_i concentrations derepresses a series of gene products which scavange trace amounts of phosphate-containing nutrient from the medium (ARGAST and BOOS 1979). One of the derepressed proteins is an anion-specific outer membrane porin (PhoE), which is immunologically related to the two general porins OmpC and OmpF (OVERBEEKE et al. 1980). PhoE appears to be important for uptake of larger phosphorylated compounds, but also allows nonspecific diffusion of nonphosphorylated compounds. Growth of *E. coli* on maltose induces the expression of a maltose/maltodextrin-specific outer membrane channel (maltoporin) which also functions as a general nonspecific porin. These examples illustrate the different physiological roles of each of these different porins.

The study of permeability properties of the different porins in vivo, in reconstituted vesicles, and in reconstituted lipid bilayers has presented convincing evidence that the outer membrane porins are not simply water-filled channels, but rather that each individual porin exhibits a solute selectivity. Selectivity is a function of the size of a solute and of its charge. The shape of the pores, which deviates significantly from a hollow cylinder (see Fig. 1; DORSET et al. 1984), possibly also influences the rate of penetration of a solute.

Rates of diffusion of uncharged and charged solutes through porin channels were determined in a swelling assay using liposomes reconstituted with purified *E. coli* porins (LUCKEY and NIKAIDO 1980a). The swelling assay gives information on the rates of diffusion of solutes through porins. In this method multilayered liposomes are reconstituted from phospholipids (e.g., egg-phosphatidylcholine), porin, and LPS. Multilayered liposomes containing the porin to be tested are made so that large solute molecules incapable of penetration through the pore are kept within the liposomes. After dilution of the vesicles into isotonic solutions of the test solutes the optical density of the vesicles is measured. If the pore is permeable to the solute, influx of the solute molecules, followed by influx of water, will occur because of the chemical potential gradient. This will lower the refractive index of the liposomes and reduce the light scattering, and therefore the optical density, of the liposome suspension. The initial rate of swelling reflects the rate of penetration of solute into the outermost layer of the multilayered vesicle (NIKAIDO 1983). Possible artifacts arising from the rapid entry of water molecules, however, have to be considered (reviewed in NIKAIDO and VAARA 1985).

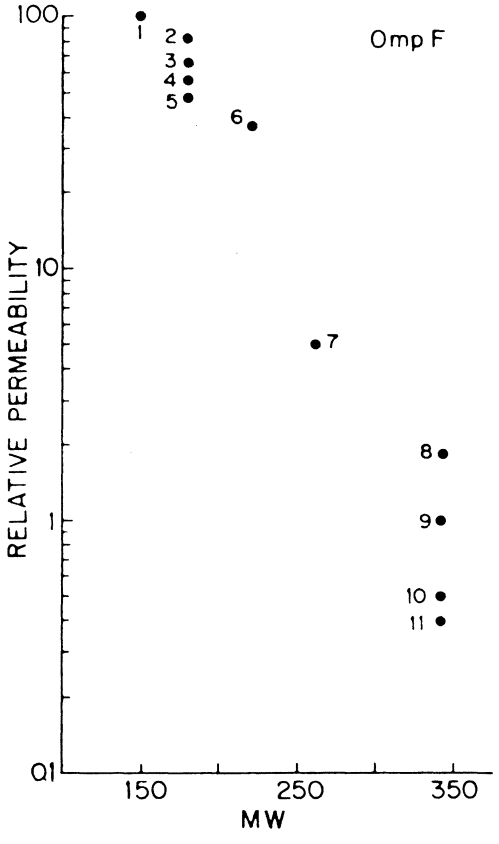

Fig. 5. Effect of solute size on the rate of diffusion of saccharides through OmpF porin channel of *E. coli*. Rates of diffusion were determined in vitro using the swelling of reconstituted liposomes. The compounds studied were as follows: *1*, L-arabinose; *2*, D-galactose; *3*, D-fructose; *4*, D-mannose; *5*, D-glucose; *6*, N-acetyl-D-glucosamine; *7*, 2,3-diacetamido-2,3-dideoxy-D-glucose; *8*, lactose; *9*, sucrose; *10*, maltose; *11*, melibiose. (Reprinted with permission of NIKAIDO and ROSENBERG 1983)

Diffusion of uncharged solutes into liposomes through purified *E. coli* porins was strongly dependent on the size of the solute (Fig. 5). The approximate nominal diameter of the pores could be estimated by fitting the data to the Renkin equation (RENKIN 1954). The problems inherent in such an estimate as well as in the pore size estimates based on single-channel conductivity measurements of porins inserted in black lipid films (see below) have been discussed (NIKAIDO and VAARA 1985). The *E. coli* porins OmpF, OmpC, and PhoE appeared to produce channels of similar size; the OmpF channel appeared to be only about 10% larger than the OmpC and PhoE channel. Nonetheless, remarkable size specificity can be observed. This molecular size effect is probably caused by the collision of solute molecules with the rims of the pore entrance and by the viscous drag exerted by the walls of the pore, as hypothesized by RENKIN (1954). The use of the Renkin equation predicts that for a solute with a Stokes radius of 0.42 nm, such as glucose, the OmpC pore, which has a radius of 0.54 nm, will have only about 60% of the permeability of the OmpF pore, which has a radius of 0.58 nm (NIKAIDO and ROSENBERG 1983). With disaccharides, such as lactose, or peptides, such as tetraalanine, even greater differences in the rate of permeation (ten fold) were observed between OmpF and OmpC channels (NIKAIDO and ROSENBERG 1983).

In this regard, the hypothesis has been put forward that synthesis of OmpF might be an adaption of enteric bacteria to life in dilute media outside of a mammalian host, since OmpF allows somewhat higher rates of solute permeation through its wider channel (reviewed in NIKAIDO and VAARA 1985).

3.2 Permeability Functions of the Specific Maltoporin

Small molecules like glucose diffuse fast enough through the OmpF and the OmpC porins, even at micromolar concentrations, to equilibrate the periplasmic solute concentration with that of the external medium. Disaccharides like maltose show only 1% of the permeability of monosaccharides through these porins (see Fig. 5). It is likely that this limitation prompted the evolution of a specific maltoporin which efficiently mediates the permeation of maltodextrins up to maltoheptaose (WANDERSMAN et al. 1979; FERENCI and BOOS 1980). The way in which maltoporin specifically increases the diffusion rates of its bulky substrates is not entirely clear. In vivo, the rate of diffusion of maltose at an external concentration of 1 μM through maltoporin is about 100 times greater than through OmpF and OmpC (SZMELCMAN et al. 1976). Maltodextrin cannot pass at all through OmpF and OmpC porins (WANDERSMAN et al. 1979).

What are the results if one compares the permeability properties of OmpF and maltoporin in in vitro studies using liposomes? Conflicting data have been published. NAKAE and ISHII (1980) reported that maltoporin and OmpF porin show approximately equal permeability for maltose in vitro. NIKAIDO and his group showed that both channels exhibit the same permeability for glucose, whereas the permeability of maltose is about 25-fold lower through OmpF than through maltoporin (LUCKEY and NIKAIDO 1970a; NIKAIDO and ROSENBERG 1981, 1983). The same difference was also seen in the swelling assay and in liposomes containing amyloglucosidase in the intravesicular space (Nikaido and Nikaido, unpublished). In the latter vesicles, the rate of entry of maltose is measured indirectly by release of glucose. Maltoporin, however, is not just a wider channel allowing entry of molecules with higher molecular weight. The permeability of lactose, for example, which differs from maltose in structure but not in molecular weight, through maltoporin is only 10% that of maltose in in vitro experiments (LUCKEY and NIKAIDO 1980a). This allows the important conclusion to be drawn that the maltoporin channel itself, even in the absence of maltose binding protein (MBP), has a remarkable chemical selectivity.

In vivo, however, there is evidence that MBP might play an essential role in determining the specificity of the maltoporin channel. Workers have isolated MBP mutants (e.g., *malE254*) which were not able to grow on maltodextrins at millimolar concentrations, although the dissociation coefficient (K_D) of the mutant MBP for binding of maltohexaose in vitro had changed only from 1 μM to 25 μM (WANDERSMAN et al. 1979). Growth on maltose was not impaired. This phenotype (Mal^+ Dex^-), which is similar to that of maltoporin-negative strains, was taken as evidence that the MBP of Dex^- mutants had lost its ability to interact with maltoporin (WANDERSMAN et al. 1979). This interaction was postulated to be essential for permeation of dextrins and maltose

at low concentration through maltoporin (see Fig. 12). Indeed, binding of solubilized maltoporin to immobilized wild-type MBP was demonstrated by using affinity columns, whereas no binding was seen if MBP from Dex$^-$ strains was used (BAVOIL and NIKAIDO 1981; BAVOIL et al. 1983).

Independent support for an MBP-maltoporin interaction has come from the work of HEUZENROEDER and REEVES (1980). They claimed that wild-type MBP inhibited the general porin activity of maltoporin. Their conclusion has gained considerable attention as an example for binding protein mediated control of porin activity. We were not convinced by the evidence presented in this study and re-examined this conclusion. We studied the effect of the presence or absence of MBP on maltoporin-mediated permeation of lactose through the outer membrane in vivo in strains devoid of the general porins OmpC and OmpF. This was done in strains with normal or four fold reduced maltoporin content. Under these conditions permeation of lactose through maltoporin is rate-limiting for the overall uptake process at low lactose concentrations. We also studied the effect of the interaction deficient *malE254*-MBP on permeation of maltose at millimolar concentrations through maltoporin in the OmpC OmpF deficient strains (BRASS et al. 1985).

Our data on maltose and lactose transport in *ompR malE* mutants indicate that (a) maltoporin is an open pore for maltose and lactose, even in the absence of interaction with wild-type MBP, and (b) the activity of maltoporin as a general porin is not inhibited by the presence of wild-type MBP. The latter conclusion is at variance with the previous report by HEUZENROEDER and REEVES and with a recent study on maltoporin integrated in planar lipid bilayers (NEUHAUS et al. 1983), in which a MBP-dependent closing of the maltoporin channel was claimed. NEUHAUS et al. reported a shift in the equilibrium of open and closed maltoporin channels to the closed state after addition of MBP, when voltage in the "physiological" direction, i.e., negative on the side containing MBP, was applied. We have shown (BRASS et al. 1985) that MBP neither interferes with, nor stimulates the function of maltoporin as a general porin in vivo, although our findings cannot disprove the concept of MBP-maltoporin interaction.

Recently, however, *ompC* and *ompF* mutants were isolated (BENSON and DECLOUX 1985) which had the remarkable ability to grow on maltodextrin even in the absence of maltoporin. These mutants also exhibited only slightly reduced maltose transport at micromolar concentrations of maltose. Interestingly, these mutants show a concomitant increase in sensitivity against a variety of hydrophobic dyes. These mutants apparently specify OmpC and OmpF channels with much greater pore diameter. These data strongly argue against the necessity of physical interaction of MBP with any porin channel to accomplish entry of maltodextrin substrates, since widening of the pore diameter and simultaneous creation of a binding site for MBP in these new OmpF and OmpC porin are highly unlikely. We conclude that the Mal$^+$ Dex$^-$ phenotype of the interaction deficient MBP mutants (e.g. *malE254*) is actually due not to a deficient interaction of their MBP with maltoporin. This phenotype is rather due to the fact that maltodextrin, which (in contrast to maltose) binds to maltoporin (FERENCI and BOOS 1980; LUCKY and NIKAIDO 1980b), cannot be pulled out

by the mutationally altered MBP due to its reduced affinity. The presence of an affinity gradient for dextrins (maltoporin < MBP) is obviously essential for the function of maltoporin as a specific pore for dextrins.

Conflicting evidence also exists about the efficiency of maltoporin as general porin. In contrast to the statement of BAVOIL and NIKAIDO (1981) that permeation of glucose through maltoporin is only 2%–10% of that through the porin channel, our kinetic data show that the general porin activity of maltoporin is equivalent to that of OmpC and OmpF. At low substrate concentration in strains with reduced maltoporin content, i.e., conditions under which permeation through the outer membrane is rate limiting for overall transport, the rate of lactose uptake was very similar in *ompR lamB*$^+$ strains and *ompR*$^+$ *lamB* strains; transport was negligible in *ompR lamB* strains (BRASS et al. 1985). Similar results were found for transport of glucose and galactose (BRASS and BAUER, unpublished).

3.3 Physiological Role of the High Porin Copy Number

What is the biological advantage of the high copy number ($\leq$100000/cell) of most of the outer membrane porin channels? We have examined this question by using maltoporin mediated permeation of maltose through the outer membrane as a test system. Until recently it has not been known which of the three processes (permeation of maltose through the outer membrane, diffusion of maltose-loaded MBP through the periplasm, or uptake through the inner membrane) represents the rate-limiting step of the multicomponent maltose transport system (see Fig. 12).

As discussed lucidly in different reviews by Nikaido, permeation of solutes through the outer membrane can only be rate-limiting for the overall uptake process at low external solute concentrations (NIKAIDO and NAKAE 1979; NIKAIDO and VAARA 1985). Diffusion through the outer membrane is a passive process described by Ficks' first law of diffusion, $V = P \times A \times \Delta c$, where V is the rate of diffusion, P is the permeability coefficient of a given solute making its way through a given porin, A is the permeable area of the membrane, and Δc is the concentration difference of the solute across the membrane. The biological advantage of any porin can only be evaluated if one keeps in mind that the rate of diffusion through this porin (V) changes linearly with Δc. At the high external concentrations of maltose which are normally used to grow bacteria (0.2% maltose = about 5 m*M*) permeation of maltose through the outer membrane maltoporin is not rate limiting. The physiological role of the high maltoporin copy number per cell can thus only be determined by careful kinetic experiments at low maltose concentrations. We measured the kinetics of maltose transport in strains with normal, two fold, and five fold reduced levels of maltoporin. Reduction of the maltoporin copy number reduces the membrane area available for permeation, the term A in the above equation.

Selective reduction of the expression of the *lamB* gene coding for maltoporin and normal expression of all other components involved in maltose transport (see Sect. 5.1) was accomplished by isolation of a Tn*10* insertion (*zjb-729*::Tn*10*)

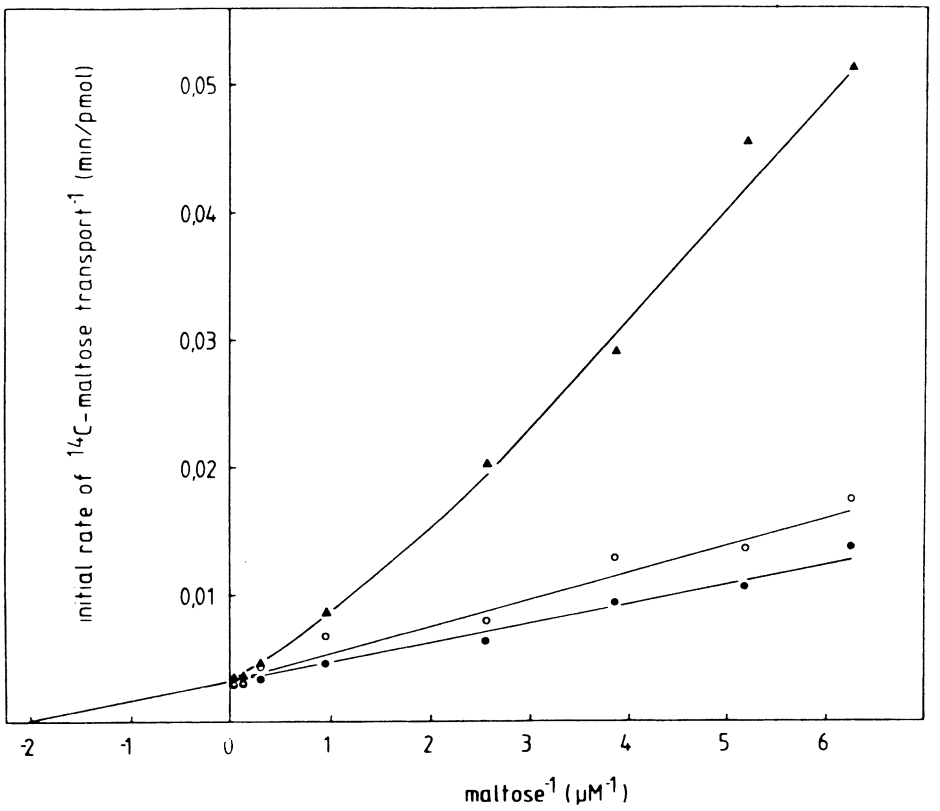

Fig. 6. The biological advantage of the high maltoporin copy number becomes apparent at low substrate concentration. Kinetics of maltose transport was measured in strains with normal (●), two-fold (○), and five-fold reduced (▲) maltoporin level at ^{14}C-maltose concentrations at 0.13–50 μM. The strain with fivefold reduced maltoporin exhibited normal transport at maltose concentrations above 1 μM and reduced transport below this value. Below 1 μM maltose, permeation through maltoporin is rate limiting for overall uptake. This is indicated by a deviation of the Lineweaver-Burk plot from linearity. At low concentrations, the plot extrapolates to the origin, reflecting the nonsaturable kinetics of permeation through porins. (Reprinted with permission of BRASS et al. 1984)

which integrated in the intergenic region (75 base pairs) between the genes *malK* and *lamB* (see Fig. 12). *zjb-729*::Tn*10* provided a new, low level promoter for *lamB* (see also Sect. 5.6).

In strains with five fold reduced maltoporin content maltose transport was unimpaired at concentrations of maltose above 1 μM. Reduction of maltose transport was only seen at concentrations below 1 μM (Fig. 6). These kinetic data precisely define the maltose concentration and the maltoporin level at which permeation through the outer membrane becomes rate limiting for transport. We conclude that even at the low concentration of 1.3×10^{-7} M, permeation of maltose through maltoporin is not rate limiting for overall maltose transport in wild-type cells with normal amounts of maltoporin. Maltoporin becomes rate limiting when the ratio of maltoporin to other maltose transport

components is reduced more than two fold. These data illustrate the selective advantage of the high copy number of maltoporin for growth on maltose, and of the general porins for growth on lower molecular weight carbon sources. This advantage becomes evident as soon as the cells are exposed to ecologically relevant, low solute concentrations. Porins were selected as an adaptive response to a famine existence.

3.4 Exclusion of Hydrophobic Solutes by Porins

Hydrophobicity retards the penetration of solutes through the wild-type OmpC, OmpF, and PhoE channels in the in vitro vesicle swelling assay (NIKAIDO and ROSENBERG 1983). What is the reason for the exclusion of small (≤ 200 daltons) hydrophobic molecules from porins? In most cases it is not the formation of aggregates or micelles, but rather the inability of hydrophobic molecules to interact with the water molecules in the porin channel and with hydrophilic residues of the porin itself which excludes hydrophobic molecules (BENZ, personal communication). Hydrophobic exclusion was also observed with intact cells (NIKAIDO et al. 1983). Interestingly, strains carrying altered OmpC and OmpF channels, which allow entry of bulky dextrins (BENSON and DECLOUX 1985), show a remarkable sensitivity against hydrophobic dyes (see above). Maltoporin, which also allows entry of maltodextrins, does not confer such a sensitivity even in the absence of MBP. Maltoporin appears to be specifically adapted to take up bulky substrates, while still excluding hydrophobic dyes (BRASS, unpublished).

3.5 Ion Selectivity of Porins

The presence of a negative charge on the solute resulted in a three fold reduction in penetration rates through OmpF and OmpC channels, whereas it accelerated the diffusion through PhoE in the vesicle swelling assay (NIKAIDO and ROSENBERG 1983). These results suggested that the *E. coli* PhoE porin is specialized in the uptake of negatively charged solutes.

A comparative study of pore size and pore selectivity of different porins from *E. coli* and other gram-negative bacteria was recently performed by BENZ et al. (1985) with the lipid bilayer technique. This approach provides the unique opportunity to study the permeability properties of a single conductive unit (i.e., the trimer) integrated into a planar phospholipid bilayer. The addition of small amounts (1–10 ng/ml) of porins to the aqueous phase bathing the membrane resulted in a stepwise increase of the membrane current at a given voltage. Steps (incorporation of active trimers) were directed upwards, whereas downward steps (inactivation of a trimer) were only rarely seen. Similar results were also found for porins of other gram-negative bacteria, and porins isolated from mitochondria and chloroplasts (BENZ 1985).

The ions used for probing the pore structure had the same relative mobilities while moving through the porin as they did while moving in the free solution.

This allowed the conclusion that the porins form large, water-filled channels. Single channel conductance could be used to estimate the effective channel diameter of the porins. Pore diameters of 1.1, 1.0, 1.1, and 1.4 nm have been calculated for the OmpF, OmpC, PhoE, and LamB porins from *E. coli,* respectively. Critical comments concerning such pore size estimates were discussed recently (reviewed in NIKAIDO and VAARA 1985).

Recent investigations have indicated that maltoporin might form two types of pores in lipid bilayer membranes: a large pore with 1.4-nm diameter and, in addition, a small pore with a diameter of about 0.6 nm (BENZ et al. 1986). The small pore is about 20-fold more abundant than the large pore. Interestingly, only the small pore could be blocked by addition of maltodextrins (e.g., maltohexaose, 10^{-4} *M*), whereas the activity of the large pore could not be inhibited by dextrins. Binding of dextrins to maltoporin (FERENCI and BOOS 1980) caused inhibition of maltoporin activity in vitro (LUCKEY and NIKAIDO 1980b) and in vivo (BRASS et al. 1985). The incorporation of both LamB pores (maltoporin) in the bilayer could be specifically inhibited by addition of preadsorbed anti-LamB-trimer antibodies (BENZ et al. 1986). The latter finding supports the assumption that these two pores might represent two different conformations of maltoporin in a bilayer. The physiological relevance of these two conformations is not clear at the moment. It was speculated that the absence or presence of MBP or the variable Donnan potential across the outer membrane could modulate the activity of maltoporin in vivo. No experimental evidence for any of these hypotheses has been found (BRASS et al. 1985 – see Sect. 3.2; NIKAIDO and VAARA 1985).

Further information on the structure of the different porins was obtained by studying the ion selectivity of the pores by zero-current potential measurements with porins incorporated in bilayers. In these experiments, small amounts of 1 *M* KCl are added to only one side of a porin-containing membrane bathed in 10 m*M* KCl. The fact that OmpC and OmpF porins allow efficient permeation of the K^+ ion and a much slower permeation of the Cl^- anion, whereas the PhoE porin favors the permeation of the anion, leads in both cases to an establishment of a membrane potential (opposite orientation) at zero-current conditions. The ion selectivities of the different porins, usually expressed as the permeability ratio of cation to anion, were 3.6, 26, 0.3, and 4.5 in KCl for the *E. coli* OmpF, OmpC, PhoE, and LamB porins, respectively (BENZ et al. 1985, 1986).

PhoE porin isolated from *Salmonella typhimurium* is also anion selective. A mutant was selected in *S. typhimurium* which constitutively expresses the *pho* regulon proteins. One of these appears as a protein at high copy number in the outer membrane. Upon measurement of its porin activity in black lipid films it exhibited, like *E. coli* PhoE porin, a marked anion selectivity (see Fig. 7) and appears to be an analogous protein.

The observed ion selectivity may be explained by the presence of negative or positive charges in or near the pores. This assumption is supported by the observation that the cation selectivity of OmpC decreases if the pH is decreased (BENZ et al. 1984). Further support for this assumption arises from results with chemically modified porins (DARVENAU et al. 1984). The question of ion selectivi-

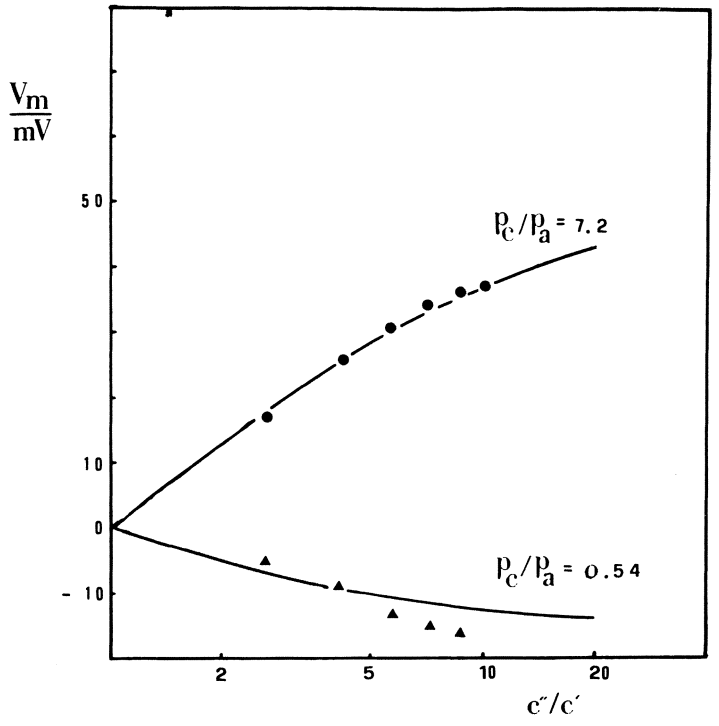

Fig. 7. Cation and anion selectivity of the OmpF and PhoE porin of *Salmonella typhimurium*. Porins were isolated from strains carrying only the OmpF porin (●) or both the OmpF and PhoE porins (▲). Porins were incorporated into black lipid films. The ion selectivity was determined by measuring the zero current potential after increasing the KCl concentration on one side of the membrane. The ion selectivity is expressed as permeability ratio of cation to anion (p_c/p_a). OmpF porin exhibits a marked cation selectivity ($p_c/p_a = 7.2$). The OmpF PhoE mixture exhibits a p_c/p_a value of 0.54, indicating an anion selectivity of PhoE. (Reprinted with permission of BAUER et al. 1985)

ty of porins is also being studied on the molecular level with hybrid proteins containing varying amounts of the PhoE porin and the OmpF porin at the NH_2- and COOH-terminal ends, respectively (Tommassen and Benz, unpublished).

3.6 Receptor-Mediated Solute Transport Through the Outer Membrane

Remarkable examples for receptor-mediated uptake processes through the outer membrane are the BtuB-mediated uptake of vitamin B12, which functions at concentrations as low as 10^{-11} *M* (KADNER and LIGGINS 1973), and the Cir-, TonA-, Fec-, and FepA-mediated uptake of ferric ion-chelator complexes (for review see NEILANDS 1982). The solubility of ferric ion is extremely low (around 10^{-18} *M*). Bacteria have therefore developed systems for uptake of this important ion by synthesis and excretion of chelators (enterochelin and others) which, after binding of ferric ions, are themselves bound and internalized by outer

membrane receptor proteins. *E. coli* cells grown under conditions of limiting ferric ion concentration derepress synthesis of chelator and several outer membrane receptor proteins (Table 1). A very interesting hypothesis proposes that production and uptake of ferric ion chelators can play an important role in bacterial infections when bacterial chelators compete with eukaryotic ferric ion binding proteins, such as transferrin in the serum or ferritin in the cells (BRAUN 1981). The observation that enterochelin production is temperature sensitive has been interpreted such that fever might be a host defense mechanism to deprive the pathogen of iron. The importance of bacterial ferric ion uptake systems for pathogenicity is further illustrated by the observation that the colicin V plasmid conferred an increased virulence to bacteria. This virulence is not due to the production of colicin, but rather is due to the fact that the plasmid carries a second ferric ion transport system which uses aerobactin as chelator (BRAUN 1981; reviewed in CROSA 1984).

4 Structure and Functions of the Periplasmic Space

The space between the outer and inner membrane is referred to as the periplasmic space. There is no clear way to define the boundaries of this space, but from a functional point of view it includes all peripherally attached proteins on the inner leaflet of the outer membrane as well as on the outer leaflet of the inner membrane. The periplasm provides a microenvironment which is intermediate between the external milieu and the vital constituents of the cells. It acts as a "homeostatic" compartment to protect the cytoplasm (STOCK et al. 1977). It allows preprocessing of food stuff and postprocessing of secreted polysaccharide chains inserted into the peptidoglycan layer (reviewed in BEVERIDGE 1981).

The periplasm contains specific classes of proteins which amount to approximately 4% of the total cell protein (NOSSAL and HEPPEL 1966) as well as various oligo- and polysaccharides (VAN GOLDE et al. 1973). The volume of the periplasmic space depends on the osmolarity of the external medium. Estimates under isotonic conditions have varied considerably, from 1%–5% (NIKAIDO and NAKAE 1979) to as much as 40% of the total cell volume (STOCK et al. 1977). The latter value seems much too high; from a recent electron microscopic study using refined embedding techniques (HOBOT et al. 1984), a periplasmic volume equivalent to 7% of the cell volume can be calculated. The proteins and oligosaccharides located in the periplasm can be specifically isolated from cells by osmotic shock after plasmolysis of the cells (see Sect. 6.1) (NEU and HEPPEL 1965). Periplasmic proteins fall into three main classes based on their function.

4.1 Protective Functions of the Periplasmic Space

One class of periplasmic proteins serves a protective role, modifying toxic compounds such as heavy metal ions or antibiotics (e.g., β-lactamase; CURTIS et al.

1972). Moreover, the periplasmic space plays an important protective role in osmoregulation of gram-negative cells. Cells grown in medium with low osmolarity synthesize and secrete membrane-derived oligosaccharides (MDO) into the periplasm (SCHULMAN and KENNEDY 1979). MDO are of low molecular weight (2000) and can comprise up to 7% of the dry weight of the cell. They contain about 9 residues of glucose and are substituted with sn-glycerol-phosphate and phosphatidylethanolamine. Since it is found that cells grown in high osmolarity media repress the synthesis of MDO, it is assumed that these oligosaccharides enable the cell to keep the osmolarity of the periplasm at a level only slightly below that of the cytoplasm (the osmotic strength of the cell interior is about 300 mos*M*). This concept implies that the osmotic pressure of the cell (about 3.5 atm) is transfered from the sensitive inner membrane to the rigid peptido-glycan-outer membrane complex. This would provide a mechanism for gram-negative bacteria to adapt to surroundings of very different osmolarity.

The periplasm is strongly anionic with respect to the external milieu; 25% of the charge is contributed by the anionic groups of the outer membrane proteins, while most of the rest of the negative charge is assumed to be due to the negatively charged MDO. The resulting Donnan potential varies with the amount of these oligosaccharides. The osmotic properties of the periplasm are dependent not only on the concentration of the synthesized MDO, but also are influenced by the MDO-induced Donnan potential.

4.2 Catabolic Functions of the Periplasmic Space

Another class of periplasmic proteins consists of enzymes which degrade large or impermeable solutes into forms which can be transported into the cell (e.g., ribonuclease, alkaline phosphatase). Other enzymes have also been found, including hexose-monophosphatase, cyclic phosphodiesterase, and endonuclease I (WETZEL et al. 1970; ANATHASWANY 1977). Recently, two new periplasmic enzyme activities have been described by the group of Boos. The first is a glycerophosphodiester phosphodiesterase, which hydrolyzes compounds such as glycerophosphoethanolamine into *sn*-glycerol-3-phosphate plus alcohol. The enzyme is coded by the *glpQ* gene, which is part of the *glpT glpQ* operon. The enzyme expands the catabolic capacity of the *glp* regulon to include a variety of glycerophosphodiesters (LARSON et al. 1982). The second is a periplasmic amylase (product of the gene *malS*) which splits amylose and dextrins which are too big to be taken up into the cytoplasm (FREUNDLIEB and BOOS 1986).

4.3 Morphogenic Functions

Most polymeric precursors for the cell wall are synthesized in, or in close apposition to, the cytoplasmic membrane. The peptidoglycan layer consists of a network in which linear amino sugar chains containing alternating residues of N-acetylglucosamine and N-acetylmuramic acid are covalently crosslinked to each other via tetrapeptides which are attached to the N-acetylmuramic acid residues (reviewed in BEVERIDGE 1981). An atomic model of murein, different

possible murein packing types, and their biological implications have recently been discussed (LABISCHINSKI and JOHANNSEN 1986). The lower limit of the mesh size of murein is given by the distance between neighboring peptidoglycan chains (1–3 nm) times the smallest distance between two crosslinked peptides connecting the same two chains (4–5 nm). The effective mesh size would depend, however, on the murein packing type, which might be different at different locations (e.g., at curved polar caps). The periplasm plays a functional role in the maintenance and expansion of the cell wall complex. Some of the enzymes catalyzing the last steps of peptidoglycan synthesis bind β-lactam antibiotics, which inactivate their catalytic activities. All penicillin-binding proteins (PBP) (except PBP-3, which is found in the inner membrane and is involded in septum formation) are found attached to both the inner and outer membrane fractions. Half of the total amount of PBP may be sacculus-located proteins, linked to the outer membrane (RODRIGUEZ-TEBAR et al. 1985). PBP catalyze energy-independent reactions and are involved with final assembly and processing of murein.

In growing cells a dynamic situation is demonstrable. At about 100 separate sites murein crosslinks are opened by murein endopeptidase and new murein strands are inserted into the pre-existing material by the murein synthetase and transpeptidase (BURMAN et al. 1983; DEPEDRO and SCHWARTZ 1981). Cooperation between different hydrolyzing and synthesizing enzymes brings about elongation and division of the cell-shaped murein sacculus without reducing its mechanical strength.

KOCH (1985) addressed the problems of how the rigid peptidoglycan, under a pressure of 3.5 atm, can enlarge and how a nonspherical, rod-shaped structure can be generated. It was suggested that cylindrical elongation using the cell turgor as driving force can only occur in two ways, either when the poles are rigid and new strands of peptidoglycan are inserted at the side walls (e.g., growth of *E. coli*) or with a rigid base and growing poles (e.g., growth of budding bacteria). However, the localized narrow growth zones proposed earlier would lead to unstable peptidoglycan (see Fig. 8; taken from KOCH et al. 1985).

As lucidly outlined in a recent review (ROTHFIELD et al. 1986), the cell division machinery must provide mechanisms to: (a) identify the mid-point of the cell; (b) initiate the changes in molecular organization that commit this site to the division process; (c) facilitate the invagination of inner membrane, murein, and outer membrane and the subsequent separation of the daughter cells; and (d) ensure that each progeny cell receives at least one copy of the bacterial chromosome during this process. Evidence has been presented that extended zones of attachment between murein and the inner and outer membranes play a role in one or more of these aspects of the division process.

At sites of cell division, these attachment sites form a characteristic paired structure consisting of two circumferential rings – the periseptal annuli – that completely surround the cell (Fig. 9) (MACALISTER et al. 1983). Serial electron micrographs of dividing cells have shown that each division septum is flanked by a pair of the periseptal annuli. The annuli might act as gaskets to seal off the division site from the remainder of the cell enevelope along the body of the cell. This would ensure that membrane or periplasmic components re-

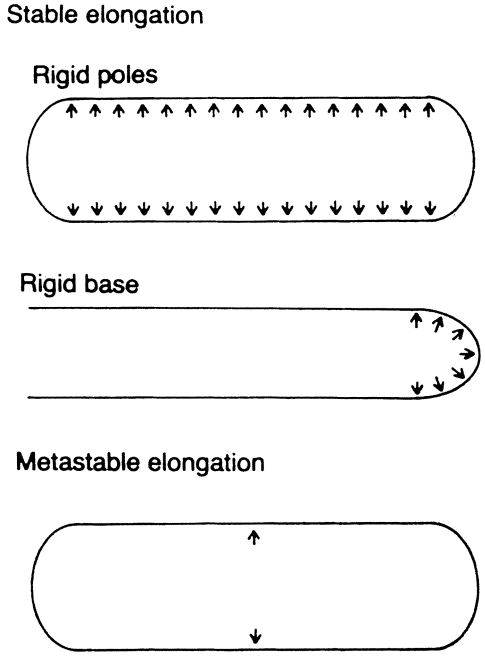

Fig. 8. Ways to achieve stable cylindrical elongation during growth. *Arrows* show sites of peptidoglycan insertion. Note that unstable growth results from a localized growth zone since spontaneous blow-outs would arise. (Reprinted with permission of KOCH 1985)

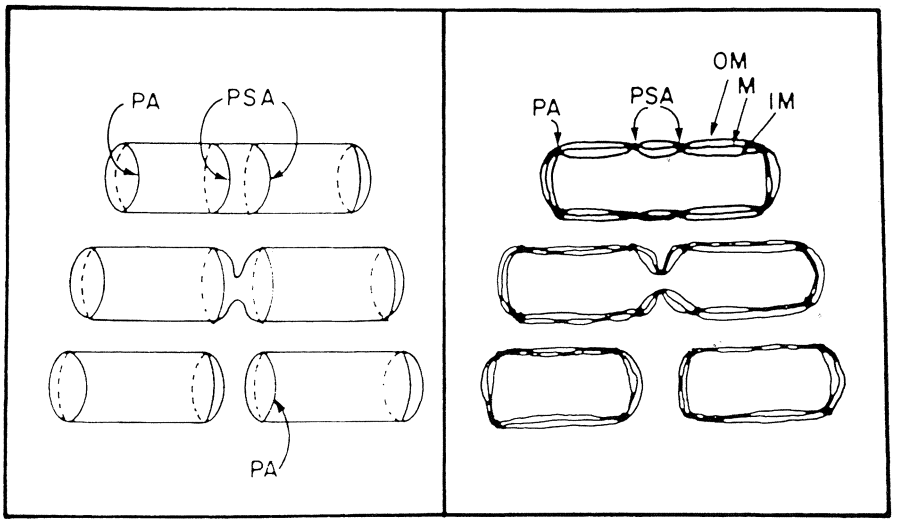

Fig. 9. Diagrammatic representation of periseptal annuli. Cells at different stages of progression through the division cycle are shown in surface views (left column) and cross-sections (right column). (Reprinted with permission of ROTHFIELD et al. 1986), *PSA*, periseptal annulus; *PA*, polar annulus; *OM*, outer membrane; *M*, murein; *IM*, inner membrane

quired for septation remain localized at the proper site during the succeeding stages of the division process. Recent evidence that periseptal annuli might indeed separate the periplasm into different compartments is presented in Chap. 10 (ROTHFIELD et al., manuscript in preparation).

4.4 Transport and Chemotactic Functions

The third group of periplasmic proteins consists of the substrate-binding proteins, which are essential components of many transport systems (BOOS 1972). Some also serve as chemotactic receptors (HAZELBAUER and ADLER 1971). Periplasmic binding proteins have been identified for a wide variety of nutrients, including amino acids, sugars, peptides, vitamins, and inorganic ions, and show high affinities for their specific substrates (see Table 2). These proteins (mol. wt. generally between 30000 and 40000) are present in the periplasm at high concentration ($10^{-4}-10^{-3}$ *M*). The pI values of the native proteins indicate that most of the binding proteins are negatively charged at neutral pH. The glutamate-aspartate and the glutamine binding proteins, however, appear to be positively charged. Most of the binding proteins have a dissociation coefficient (K_D) for their substrate in the range between 10^{-7} *M* and 10^{-6} *M*. In most cases a similar K_m of the respective transport system is found. All binding protein tested so far showed a pronounced conformational change upon binding of substrate, which could be assayed as a change in the fluorescence spectrum, or by other techniques. Three sugar binding proteins (galactose- ribose-, and maltose-binding proteins) and probably a dipeptide-binding protein, but none of the numerous other binding proteins, function as chemoreceptors in chemotaxis (see Chap. 9). All periplasmic proteins, including the binding proteins, are synthesized as precursors on membrane-bound ribosomes. They are processed cotranslationally into the mature protein by cleavage of the signal sequence during secretion into the periplasm (see Sect. 5.7).

Several binding proteins have been purified and their amino acid sequences determined. Comparison of the amino acid sequence of maltose-binding protein (MBP) with that of arabinose-, galactose-, and ribose-binding proteins revealed interesting homologies in two main regions. Each of these homologous regions is about 60 amino acids long and separated by a stretch of about 110 nonhomologous amino acids (DUPLAY et al. 1984; see Fig. 10). The sequence similarity may correspond to structural and functional similarities between these binding proteins. MBP appears to have an extra segment of 80 amino acids at its NH_2 terminus, relative to the three other proteins.

Some binding proteins have also been crystallized and their three-dimensional structure deduced at high resolution by X-ray crystallographic techniques. A common structural feature of the different binding proteins which have been characterized at 3 Å resolution has since emerged (SAPER and QUIOCHO 1983). All binding proteins examined, including those specific for L-arabinose, D-galactose, and leucine-isoleucine-valine, are elongated by the presence of two domains with similar secondary structure (Fig. 11). The elipsoidal dimensions of these molecules are about $40 \times 35 \times 70$ Å. The substrate binding sites are located in

Table 2. Periplasmic binding proteins. (From SWEET 1983, modified)

Binding protein	Organism	Yield		Mol wt	pI	K_D (μM)	Conformational change	Chemo-receptor[c]	Crystals[d]	References[e]
		mg/100 g cells	molecules/cell							
Sugars										
Arabinose	*E. coli* B/r	83	–	33200[b]	ND	0.3	NMR	no	MPD	[1–3]
Galactose	*E. coli* K12	20	10^4	33000	ND	0.48	Fluorescence	yes	PEG	[4–8]
	S. typhimurium LT2	2[a]	10^3	33000	ND	0.38	Fluorescence	yes	PEG	
Maltose, Maltodextrius	*E. coli* K12	40	3×10^4	40000	4.2	1.5	Fluorescence	yes	PEG	[8–10]
Ribose	*E. coli* W	–	–	29500	6.6	0.13	ND	yes	–	[5, 11, 12, 14]
	S. typhimurium LT2	14	–	29000	7.3	0.33	CD, fluorescence	yes	MPD, PEG	
Xylose	*E. coli* K-12	ND	ND	37000	7.4	0.6	Fluorescence	NR	–	[13]
Amino acids, derivatives and peptides										
Arginine	*E. coli* K12	–	–	27700	5.1	0.03	ND	no	–	[15]
Cystine	*E. coli* W	15	–	27000	4.8	0.01	ND	no	–	[16]
Glutamate, Aspartate	*E. coli* K12	–	–	31000	9.7	0.8, 1.2	Fluorescence	no	–	[17]
Glutamine	*E. coli* K12	50	–	26000	8.6	0.03	Fluorescence	no	–	[18]
Histidine	*S. typhimurium* LT2	–	2.3×10^3	26104[b]	5.5	0.15	NMR, fluorescence	no	–	[19–22]
Lysine, Arginine, Ornithine	*S. typhimurium* LT2	–	–	27000	5.1	3.0, 1.5, 5.0	ND	no	–	[23]
Leucine	*E. coli* 7	7	–	36000	4.5	0.7	ND	no	AS	[17, 24, 25]
Leucine, Isoleucine, Valine	*E. coli* K12	43	10^4	36000	5.2	1.0, 1.0, 1.0	Microcalorimetry	no	PEG	[25–27]
Glycine-Betaine	*E. coli* K-12	ND	ND	32000	ND	1.4	ND	NR	ND	[28]
	S. typhimurium LT2	ND	ND	31000	ND	1	ND	NR	ND	[29]
Oligopeptides (2–6 amino acid residues)	*E. coli* K-12		$>3 \times 10^4$	58000	ND	ND	ND	no	ND	[30, 31]
	S. typhimurium LT2		1×10^4	58000	ND	ND				
Dipeptides	*E. coli* K12		ND	49000	ND	ND	ND	yes	ND	[32]
	S. typhimurium LT2		ND	49000	ND	ND	ND	no	ND	[32]

Other metabolites										
Succinate, Malate Fumarate (Lactate)	*E. coli* K-12	2–4	–	15000	ND	40, 55, 34	NR	NR	–	[33]
Tricarboxylate	*S. typhimurium* LT2	100	2×10^5	28600	6.1	0.14	ND	NR	AS	[34]
sn-Glycerol-3-Phosphate	*E. coli* K-12		–	45000	7.0	0.2	Fluorescence	NR	–	[35]
Ions										
Phosphate	*E. coli* K10	–	–	42000	ND	0.8	ND	NR	–	[36]
Sulfate	*S. typhimurium* LT2	–	10^4	34667[b]	ND	20	CD, ORD	NR	MPD	[37–39]
Vitamins										
Thiamine	*E. coli* Crooke's	–	–	40000	ND	0.05	NR	NR	–	[40]

ND, not determined. *NR*, not reported

[a] Lower *S. typhimurium* yield correlates with lower chemostactic response [4]

[b] From sequence

[c] Reported that indicated sugar BP act as chemoreceptors, but that amino acid BP do not [14]

[d] Crystallization using 2-methyl-2,4-pentanediol (MPD), polyethylene glycol (PEG), or ammonium sulfate (AS)

[e] See following list of references:

1. Parsons and Hogg (1974)
2. Quiocho et al. (1977)
3. Clark et al. (1982)
4. Zukin et al. (1977)
5. Zukin et al. (1979)
6. Boss et al. (1972)
7. Alber et al. (1981)
8. Quiocho et al. (1979)
9. Kellerman and Szmelcman (1974)
10. Szmelcman et al. (1976)
11. Willis and Furlong (1974)
12. Aksamit and Koshland (1972)
13. Ahlem et al. (1982)
14. Mowbray and Petsko (1982)
15. Rosen (1973)
16. Berger and Heppel (1972)
17. Willis and Furlong (1975)
18. Weiner and Heppel (1971)
19. Lever (1972)
20. Hogg (1981)
21. Robertson et al. (1977)
22. Higgins et al. (1982)
23. Rosen (1971)
24. Furlong and Weiner (1970)
25. Oxender et al. (1980)
26. Penrose et al. (1968)
27. Meador and Chiocho (1978)
28. May et al. (in press)
29. Higgins et al. (in press)
30. Higgins (1984)
31. Higgins and Hardie (1983)
32. Manson et al. (1986)
33. Lo and Sanwal (1975)
34. Sweet et al. (1983)
35. Argast and Boos (1979)
36. Medveczky and Rosenberg (1970)
37. Pardee et al. (1966)
38. Isihara and Hogg (1980)
39. Langridge et al. (1970)
40. Griffith and Leach (1973)

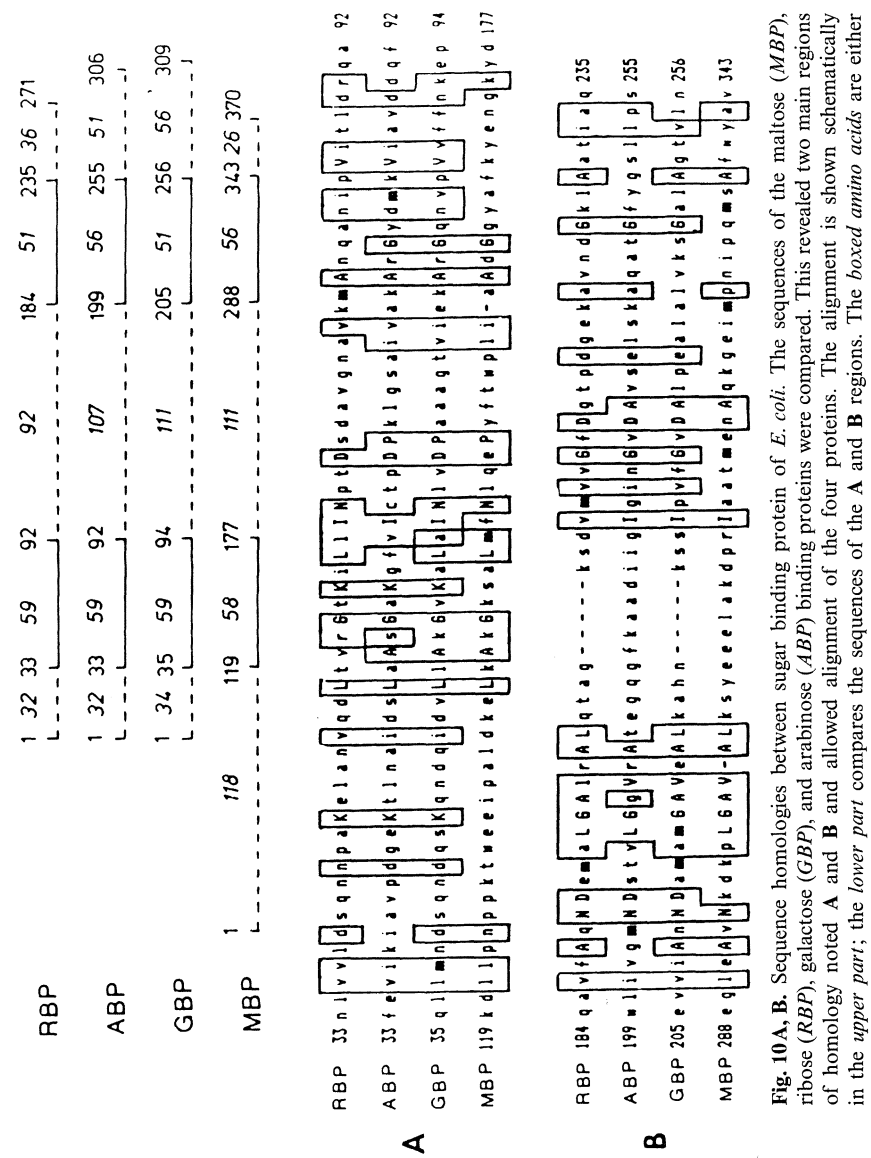

Fig. 10 A, B. Sequence homologies between sugar binding protein of *E. coli*. The sequences of the maltose (*MBP*), ribose (*RBP*), galactose (*GBP*), and arabinose (*ABP*) binding proteins were compared. This revealed two main regions of homology noted **A** and **B** and allowed alignment of the four proteins. The alignment is shown schematically in the *upper part*; the *lower part* compares the sequences of the **A** and **B** regions. The *boxed amino acids* are either identical or belong to the same class of conservative replacement. (Reprinted with permission of DUPLAY et al. 1984)

a cleft formed between the two domains. The bilobate structure, which also holds for MBP (QUIOCHO et al. 1979), appears to be important in the substrate-induced conformational change. This change leads to a more compact structure after closing the binding cleft by a hinge-like motion of the two domains through 18° relative to each other. The substrate is bound by extensive interactions with aminoacid residues from opposite walls of both domains and is completely inaccessible to the solvent. The lobes must separate after interaction with their respective inner membrane transport components to allow the sugar to be released for translocation across the membrane (see Fig. 12).

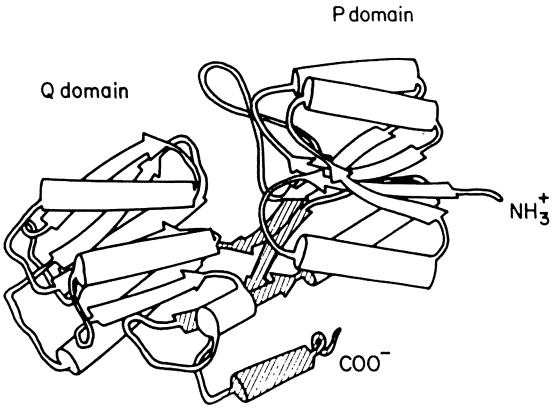

Fig. 11. Schematic drawing of the molecular conformation of L-arabinose-binding protein from *E. coli*. The α-helices and β-strands are represented by *cylinders* and *arrows*, respectively. The hatched represents the hinge. (Reprinted with permission of MAO and MCCAMMON 1983)

Since other transport systems exist which work without binding proteins and still exhibit similar high V_{max} and low K_m values, the requirement for binding protein and its specific role in delivering substrate to the membrane transport proteins is not clear. Various arguments have been advanced to explain the role of the binding proteins in transport and have been discussed in detail (reviewed by HENGGE and BOOS 1983). These authors have presented persuasive arguments suggesting that the three most common explanations – (a) that the binding protein enhances diffusion of the substrate through the periplasm, (b) that binding proteins increase the concentration of substrate in the periplasm, or (c) that they can enhance the affinity of otherwise binding-protein-independent transport systems – are inconsistent with available information. A more realistic explanation might be that these proteins increase the rate of entry of a specific substrate through the outer membrane porins by immediate binding. This should strongly reduce any exit reaction back into the medium, provided the bound substrate can then be delivered to the inner membrane transport components.

4.5 The Concept of the "Periplasmic Gel"

Earlier electron microscopic studies of thin sections from *E. coli* envelopes have indicated a free space between the inner membrane and the peptidoglycan layer. A recent report (HOBOT et al. 1984) suggests that the entire periplasm may be filled with a highly hydrated peptidoglycan gel. This gel is probably more crosslinked in the part underlying the outer membrane and less crosslinked towards the cytoplasmic membrane. It would have a high water content and large pores through which proteins could diffuse (see Sect. 4.3). The inner, non-crosslinked part of the gel is believed to consist of freely moving polysaccharide chains, oligosaccharides, and periplasmic proteins, forming a highly viscous

solution similar in concentration to the outer crosslinked part (HOBOT et al. 1984). The concentration of the periplasmic binding proteins can be estimated to be around 100 mg/ml; MBP alone is present at a concentration of 1 m*M* in maltose-induced cells (40 mg/ml) (DIETZEL et al. 1978). Cells grown in dilute media contain, in addition, appreciable amounts of MDO (see Sect. 4.1).

Potential advantages of the suggested periplasmic gel have been discussed (HOBOT et al. 1984). It was assumed that concentration and charge gradients could build up in the periplasm in response to the membrane potential across the cytoplasmic membrane. It was postulated that these gradients might control the opening of porins and allow a simple mechanism of binding protein transport. It was assumed that binding proteins would move like "shuttle proteins" in the gradient towards the cytoplasmic membrane and back again after transfer of the substrate which, during this transfer, undergoes a change in its conformation and electrical charge. These attractive considerations, however, deserve a critical discussion.

Planar membranes reconstituted with native outer membranes, a system which clearly reflects the native state of the porin, requires about 130 mV transmembrane potential to induce channel closure (SCHINDLER and ROSENBUSCH 1978). However, Donnan potentials which could potentially regulate porin activity never exceed 80 mV, even in cells grown in medium of low ionic strength. Thus, the significance of closure of porins, which could not be observed in *E. coli* porins in vivo (BENZ et al. 1978) and could not be measured in intact cells (HELLMAN and NIKAIDO, submitted) remains unclear.

The attractive shuttle protein hypothesis is probably not correct since the change in pI of the protein after delivering the substrate is much too small (0.1–0.2 pH units for *E. coli* GBP, e.g.) to account for a change in movement in a periplasmic potential gradient. Moreover, binding proteins exhibit quite different pI's ranging from 4.2 for MBP to 9.7 for glutamate-aspartate binding protein (see Table 2), which does not fit with the shuttle idea. The pI's of different maltose-binding proteins isolated from *E. coli, S. typhimurium, Klebsiella pneumoniae,* and *Enterobacter aerogenes* are 4.9, 5.4, 6.3, and 8.9, respectively, and yet all these proteins could be used for reconstitution of maltose transport and chemotaxis in malE deletion strains of *E. coli* (DAHL and MANSON, 1986) (see also Sect. 9.7).

Until recently it was assumed that periplasmic proteins could diffuse within the periplasmic space as within an aqueous solution. The pores in the peptidoglycan network were assumed to be wide enough to allow free movement of proteins, since periplasmic proteins can easily be isolated by the osmotic shock procedure after permeabilization of the outer membrane (see Sect. 6.1). We have developed a method to measure the lateral mobility of proteins in the periplasm (BRASS et al. 1986). We have introduced fluorescently labeled MBP into the periplasm of Ca^{2+}-treated cells and studied its periplasmic mobility with the FRAP (fluorescence recovery after photobleaching) technique. We found that the rate of lateral diffusion of MBP introduced into the periplasm is unexpectedly slow, about 100-fold slower than its rate of diffusion in aqueous solution. Possible reasons for the remarkable retardation of MBP are a very high periplasmic viscosity or ionic interactions of the protein with the peptido-

glycan or the membranes. In a subsequent study using temperature-sensitive cell division mutants we observed that the periplasm might be divided into different compartments by the periseptal annuli, which appear to function as tight barriers for long-distance movements of protein at potential division sites and at pole caps (ROTHFIELD et al., manuscript in preparation) (see Chap. 10). The FRAP technique, which is applicable for all sorts of macromolecules, should allow detailed studies of periplasmic viscosity, pore size of murein, and the possible influence of membrane potential on the mobility of periplasmic components.

5 The Binding Protein-Dependent Maltose-Maltodextrin Transport System

We have used the maltose transport system, one of the best characterized bacterial transport systems, as a model system to study the nature of the periplasmic compartment (see Sect. 4.5 and Chap. 10), and as a sensitive indicator system for Ca^{2+}-induced changes in the outer membrane permeability (see Sect. 6.2). We have also studied the expression of one of its genes (*lamB*) under a foreign, transposon-Tn*10*-dependent promoter (see Sects. 3.3 and 5.6), and have developed a technique for reconstitution of maltose transport and chemotaxis (see Chaps. 7, 8, 9). The following sections summarize some of the interesting aspects of this multifunctional system.

5.1 Genetics and Functions of the Five Cell Envelope Proteins Involved in Maltose/Maltodextrin Transport

The maltose/maltodextrin transport system is very efficient. It allows rapid uptake of bulky substrates (up to 1260 daltons) at low external concentration ($K_m = 1$–$2\ \mu M$; $V_{max} = 500$ pmol min^{-1} per 3×10^{-7} cells). Accumulation factors of the unmodified substrate up to $1:10^5$ inside the cell can be achieved. The system is comprised of five different proteins localized in three different layers of the cell envelope: the outer membrane, the periplasmic space, and the inner membrane (see Fig. 12). The genes for these proteins are clustered in the *malB* region located at 91 min on the bacterial chromosome. The *malB* region consists of two divergent operons (HOFNUNG 1974) transcribed from promoters P_L and P_R (also called P*malE* and P*malK*; RAIBAUD et al. 1979) (Fig. 12).

The product of the *lamB* gene, maltoporin (also known as receptor for phage λ), facilitates permeation of maltose and maltodextrins through the outer membrane. Maltose-inducible outer membrane proteins which seem homologous to the LamB protein have been detected in most enterobacterial species (SCHWARTZ and LE MINOR 1975). MBP, the product of the *malE* gene, is present in the periplasm at a relatively high concentration (1 m*M*; DIETZEL et al. 1978). Interestingly, its existence was first predicted in a study on bacterial chemotaxis (HAZELBAUER and ADLER 1971). Later, MBP was isolated and identified as an essential component of the maltose transport system (KELLERMAN and

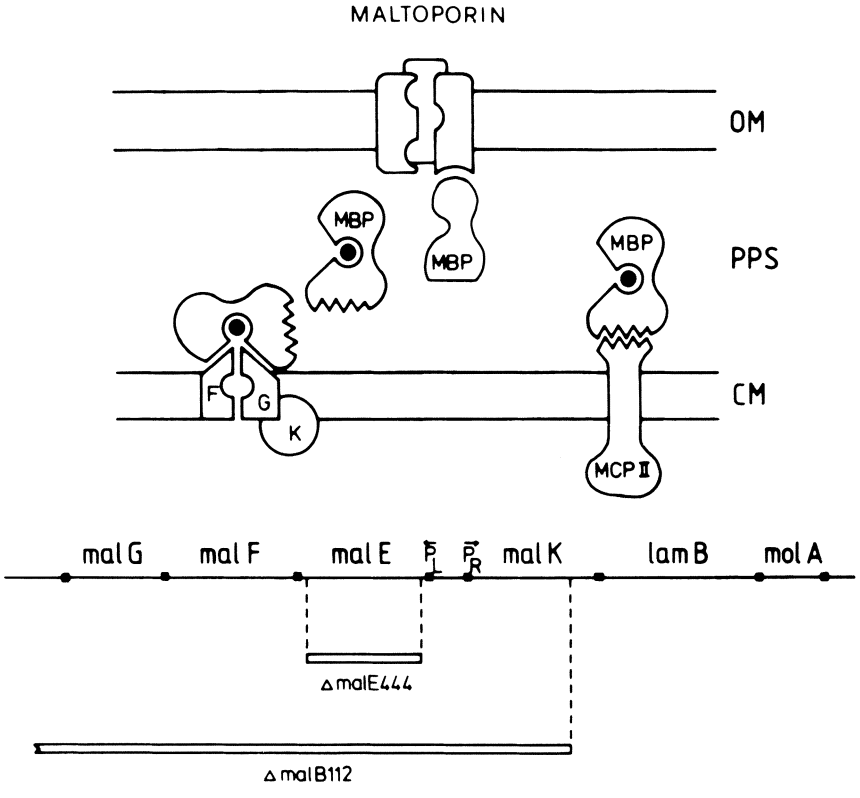

Fig. 12. The maltose transport system. The genetic organization of the *malB* region as well as the localization and the interaction of *malB* gene products is shown. The *malB* region consists of two divergent operons transcribed from promoters P_L and P_R. The *malE* gene codes for *MBP*, *lamB* codes for maltoporin (or λ receptor), and *malF, G, K* code for the cytoplasmic membrane components of the transport system. The *molA* gene, whose function is unknown, is expressed as part of the *malK-lamB* operon (GILSON 1983). The *malE444* deletion is nonpolar and lies entirely within *malE*. The *malB112* deletion extends from *malK* through *malG*. Known or suspected protein interactions within the periplasmic space (*PPS*) are shown in the *upper part* of the figure. Maltoporin, in conjunction with MBP, facilitates permeation of maltose and maltodextrins through the outer membrane (*OM*). MBP undergoes a conformational change upon binding substrate. The substrate-bound form can interact either with the complex formed by the *malF, G,* and *K* gene products to effect active uptake of maltose across the cytoplasmic membrane or with methyl-accepting chemotaxis protein II (*MCPII*), product of the *tar* gene, to initiate the maltose chemotactic response. (Adapted from BRASS and MANSON 1984; HENGGE and BOOS 1983)

SZMELCMAN 1974). MBP carries a high affinity binding site for maltose (SCHWARTZ et al. 1976; $K_D = 1\text{–}2\ \mu M$) and for maltodextrins (WANDERSMAN et al. 1979; FERENCI and BOOS 1980). This (and potentially also other substrate binding sites of this system) can be specifically labelled in photoaffinity experiments with maltooligosaccharides carrying a 3-azi-1-methoxybutyl group at their reducing end (THIEME et al., in press), MBP probably functions by trapping maltose in the periplasm and delivering it to the inner membrane components (see Fig. 12). MBP might increase the net maltoporin-mediated influx of sub-

strate through the outer membrane by reducing the free periplasmic concentration of substrate, thus reducing maltoporin-mediated exit of substrate from the periplasm into the external medium.

The efficiency of substrate binding in vitro decreases progressively at protein concentrations higher than 5 mg/ml and is reduced three- to fivefold at 40 mg/ml, the in vivo concentration estimated for MBP. Interestingly, binding can be stimulated under these conditions by addition of detergents (Triton 0.1%) or high concentrations (300 m*M*) of salt (BRASS and BOOS unpublished). It is not clear, however, if this finding indicates a multimerization, which has been reported for MBP and other binding proteins (ANTONOV et al. 1976; RICHARME 1982b, RASCHED et al. 1976; MOWBRAY and PETSKO 1983).

The products of the *malF, G,* and *K* genes are thought to form a protein complex associated with the cytoplasmic membrane; this complex is believed to interact with maltose-loaded MBP to mediate active transport of maltose across the inner membrane (reviewed in HENGGE and BOOS 1983). Very similar structural principles were found for other binding-protein-dependent transport systems (reviewed in AMES-FERRO-LUZZI 1984).

The *malF, G,* and *K* gene products were first identified by the use of the gene fusion technique. The *malF-lacZ* hybrid protein was purified by monitoring its β-galactosidase activity. Antibodies raised against the hybrid protein were then used to isolate the MalF protein from wild-type inner membranes (SHUMAN et al. 1980). By SDS-polyacrylamide gel electrophoresis, the protein exhibits an apparent molecular weight of 40000. Its molecular weight deduced from DNA sequence analysis, however, is 57000 (FROSHAUER and BECKWITH 1984). Secondary structure predictions indicate that the protein is very hydrophobic and spans the membrane 6–8 times.

The MalK protein was identified as an inner membrane associated protein in a manner similar to MalF. Interestingly, in *malG* mutants MalK was found in the cytoplasm (SHUMAN and SILHAVY 1981). Therefore, and because its sequence resembles that of a soluble globular protein (GILSON et al. 1982a), MalK is judged to be a peripheral membrane protein, anchored to the membrane via MalG (20000 daltons). It is thought to be involved in energization of the maltose transport system (see Sect. 5.3). Transient inhibition of the maltose transport activity is observed when maltose and glucose are present in the medium at the same time (POSTMA 1981). MalK might be the target for the inhibitory activity exerted by the phosphotransferase system, since mutants resistant to the transient inhibitory effect of glucose were found to map in MalK (SCHWARTZ and SAIER 1983, unpublished).

5.2 Transport of Substrate Across the Inner Membrane

The common feature of all binding-protein-dependent transport systems, including the maltose transport system, is that the inner membrane components do not take up free substrate, but rather only substrate delivered by binding protein. One explanation for this phenomenon is that a hidden binding site in the inner membrane becomes accessible only after interaction with substrate-loaded bind-

ing protein. In the case of the maltose transport system, interaction of MBP with the MalF, G, and K proteins could perhaps trigger a change in the conformation of these proteins which allows transfer of substrate from the periplasm to the inner membrane binding site. The existence of an inner membrane substrate binding site for the MalF, G, and K complex has been postulated (SHUMAN 1982). MBP-independent suppressor mutations mapping in *malF* oder *malG* were selected, and strains carrying these mutations grew on maltose and exhibited a 1000-fold increase in the K_m of maltose transport (SHUMAN 1982). On the basis of a recent study (TREPTOW and SHUMAN 1985) with 5 MBP-independent suppressor mutants, which revealed only very weak and unspecific inhibition of ^{14}C-maltose transport by other substrates of the transport system, we feel that the existence of such a binding site for maltose in the inner membrane complex has not yet been proven. Binding studies with the newly developed photoaffinity substrates (see Sect. 5.1) should help to clarify this question.

The crucial point for any model of maltose transport, the interaction of MBP with the inner membrane MalF, G, and K proteins, also has not yet been unequivocally proven. The reason for this may very well be that tight binding of maltose-loaded MBP to these proteins cannot be expected, since removal of substrate during the process of transport should lead to a very short lifetime of such a complex. We showed that transport of *malE* mutants lacking MBP could be reconstituted in Ca^{2+}-treated cells by addition of exogenous MBP (BRASS et al. 1983; see Chap. 7). This result shows once more that it is the binding-protein-substrate complex, and not the free substrate, that is recognized by the inner membrane transport components.

In the case of binding-protein-dependent histidine transport, support for the concept of binding protein-membrane protein interaction was provided by genetic analysis of suppressor mutants. A transport-defective histidine-binding protein mutant (*hisJ*) was isolated which produced a binding protein still able to bind the substrate. A transport-positive suppressor mutant in the *hisP* gene, which codes for a putative inner membrane component, was found that suppressed the binding protein defect (AMES and NIKAIDO 1978; AMES and SPUDICH 1976). Meanwhile, however, this picture has been complicated by the available sequence data, which show that the level of hydrophobicity of the HisP protein is that of a soluble globular proptein (GILSON et al. 1982a). The same work revealed an extensive homology between HisP and the MalK protein, also known as a peripheral transport component (see above). This indicates a possible evolution of *hisP* and *malK* genes from a common ancestral transport gene. Thus, HisP could be a peripheral protein, possibly located on the interior of the cytoplasmic membrane. If this is true, the model for histidine transport based on the suppression data, which assumes direct interaction of HisJ and HisP, must be revised (reviewed in AMES 1984).

5.3 Energization of the Maltose Transport System

Periplasmic binding-protein-dependent transport systems such as the maltose transport system all exhibit remarkably high substrate accumulation ratios. The uptake of substrate which is not chemically modified (as it is in the case of

group translocation transport systems, e.g., the bacterial phosphotransferase system, PTS) can lead to cytoplasmic concentrations which are 10^5-fold higher than the external concentration (μM). The energetics of these systems is not fully understood. In *uncA* mutants (deficient in the membrane-bound ATPase converting proton motive force into high energy phosphate bonds), periplasmic binding-protein-dependent transport systems, such as the maltose transport system, are strongly inhibited by arsenate. The systems are stimulated by glycolytic carbon sources in the presence of uncouplers (BERGER 1973; BERGER and HEPPEL 1974; FERENCI et al. 1977). Based on these findings it was assumed that ATP or a related phosphorylated compound could provide the driving energy for binding-protein-dependent accumulation of substrate.

Nevertheless, as discussed recently (HENGGE and BOOS 1983), conflicting evidence exists regarding the mechanism of energization of binding-protein-dependent transport systems. The activity of the GBP-dependent transport system was strongly reduced by DCCD, an inhibitor of the proton translocating ATPase in a respiration deficient mutant (SINGH and BRAGG 1977, 1979). In addition, uncouplers inhibited substrate uptake under conditions where ATP levels were high but proton motive force was abolished (FERENCI et al. 1977).

To clarify this situation, reconstitution experiments were performed with inner membrane vesicles from mutants lacking binding protein. Reconstitution of binding-protein-dependent transport of glutamine (HUNT and HONG 1981) and methyl-β-D-galactoside (ROTMAN and GUZMAN 1984) was achieved by addition of the respective binding proteins. It has not yet been possible to achieve reconstitution of maltose transport in such a vesicle system (Nikaido, personal communication). The ability of various substances to stimulate uptake in reconstituted vesicles was tested. Glutamine transport was stimulated by addition of pyruvate or succinate to vesicles containing intravesicular NAD^+. In contrast, no dependence on NAD^+ for the uptake of methyl-β-D-galactoside into vesicles was observed for stimulation by pyruvate, succinate, glycerol, lactate, or malic acid (ROTMAN and GUZMAN 1984). Although there is not an easily discernable pattern which could be concluded from all these results, it is clear that ATP and acetyl CoA did not stimulate uptake in any system.

The MalK protein is assumed to be involved in the energization of the maltose transport system. Arguments based on the known sequence of *malK* have been advanced for both possible modes of energization. On the one hand, *malK* displays homology with *ndh*, the structural gene for the respiratory NADH dehydrogenase (YOUNG et al. 1981; GILSON et al. 1982a, b). This suggests that energization of the system might be involved in dehydrogenation reactions. On the other hand, MalK appears to contain an ATP-binding site, arguing for an ATPase function of this protein (HIGGINS et al. 1985). Possibly there is a dual energization of maltose transport, involving high energy phosphoesters and respiration driven oxido-reduction reactions.

5.4 Enzymes Involved in Metabolism of Maltose and Maltodextrins

The metabolism of maltose and higher maltodextrins (reviewed by SCHWARTZ 1986) requires two specific enzymes, amylomaltase and maltodextrin phospho-

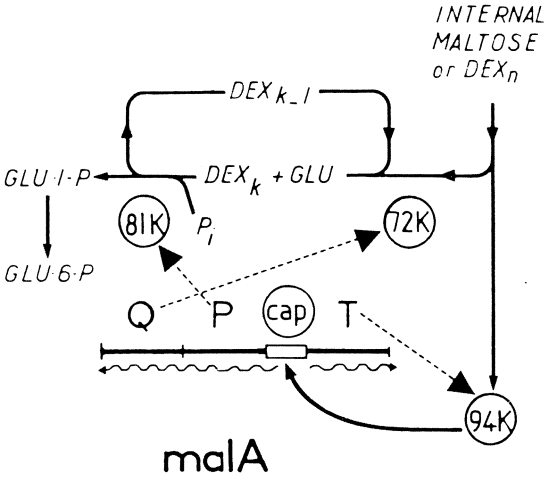

Fig. 13. Metabolism of maltose and maltodextrins in *E. coli.* The genetic organization of the *malA* region (75 min) as well as the functions of its three gene products involved in maltodextrin metabolism and regulation of the *malA*-operons are indicated. The *malA* region consists of two divergent operons *malPQ* and *malT*. *malT* codes for a 94000 activator protein which, after binding of maltose and in conjunction with the catabolite activator protein (*CAP*) facilitates transcription of the *mal* operons (CAP is dispensible for *malPQ*). *malP* codes for maltodextrin phosphorylase (two identical subunits of 81000), *malQ* codes for amylomaltase (72000 daltons). For further details see Sect. 5.4 and 5.5. (Adapted from HOFNUNG 1982)

rylase. These enzymes are the products of the genes *malQ* and *malP*, respectively, which are located in the *malA* region at 75 min on the *E. coli* genetic map (Fig. 13). *MalQ* mutants are unable to grow on maltose (SCHWARTZ 1967) and for still unknown reasons are even sensitive to maltose (reviewed in SCHWARTZ 1986). On rich media containing maltose, *malQ* strains give rise to secondary mutations in *malT* (see below) and *malK* which prevent entry of maltose (RAIBAUD et al. 1979).

Amylomaltase is not a hydrolase like β-galactosidase, but rather liberates free glucose from dextrins as it simultaneously transfers the nonreducing glucosyl unit to growing dextrin chains (MONOD and TORRIANI 1950) (see Fig. 13). Maltose itself cannot be split but can function as an acceptor molecule. Strains devoid of the maltodextrin phosphorylase accumulate large amounts (up to 50% of the cellular content) of long $\alpha(1-4)$-polyglucan (SCHWARTZ 1967). In wild-type cells, however, this enzyme, in the presence of inorganic phosphate, catalyzes the phosphorylytic splitting of dextrin chains, resulting in glucose-1-phosphate and a dextrin reduced by 1 glucose unit. The energy of the $\alpha(1-4)$-glucosyl bond is thus used to establish the high energy phosphoester bond in glucose-1-phosphate.

Amylomaltase is a monomer of 71000 daltons (PALMER et al. 1976); maltodextrin phosphorylase is a dimer of two identical subunits of 81000 daltons. Its amino acid sequence displays a high degree of homology (60% at the level of chemically related amino acids) with that of the rabbit muscle glycogen

phosphorylase (PALM et al. 1985). Homology is low at the amino terminal end, however, which is known to bind to the allosteric effector 5′-AMP in eukaryotic phosphorylases. The bacterial enzyme is not allosterically regulated.

Recently, a new maltose inducible gene (*malS*; 80 min) has been discovered. It codes for a periplasmic α-amylase. This enzyme hydrolyzes amylose and maltodextrins but not cyclic hexaose, which is split by the cytoplasmic amylomaltase (*malQ* product) (FREUNDLIEB and BOOS 1986). A probable function of this periplasmic enzyme is degradation of dextrins larger than maltoheptaose, which still pass through maltoporin (i.e., induce a chemotactic response – Manson unpublished) but are too long to be taken up through the inner membrane.

5.5 Regulation of the Maltose Regulon

In bacteria, initiation of transcription at many promoters requires activator proteins in addition to RNA polymerase. They facilitate binding of the RNA polymerase at specific promoters by a local change in DNA conformation. The genes responsible for maltose transport and metabolism are transcribed at a basal level even in the absence of the inducer maltose; tenfold induction of transport is observed after growth in maltose.

Transcription of the *malB* region (91 min) as well as the expression of the *malA* region (75 min) is under positive control of the *malT* gene product. This was shown by the use of *malB* and *malA* gene fusions (DÉBARBOUILLÉ et al. 1978; SCHWARTZ et al. 1981). The *malT* gene, which is located in the *malA* region (Fig. 13), was defined by mutations which all drastically reduce the induced (and the noninduced) expression of the *mal* operons (SCHWARTZ 1967). $MalT^-$ mutants are recessive to $malT^+$ (HOFNUNG et al. 1971). $MalT^c$ mutations allowing constitutive expression of the *mal* operons were selected by screening for high expression of the $malP$–$lacZ^+$ hybrid operon in the absence of maltose (DÉBARBOUILLÉ et al. 1978). Some lines of evidence indicate that an unknown metabolic product of maltose rather than maltose itself might be the true inducer. The reason why *malK* mutants show a constitutive expression of the maltose regulon is not understood. The hypothesis was put forward that these strains retain the unknown endogenous inducer, which might leak out in wild-type cells. A recent study concerned with this question indicates the presence of different endogenous inducers, dependent on the osmolarity of the growth medium (BUKAU et al. 1986). The properties of all known alleles of *malT* were explained by assuming that the MalT protein exists in two conformations at equilibrium, one of which stimulates transcription, the other not (reviewed in SCHWARTZ 1986). The unknown inducer would displace the conformational equilibrium in favor of the active form. The MalT protein has been purified as a 94000 dalton polypeptide which tends to precipitate (DÉBARBOUILLÉ et al. 1982), with the resolubilized protein having lost its activity. A few hundred MalT proteins are synthesized per generation in wild-type cells.

The expression of all *mal*-regulon operons is completely repressed in glucose-grown cells, which exhibit reduced cAMP levels (PETERKOFSKY and GRAZDAR 1974). *crp* and *cya* mutants lacking a functional catabolite activator protein

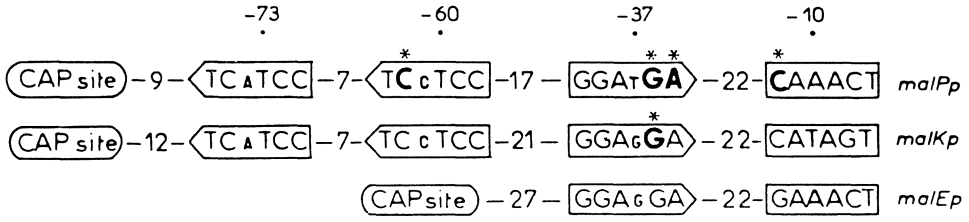

Fig. 14. Structure of the *malT*-controlled promoters. The only nucleotides shown are those in the Pribnow boxes and in the hexamers that are proposed to constitute binding sites for the MalT activator protein. There are three MalT binding sites at the $malK_p$ and $malP_p$ and one at the $malE_p$ promoter in different orientations (*arrow-like boxes*). In addition, *CAP sites* are indicated; that of $malP_p$ is dispensible. *Numbers* indicate the distance (nucleotides) between the sequences; *small letters*, nucleotides completely conserved in all MalT binding sites; *stars*, nucleotides whose alteration decreases promoter activity; *numbers above*, positions of nucleotides with respect to the transcription startpoint in $malP_p$. (Modified from RAIBAUD et al. 1985)

and adenylate cyclase, respectively, are unable to grow on maltose (SCHWARTZ and BECKWITH 1970; PERLMAN and PASTAN 1969).

Transcription initiated at 3 of the 4 *mal* regulon promoters (*malEp; malKp,* and *malTp*) is dependent on the presence of the cAMP-loaded catabolic activator protein (CAP). The fourth promoter, *malQp*, contains a nonfunctional CAP binding site and is only indirectly CAP dependent, mediated through a cAMP-CAP modulation of *malT* expression (CHAPON 1982; RAIBAUD et al. 1985). A possible working model is that CAP binding at the *malA* and *malB* promoters facilitates binding of MalT, which in turn allows transcription by RNA polymerase.

A recent study (RAIBAUD et al. 1985) compares the nucleotide sequence of the three MalT-regulated promoters *malPp, malKp,* and *malEp*. Possible CAP and MalT binding sites are indicated (see Fig. 14). All promoters contain CAP binding sites, although that of *malPp* appears dispensable since deletion of this site does not influence expression of *malP*. Interestingly, three MalT binding sites are found at the *malPp* and the *malKp* promoters, each containing one copy of the sequence 5′–GGA$^{G}_{T}$ GGA–3′. The sequence is present once in one orientation in the −37 region and twice in the other orientation in regions −60 and −73. The same sequence is present only once in the *malEp* promoter. It has been suggested that these repetitive sequences indicate that the MalT protein molecules cooperate after they are bound to DNA, and that the *malEp* promoter, which exhibits only one MalT binding site, may be functionally coupled to the *malKp* promoter.

5.6 Selective Expression of *lamB* Under a Foreign Transposon-Tn*10*-Dependent Promoter

We isolated a Tn*10* insertion within the *malK-lamB* operon (*zjb-729*::Tn*10*) which allowed selective reduction of the expression of the *lamB* gene without affecting expression of the *malB* genes. Insertion mutants normally inactivate

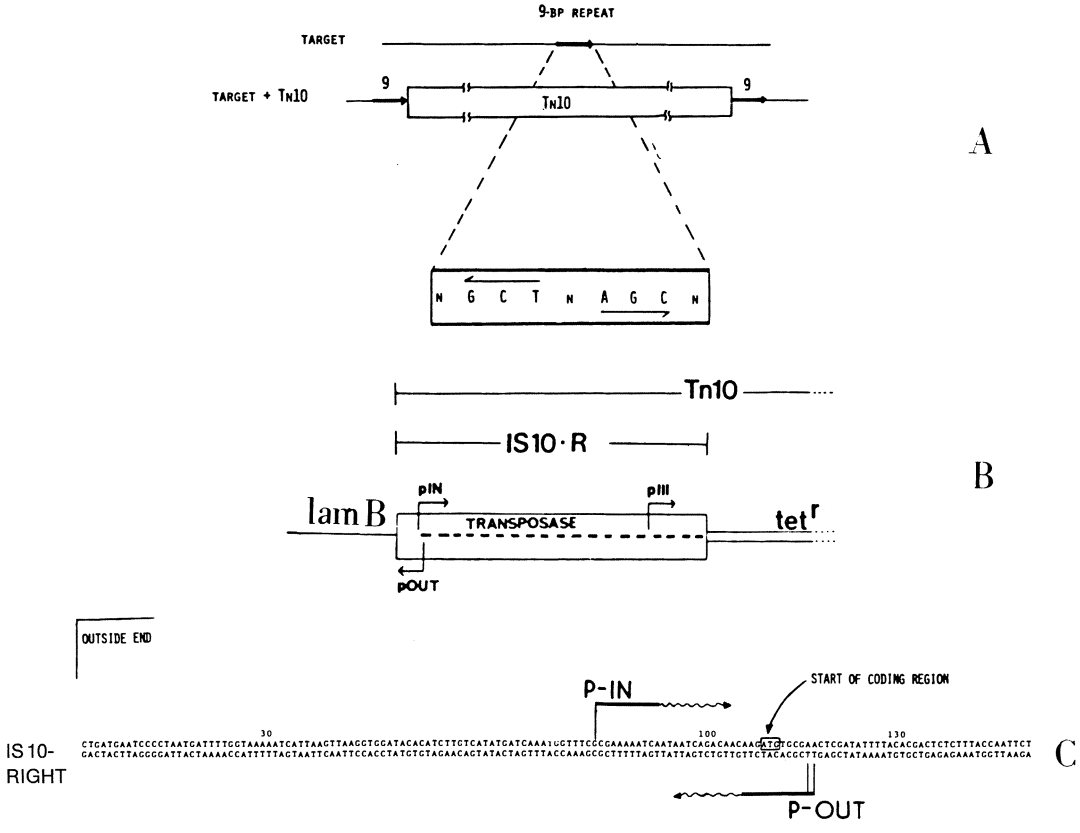

Fig. 15A–C. Structure and sequence of IS-10-right of transposon Tn*10*. Transposon Tn*10* is 9300 base pairs long. (**A**) It inserts into target DNA at hot spots that share a symmetrical 6 base pair consensus sequence. This sequence is symmetrically located within the 9 base pair target DNA sequence that is duplicated during Tn*10* insertion. Tn*10* has inverted repeats of an insertion sequence at its ends. IS-10-right provides the Tn*10*-encoded transposase, necessary for normal Tn*10*-transposition (**B**). Expression of this enzyme is regulated by two overlapping promoters, p-IN reading into the transposase gene and p-OUT reading out (**C**). Inhibition of translation of transposase is assumed to occur by direct pairing between the transposase mRNA and the complementary RNA specified by the pOUT promoter. In rare situations, if no *rho*-sites are present, pOUT can read into adjacent genes, in our case *lamB* (**B**). (Modified from KLECKNER et al. 1983; SIMONS and KLECKNER 1983; and HALLING et al. 1982)

the gene in which they insert and have strong polar effects on downstream genes. Here, however, we found a Tn*10* insertion with a very peculiar localization, in the 75 base pair intergenic region between the genes *malK* and *lamB*. The *malK-lamB* boundary is a preferential site for F-factor integration and for deletion endpoints (HOFNUNG et al. 1974), suggesting that this interval may contain sequences favoring DNA rearrangements. Tn*10* inserts at high efficiency into favored sites, which occur an average of every 1000 base pairs of target DNA (reviewed in KLECKNER 1983). Tn*10* also recognizes a large number of sites less specifically but at a much lower efficiency. The insertion hot spots

share a symmetrical base pair consensus sequence (see Fig. 15A). A related sequence is found between *lamB* and *malK*.

The insertion *zjb-729*::Tn10 allows *malT*-independent low-level expression of the *lamB* gene from a Tn*10* promoter (probably pOUT) at 20%–25% that of a $malT^c$ constitutive strain (BRASS et al. 1984; see also Sect. 3.3). The properties of *zjb-729*::Tn*10* suggest its use in situations in which it is desirable to infect *malT* or polar *malK* mutations, or in glucose grown cells, with phage having λ host range. This Tn*10* can potentially also be used to study the expression and function of the *E. coli lamB* product in other enteric bacteria. Activation of adjacent genes by Tn*10* has also been described recently for the *ilv* and *his* operons of *S. typhimurium* (BLAZEY and BURNS 1982; CIAMPI et al. 1982).

Tn*10*-dependent expression of *lamB* was not induced by tetracycline. This lack of induction is expected if transcription comes from pOUT, a strong outward-directed promoter found near the outside end of IS10-right and IS10-left of Tn*10*. It is believed that pOUT modulates the expression of the transposase gene responsible for transposition of the Tn*10* element. The transcript specified by pOUT is postulated to inhibit translation of the coding region of the transposase gene by pairing directly with the message of pIN, the transposase promoter (see Fig. 15B, C). Regulation of gene expression by antisense RNA is a new mechanism which was first described for the Tn*10* elements. [A related mechanism, translational inhibition by a complementary RNA transcript (micRNA), was recently postulated as being involved in osmoregulation of the OmpF and OmpC outer membrane porins (MIZUNO et al. 1984).]

5.7 Secretion of Maltose Binding Protein and Maltoporin

Proteins are synthesized on ribosomes in the cytoplasm. All proteins of the cell envelope have to be translocated through membranes. How does the cell discriminate between proteins meant to stay in the cytoplasm and those destined for export? How does the cell guide the secreted protein to the appropriate layers of the cell envelope? Porins stuck in the inner membrane would probably kill the cell, and MBP sent to the outer membrane could stimulate neither transport nor chemotaxis. For the right localization, the amino acid sequence of an exported protein must contain topological information. It is generally assumed that the NH_2-terminal signal sequence plays an essential role in protein sorting. This sequence (between 15–30 amino acids long) is found transiently on precursors of excreted proteins during their synthesis. All periplasmic proteins and all outer membrane proteins studied so far are synthesized as precursors containing a signal sequence. In addition to the signal sequence, regions of the mature protein may contribute to a proper localization. It has been shown that export of proteins requires energy. The form of energy required is an electrochemical potential gradient and not ATP (DATE et al. 1980).

Because its components are localized in three different layers of the cell envelope, the maltose transport system has been used as an attractive model system for numerous studies on prokaryotic secretion processes. This area has been extensively reviewed in the last years (MICHAELIS and BECKWITH 1982;

BENSON et al. 1985). MBP and maltoporin are synthesized with an NH_2-terminal signal sequence. Precursor MBP and maltoporin molecules are processed co-translationally. The signal sequence of MBP (26 residues) is split off by an inner membrane signal peptidase, but only after most of the nascent chain has been synthesized (JOSEFSSON and RANDALL 1981). Maltoporin is probably also processed by an inner membrane peptidase.

There is little homology among the amino acid sequences of signal peptides. They are, however, quite similar with respect to polarity. All of them have a short N-terminal hydrophilic portion (2–8 mainly basic amino acids), followed by a long stretch of hydrophobic amino acids. Inouye and coworkers have proposed that the basic residues at the extreme NH_2 terminus of the signal sequence are responsible for the initial interaction of the nascent polypeptide with the negatively charged surface of the cytoplasmic membrane. Subsequently, the hydrophobic core loops into the lipid bilayer to initiate protein translocation across this membrane (DIRIENZO et al. 1978) (see Fig. 16).

Gene fusions have been used to study protein export. Mutants defective in export could easily be selected by taking advantage of the unusual properties of certain *lamB-lacZ* or *malE-lacZ* fusion-bearing strains. Fusion strains sensitive to induction by maltose (probably because large amounts of hybrid proteins plug the inner membrane export machinery) or fusion strains with a Lac^- phenotype (because insertion of the hybrid LamB-LacZ protein into the outer membrane interferes with formation of β-galactosidase tetramers) were used as starting strains. Maltose resistant suppressor mutations that relieve overproduction lethality or lead to a Lac^+ phenotype were found. They could be identified as signal sequence mutations. These mutant strains no longer secrete the hybrid protein but accumulate unprocessed precursors in the cytoplasm. In addition, unlinked suppressor mutations were found in genes specifying proteins of a supposed export machinery (see Fig. 16; genes *secB, prlE*). The location of membrane-associated hybrid proteins was also altered in these second-site suppressor mutants.

Signal sequence mutations that alter the hydrophobic core (e.g., introduction of a charged residue) adversely affected MBP export (BEDOUELLE et al. 1980). This supports the idea that the hydrophobic nature of the signal sequence is essential. The same holds true for the *lamB* signal sequence (EMR et al. 1980). Mutations in the hydrophilic region of the signal sequence which change positively charged arginine to uncharged serine strongly reduce *lamB* expression (HALL et al. 1983). The finding that outer membrane protein synthesis is reduced by mutations which affect initiation of export clearly demonstrates that synthesis and export are tightly coupled.

Signal sequence mutations crossed into a wild-type *lamB* gene block the export of LamB. Cells carrying these mutations are therefore unable to grow on dextrins. Using such Dex^- strains, and comparable *malE* signal sequence mutations (Mal^-), it has been possible to identify second-site mutations that suppress the Dex^- and Mal^- phenotypes (reviewed in BENSON et al. 1985). Certain alleles of *prlA* phenotypically suppress export-defective signal sequence mutations in *lamB* and *malE* (EMR and BASSFORD 1982), as well as in *ompF* and *phoA* (*phoA* codes for the periplasmic alkaline phosphatase). The *prlA*

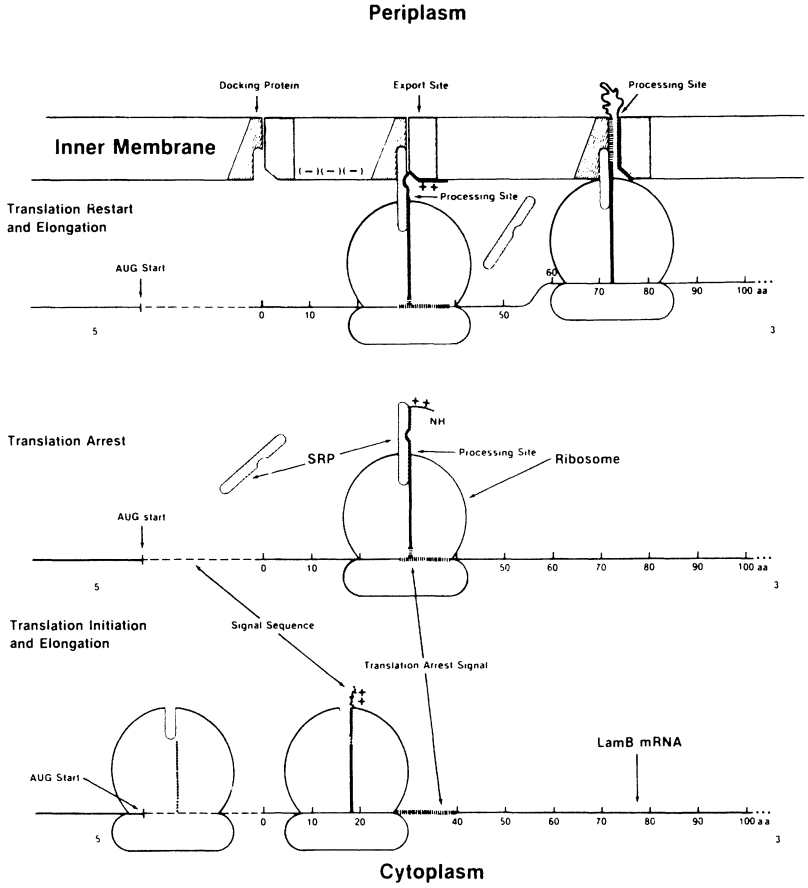

Fig. 16. Initiation of maltoporin secretion. According to the signal sequence hypothesis translation of maltoporin monomers is initiated by soluble ribosomes. Translation proceeds only until the signal sequence emerges and is recognized by a component of the export machinery, the signal recognition particle, *SRP* (the *E. coli* PrlA protein could be part of a hypothetical *E. coli*-SRP, which has so far only been proven in eukaryotes). Interaction of the polysome with hypothetical docking proteins releases translation arrest and the signal sequence loops into the inner membrane bilayer. The *E. coli* SecA protein could be part of the prokaryotic equivalent of docking protein which relieves translation arrest imposed by SRP. As synthesis proceeds, the nascent chain is transferred vectorially across the membrane. After cleavage of the signal sequence at the processing site, the protein is discharged on the opposite side of the membrane. (Reproduced with permission of the Annual Review of Biochemistry, Vol 54, © 1985 by Annual Reviews Inc. from BENSON et al.,1985)

mutations do not cause any growth defect and do not alter protein export. *prlA* maps at 72 min, at the end of an operon specifying ribosomal proteins. The *prlA* gene product has recently been detected as an integral inner membrane protein of 50000 daltons (AKIYAMA and ITO 1985). The observed allele specificity between different *prlA* alleles and various *lamB* and *malE* signal sequence mutations suggests a direct interaction of the signal sequence and the PrlA protein. Similar selection procedures have revealed a number of other genes whose prod-

ucts may be components of the cellular export machinery. In many cases, however (except *prlA* and *secA*) the evidence for involvement is weak (reviewed in BENSON et al. 1985). PrlA could be a component of the prokaryotic equivalent of the signal recognition particle (SRP), which is known to be part of the eukaryotic export machinery (WALTER and BLOBEL 1982). However, care has to be taken in the interpretation of such export defective mutations. The *secA* phenotype (block of synthesis and export of secreted proteins) was thought to indicate that the SecA protein could be required to relieve translational arrest after contact of the SRP-bound signal sequences with the inner membrane export machinery (see Fig. 16). Recently, however, it became clear that synthesis inhibition in *secA* amber mutants is only a secondary consequence of its primary export deficiency due to strongly reduced cAMP levels. Addition of cAMP restored synthesis but did not restore secretion of MBP and maltoporin in *secA* cells (STRAUCH et al. 1986).

What signal directs maltoporin into the outer membrane and MBP into the periplasm? It was suggested that a specific interaction of outer membrane proteins with LPS might function both as the requirement for proper translocation of proteins (e.g., OmpA) into the outer membrane and as a determinant of stable interaction of this protein in the outer membrane (DATTA et al 1977). A recent study showed however, that OmpA finds its way into the outer membrane even in the absence of synthesis and excretion of LPS (RICK et al. 1983). Thus, LPS is probably not involved in the sorting of secreted proteins. Recent results with hybrid proteins from strains carrying different *lamB-lacZ* fusions suggest that the first 49 amino acids of the mature maltoporin protein might contain the information for localization. Hybrid proteins with 39 or 43 amino acids of the mature LamB protein are not exported to the outer membrane, whereas a hybrid protein that is only slightly larger, containing 49 amino acids of the mature LamB, is found in the outer membrane (BENSON et al. 1984). These fusions locate the protein sequence that appears to guide LamB to the outer membrane to a region of amino acid homology shared by other major outer membrane proteins (NIKAIDO and Wu 1984).

A different approach was used to determine the nature of information required for export and sorting of the OmpA protein into the outer membrane. The location of the gene products of overlapping in frame deletions in the *ompA* gene was studied (FREUDL et al. 1985). Proteins lacking the amino acids 4–45, 43–84, 46–227, 86–227 or 160–325 of the mature protein were all efficiently translocated across the plasma membrane. The first two proteins were found in the outer membrane, the others in the periplasmic space. These data show that the information for export and localization of the OmpA protein lies within the protein conformation. The existence of a common sorting signal (first 14 amino acids; NIKAIDO and WU 1984) is doubtful, since this region is not essential for routing of OmpA.

A selective suppression of synthesis and export of some periplasmic proteins including MBP was recently described in strains that overproduce a C-terminal truncated periplasmic enzyme (GlpQ′). Inhibition of synthesis and secretion in these cells, which, in contrast to *secA* cells, exhibit normal cAMP levels provides evidence for a secretion-translation coupling of MBP. Interestingly,

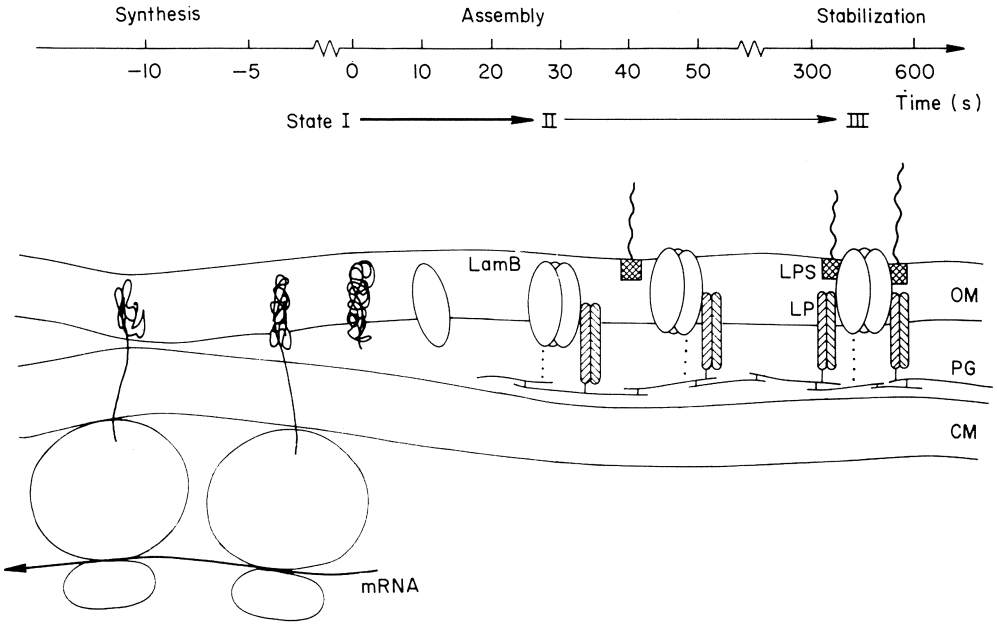

Fig. 17. Schematic representation of the consecutive stages in assembly of maltoporin. The synthesis of newly induced maltoporin monomers by membrane-bound ribosomes at sites of adhesion of outer and inner membrane takes about 30 s; after 60 s the first monomers could be detected in cell lysates by immunoprecipitation (*state I*). An additional delay of 30–50 s was observed until maltoporin trimer was exposed at the cell surface (*state II*). Stabilization of newly assembled trimers by interaction with outer membrane lipoprotein, LPS, and peptidoglycan could be followed by a change in the temperature needed to solubilize maltoporin (from 37° C to 70° C). Maturation to *state III* trimers is slow ($T_{1/2} = 6$ min). (Reprinted with permission of VOS-SCHEPERKEUTER and WITHOLT 1984)

secretion of outer membrane proteins including maltoporin was not affected. It is proposed that GlpQ′ blocks a yet unknown component of the secretory machinery, which is involved in sorting of exported periplasmic and outer membrane proteins (HENGGE and BOOS, in press).

Is LamB secreted all over the cell or preferentially at the septum? An early study using λ phage adsorption as a label for newly inserted LamB protein indicated specific secretion sites at the septum of dividing cells (RYTER et al. 1975). A new study, using anti-LamB trimer antiserum in combination with *Staphylococcus*-protein A – gold complexes, revealed that LamB is inserted at more than 200 randomly located sites per cell (VOS-SCHEPERKEUTER and WITHOLT 1984; VOS-SCHEPERKEUTER et al. 1984). It was suggested by these authors that the earlier results may have reflected differences in accessibility of LamB to the virus probe rather than differences in the location of outer membrane insertion sites. Direkt insertion via transient zones of adhesion has been proposed. A maturation process could be resolved whereby newly synthesized LamB monomers are converted into stable, functional maltoporin trimers which interact with peptidoglycan, LPS, and lipoprotein (see Fig. 17).

6 Induced Changes of the Outer Membrane Barrier Function

The outer membrane permeability barrier can be overcome by a variety of treatments with different agents like EDTA and cations like Ca^{2+}. These techniques, developed within the last 20 years, have been extremely useful in numerous biochemical studies of the transport function of the cell envelope. They also have proved essential for genetic engineering by permitting uptake of large DNA molecules through the Ca^{2+}-permeabilized outer and inner membranes of gram-negative bacteria.

6.1 Effects of EDTA on the Outer Membrane

The importance of divalent cations to outer membrane assembly and maintenance was already shown more than 20 years ago by Repaske (Repaske 1958) and by Leive, who reported a nonspecific increase in the outer membrane permeability by treatment of cells with EDTA in the presence of Tris buffer (Leive 1965). It was shown that EDTA removes up to 50% of the cellullar LPS content, presumably by complexing the divalent cations which are involved in LPS-LPS interactions. Removal of the neutralizing cations from the negatively charged LPS molecules by EDTA was thought to destabilize the membrane structure by electrostatic repulsion of the LPS monomers. The synergistic effect of Tris was explained by proposing that this bulky primary amine would further destabilize the outer membrane by replacing other cations bound to LPS (Nikaido and Vaara 1985). Based on the observation of a strongly increased outer membrane permeability to β-lactam antibiotics in cells incubated in 200 m*M* NaCl it was shown that also other monovalent cations destabilize the outer membrane architecture, probably also by squeezing out the crossbridging divalent cations from binding sites in LPS (Homma and Nakae 1982) (see also Sect. 7.3). Tris is known to bind to LPS and dissociates LPS aggregates. Treatment with 100 m*M* Tris alone is sufficient to release LPS from *S. typhimurium* (Vaara and Hukari unpublished) and periplasmic alkaline phosphatase from *E. coli* into the cell-free supernatant (Brass unpublished).

Treatment with EDTA and Tris-buffer allows lysozyme access to the peptidoglycan matrix, resulting in the production of spheroplasts (Repaske 1958). EDTA-Tris treatment renders *E. coli* cells sensitive to a number of hydrophobic compounds, like actinomycin D (Leive 1974). Nikaido and Nakae proposed that the space occupied by the released LPS might be filled by phospholipids (Nikaido and Nakae 1979). Hydrolysis of phospholipids by an endogenous phospholipase was found to enhance the reorganization of the outer membrane responsible for the actinomycin D-sensitive phenotype (Hardaway and Buller 1979).

EDTA-Tris treatment, in combination with a cold osmotic shock (by dilution) of cells preincubated with 20% sucrose, allows exit and selective isolation of the periplasmic proteins and periplasmic oligosaccharides (MDOs) localized between the outer and inner membrane (Neu and Heppel 1965; Schulman and Kennedy 1979). This simple technique has greatly influenced the progress

in studies about bacterial transport systems. A possible mechanism for outer membrane permeabilization during osmotic shock has been suggested in a recent review (NIKAIDO and VAARA 1985). During preincubation the periplasm is filled with sucrose, which produces plasmolysis. Upon dilution, the osmotic imbalance of this system is corrected by efflux of sucrose through porins and influx of water. Because the former process is slow, whereas entrance of water is rapid, the outer membrane might be ruptured. However, since osmotic shock can also be performed with high concentrations of NaCl (500 m*M*; WRISSENBERG and BOOS unpublished), which should diffuse through the porins much faster than sucrose, other explanations might still also be possible.

EDTA-induced permeabilization of the outer membrane allowed access into the periplasm to a number of proteins, such as lysozyme (reviewed by LEIVE 1974) or colicin K (in a mutant lacking the outer membrane receptor for this colicin; TILBY et al. 1978). Entry in the presence of EDTA, however, appears to be restricted to those proteins which have a high affinity for some periplasmic components or the inner membrane. In spite of a very sensitive test system (see below) only negligible import (and/or retention) of periplasmic binding proteins could be observed (BRASS unpublished). EDTA treatment should be performed in a highly standardized manner, otherwise dramatic effects on cytoplasmic components and cellular metabolism have to be expected (LEIVE and KOLLIN 1967).

6.2 Ca^{2+}-Induced Permeabilization of the Outer Membrane

Unexpectedly, the structure of the outer membrane can also be destabilized by saturation with divalent cations. Ca^{2+} in particular appears to induce a nonspecific permeability. A simple explanation for this effect might be that incubation of the cells in high concentrations (m*M*) of divalent cations might overload LPS with positive charge and these "overdressed" LPS molecules repel one another. As described below, however, this simple picture is not complete.

We found that *lamB* and *malE* mutants, defective in diffeent components of the maltose transport system of *E. coli,* represent a sensitive test system to measure Ca^{2+}-induced permeabilization of the outer membrane. The impaired maltose transport of *lamB* cells (SZMELCMAN et al. 1976) could be partly restored by pretreatment of the cells with 25 m*M* Ca^{2+} (see Fig. 18). The induced permeability increase persisted for some time ($t_{1/2} = 30$ min) after transfer of the cells to Ca^{2+}-free medium at room temperature.

The Ca^{2+}-induced increase of outer membrane permeability was shown by us not to be due to a modified specificity of the general porins OmpF and OmpC, but rather due to microlesions in the outer membrane, since we found that even macromolecules like amylopectin (20000 daltons), lysozyme, and antibodies could easily be introduced into the periplasm. Incubation of Ca^{2+}-treated cells in the presence of periplasmic binding protein resulted in uptake of these proteins into the periplasm. Maltose transport could be reconstituted in *malE malT*^{c} mutants lacking the periplasmic MBP, but expressing all other maltose

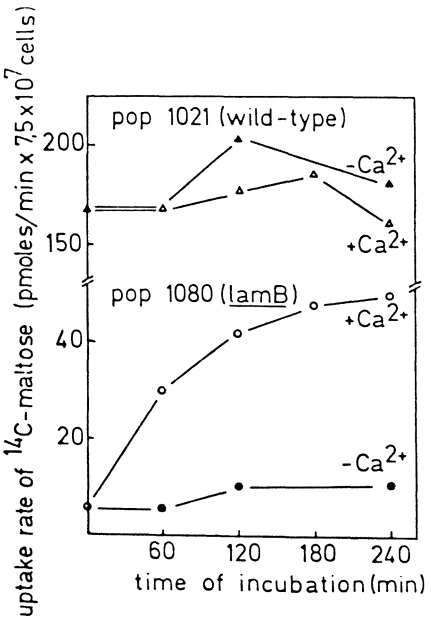

Fig. 18. Ca^{2+} induced permeabilization of the outer membrane as measured by restoration of maltose transport in *lamB* strains. Transport of maltose in *lamB* strains missing maltoporin is 100-fold reduced compared to wild-type at micromolar concentrations. Transport can be partially restored by pretreatment of the cells in Tris buffer containing 25 m*M* Ca^{2+} at room temperature. Maltose transport (measured after washing and transfer of the cells into minimal salt medium containing in addition 6×10^6 *M* ^{14}C-maltose) increased in cells pretreated for increasing times in Ca^{2+}, indicating a permeabilization of the outer membrane permeability barrier. (Reprinted with permission of BRASS et al. 1981)

transport components constitutively (BRASS et al. 1981) (see Fig. 22). The optimal conditions for Ca^{2+}-induced permeabilization of the outer membrane are discussed in detail in Sect. 7.3 and Chap. 8. Ca^{2+}-induced permeabilization of the outer membrane was also suggested by the pioneering report of Mandel and Higa on Ca^{2+}-induced competence of *E. coli* for DNA uptake, and from the observation that Ca^{2+}-treated cells show an increased sensitivity to hydrophobic antibiotics like actinomycin D, nigericin, and valinomycin (MANDEL and HIGA 1970; TAKETO 1974; SABELNIKOV and DOMRADSKY 1981) (see also Chapt. 8).

6.3 Effects of Polymyxin and Other Polycationic Compounds on the Outer Membrane

Interestingly, many membrane-active agents effective against gram-negative bacteria are cationic. These include oligopeptide antibiotics like polymyxin, tyrocidine, or gramicidin, quaternary ammonium disinfectants like chlorohexidine, and aminoglycoside antibiotics like streptomycin (reviewed in HANCOCK 1984). These polycations bind to LPS and alter its packing arrangement (PETERSEN et al. 1985). There is growing evidence that certain cationic host defense factors increase the permeability of the bacterial outer membrane (NIKAIDO and VAARA 1985). One example is the arginine-rich protein protamine, which is inhibitory to gram-negative cells (VAARA 1981). Another example is the bactericidal, permeability-increasing protein which is found in polymorphonuclear leukocytes (ELSBACH and WEISS 1983). The interesting topic of bactericidal cationic peptides involved in bacterial antagonism and host defense has been reviewed recently

(SAHL 1985). The mode of action of one bactericidal peptide, polymyxin, is described in detail below. Polymyxin is an amphipathic, polycationic decapeptide carrying five positively charged groups and a hydrophobic fatty acid tail. Permeabilization of the outer membrane of *S. typhimurium* by polymyxin was observed (CERNY and TEUBER 1971; VAARA and VAARA 1981). Sensitivity of the cells to lysozyme and loss of periplasmic proteins were observed. Initial binding to the outer membrane appears to be an interaction of polymyxin with the anionic divalent-cation binding site in the KDO group of LPS (SCHINDLER and OSBORN 1979). Polymyxin appears to displace bound cations competitively, since its effects are blocked by higher concentrations of magnesium. The structural alterations of the outer membrane of polymyxin – treated cells which were see in the electron microscope were explained as LPS-polymyxin associations following intercalation of polymyxin into the outer leaflet of the outer membrane (LUNATAMAA et al. 1976). After self-promoted permeabilization of the outer membrane, the antibiotic integrates into the inner membrane via its hydrophobic tail, thereby destroying the barrier function of this membrane.

The dual target specificity of polymyxin for the outer and inner membrane could be resolved by papain cleavage of polymyxin, which results in a polymyxin nonapeptide (PMBN) which has lost its N-terminal diamino-butyric acid residue with its attached fatty acid. PMBN alone, like polylysin, another polycationic agent, has only very low antibactericidal activity against *S. typhimurium* and *E. coli,* but enhances sensitivity to hydrophobic agents like novobiocin, erythromycin, actinomycin D, and the lethal effects of the complement cascade (VAARA and VAARA 1983).

With our sensitive reconstitution assay (see Sect. 7.2) we tested whether PMBN-induced permeabilization of the outer membrane allows penetration of macromolecules through the outer membrane. The fact that *malE* mutants treated with PMBN in the presence of MBP could (in contrast to Ca^{2+}-treated cells) not be reconstituted for maltose transport (BUKAU and BRASS unpublished) shows that polymyxin-induced outer membrane lesions are much smaller than Ca^{2+}-induced lesions.

Isolated LPS from polymyxin resistant strains of *S. typhimurium* (*pmrA*) showed a novel type of alteration in lipid A structure. The ester-linked phosphate in lipid A is replaced by 4-amino-L-arabinose. This substitution eliminates a negative charge and introduces a positive charge (VAARA et al. 1981). This explains the decreased binding of polymyxin to the mutant LPS. Some polymyxin resistant mutants (*pmx*) are also resistant to the action of other polycationic compounds, like polylysine, and also become refractory to EDTA (reviewed in HANCOCK 1984; NIKAIDO and VAARA 1985).

Polycationic aminoglycoside antibiotics like streptomycin and gentamycin apparently enter the periplasm of *Pseudomonas aeruginosa* not via porins, but in a self-promoted pathway. It was suggested that this involves displacement of divalent cations from LPS, thus destroying the LPS crossbridging and destabilizing the outer membrane (reviewed in HANCOCK 1984). This idea is supported by recent experiments in which binding of different polycationic aminoglycoside antibiotics and polymyxin to LPS of *Pseudomonas aeruginosa* was measured by the displacement of a cationic spin probe from LPS into the aqueous environ-

ment upon addition of competitive cations. The more highly charged cations were found to be more effective at displacing the probe. The relative affinity of several antibiotics for LPS correlated with their ability to increase outer membrane permeability (PETERSON et al. 1985). Permeabilization of the outer membrane by aminoglycosides results in enhanced sensitivity to lysozyme, β-lactams, and hydrophobic dyes, and possibly also in the uptake of the antibiotic itself.

7 Reconstitution of Binding-Protein-Dependent Transport

Much of the evidence for the involvement of periplasmic binding proteins as essential substrate recognition components of specific transport systems (and as chemoreceptors in chemotaxis) is based on genetic data. The direct biochemical proof for involvement of binding proteins requires a demonstration of restoration of transport by addition of a purified binding protein to a system lacking this component.

Moreover, reconstitution experiments are essential for understanding the mechanism of action of binding protein dependent transport systems, since they allow transport studies in simpler systems such as membrane vesicles and finally (but far in the future) in a liposome system.

7.1 Reconstitution Experiments with Shocked Cells, Spheroplasts, and Vesicles

In early reconstitution studies, binding protein was removed from the periplasm by the osmotic shock procedure (NEU and HEPPEL 1965). The stimulation of transport which was seen after addition of binding protein to shocked cells was not due to transport reconstitution, but rather to artifacts arising from residual and resynthesized binding protein (RAE et al. 1976; GERDES et al. 1977). Successful reconstitution of phosphate, glutamate, and ribose transport in *E. coli* was reported by addition of the respective binding proteins to spheroplasts (BARASH and HALPERN 1971; GERDES et al. 1977; GALLOWAY and FURLONG 1979). Reconstitution using spheroplasts derived from a binding protein deficient mutant, however, has been reported only in the case of the ribose transport system (ROBB and FURLONG 1980). Several disadvantages are inherent in the spheroplast procedure: the preparation of spheroplasts is laborious, the quality of preparations is variable, and transport assays have to be performed in the presence of high concentrations of binding protein and of sucrose to prevent lysis of the spheroplasts.

An important development in reconstitution procedures was achieved when inner membrane vesicles were used instead of spheroplasts. This interesting technique was developed to allow studies of the energization of binding protein mediated transport systems (HUNT and HONG 1981, 1983; ROTMAN and GUZMAN 1984). A final conclusion about the nature of the energy coupling could, how-

ever, not yet be drawn from these studies, since it turned out that the vesicles are capable of metabolism (HUNT and HONG 1983). Another problem with these vesicle reconstitution experiments is the very low level of reconstituted transport, which is in the order of 1% of that of a corresponding suspension of intact cells (reviewed by AMES, in press).

7.2 Reconstitution by Import of Exogenous Binding Protein into the Periplasm of Ca^{2+}-Treated Cells

We have described a new method for reconstitution of binding protein mediated transport in whole cells, which is based on the reversible Ca^{2+}-induced permeabilization of the outer membrane (see Sect. 6.2). The introduction of exogenous binding protein into the periplasm of binding protein deficient mutants is accomplished by incubating cells and binding protein in the presence of 300 m*M* Ca^{2+} at 0° C. Several advantages of this approach are evident:

(a) Reconstitution experiments with whole cells are highly reproducible, much simpler than those with spheroplasts and vesicles, and reflect a more natural situation (for comparison see (Fig. 19).

(b) Due to resealing of the outer membrane after transfer of the cells into Ca^{2+}-free medium, extracellular binding protein can be washed off prior to the transport assay; the cells retain their reconstituted transport ability for hours.

(c) The efficiency of the technique as judged by the K_m and V_{max} values of reconstituted maltose transport is high: K_m is identical with the wild-type value, V_{max} is about 30% of the wild-type value (see Sect. 7.4).

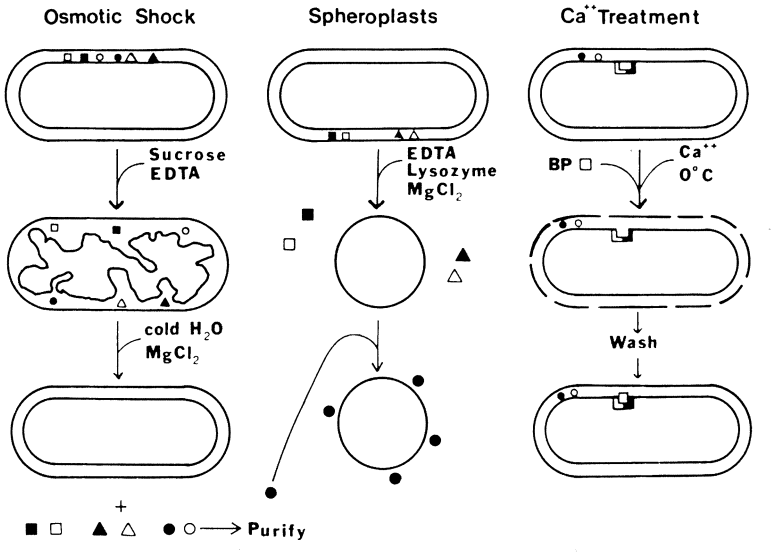

Fig. 19. Comparison of the different approaches used to reconstitute binding-protein-dependent transport in spheroplasts and whole, Ca^{2+}-treated cells. (Reprinted with permission of FURLONG and IIDA 1984)

(d) The technique also allows reconstitution of binding protein mediated chemotaxis (see Chap. 9).

The whole cell reconstitution approach with Ca^{2+}-treated cells also has, of course, the disadvantage of being a more complicated system than the vesicle system especially. This might limit its applicability in some instances.

7.3 Conditions Affecting Entry and Function of Exogenous MBP in Ca^{2+}-Treated Cells

Permeabilization of the outer membrane as measured by reconstitution of maltose transport in *Δ malE444 malT*^{c} mutants of *E. coli* after addition of exogenous MBP is dependent on the presence of high concentrations of divalent cations (BRASS et al. 1983). While treatment of *E. coli* with moderate concentrations (200 m*M*) NaCl caused a dramatic increase in the nonporin mediated diffusion rate of β-lactam antibiotics through the destabilized outer membrane (HOMMA and NAKAE 1982), monovalent cations turned out to be completely inefficient in promoting entry of MBP into the periplasm. Besides Ca^{2+}, other divalent cations such as Ba^{2+}, Sr^{2+}, Mg^{2+}, and Ni^{2+} are also able to permeabilize, with the efficiency decreasing in that order (BUKAU et al. 1985).

The calcium concentration in the reconstitution mixture is a critical factor. Reconstituted transport activity increases linearly with the Ca^{2+} concentration up to 400 m*M* when cells are pretreated with MBP at 0° C. The viability of the cell remains high (80%) at all Ca^{2+} concentrations tested. Recently, it has become clear that the plasmolysis of the cells caused by these high Ca^{2+} concentrations is a prerequisite for optimal reconstitution (BUKAU et al. 1985). The effect of plasmolysis on the outer and inner membranes is discussed in more detail in Chap. 8.

Interestingly, Ca^{2+} by itself is inefficient in promoting reconstitution. The combined action of Ca^{2+} and phosphate ions appears to be necessary (BUKAU et al. 1985). Cells cannot be reconstituted when the phosphate-containing growth medium is washed away with Tris buffer or NaCl, prior to the addition of Ca^{2+}. Compared to cells washed in Tris alone, cells washed in Tris plus increasing phosphate concentrations (pH 7.5) show a dramatic increase in transport activity. The phosphate optimum in this pretreatment is around 100 m*M* (see Fig. 20). Thus, it is perhaps more accurate to speak of calcium-phosphate-induced, rather than Ca^{2+}-induced reconstitution. The way in which Ca^{2+} and phosphate ions act together to permeabilize the outer membrane is discussed in Chap. 8.

Temperature strongly influences reconstitution, which becomes dramatically better when the temperature is lowered from 20° C to 0° C. The low temperature optimum indicates that a low fluidity of LPS and phospholipid molecules is essential for outer membrane permeabilization. Differential scanning calorimetry with whole cells of *E. coli* showed only one thermal transition melting of the lipids between −5° C and 10° C for both membranes (MELCHIOR and STEIM 1976) (isolated outer membranes exhibit artificially high transition temperatures due to the redistribution of lipids mentioned above, see Sect. 2.1). The

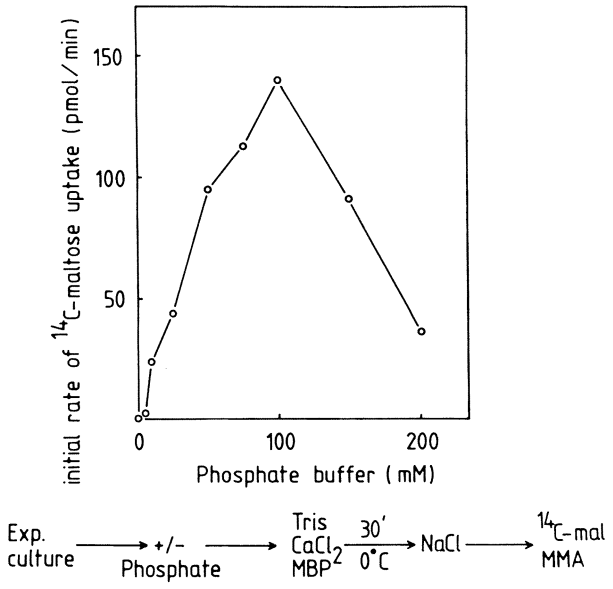

Fig. 20. A combined action of phosphate and calcium-ions is essential for reconstitution. An exponential culture of a nonpolar *malE444* mutant strain lacking MBP was pretreated with Tris buffer without or with increasing concentrations of potassium phosphate. After centrifugation, the cells were resuspended in the reconstitution mixture (100 m*M* Tris buffer, pH 7.5; 300 m*M* Ca^{2+}; 0.25 m*M* MBP) and incubated for 30 min at 0° C. After washing in NaCl initial rates of reconstituted maltose transport were measured at 6 μ*M* ^{14}C-maltose in minimal medium A. (Modified from BUKAU et al. 1985)

fluidity of LPS is probably reduced much below its normal value at 0° C in the Ca^{2+}-treated cells due to binding of Ca^{2+} or calcium-phosphate to LPS. Ca^{2+}- and calcium-phosphate-induced phase transitions and separation of phospholipid mixtures are well documented (OHNISHI and ITO 1974; FRALEY et al. 1980).

MBP enters the periplasm rapidly; high rates of reconstituted transport are already seen in cells incubated with MBP for less than 1 min. Cells incubated for longer periods (up to 30 min) show only slightly higher transport activity. The reconstituted transport activity remains stable over the next 2 h, even after repeated washing of the cells. The rapid entry of MBP and its very slow exit after shift of the cells into NaCl or minimal medium indicate either that the outer membrane reseals immediately after removal of Ca^{2+} or that there are high-affinity binding sites for MBP in the periplasm (BRASS et al. 1983). This question was further pursued using *malE* signal sequence mutants with strongly reduced periplasmic MBP content (7% of the wild-type amount; BEDOUELLE et al. 1980; MANSON et al. 1985) in which MBP is fully active but rate limiting for maltose transport. These mutants show only a 40% decrease of maltose transport activity during an incubation in Ca^{2+} under standard conditions for 60 min (BUKAU 1986). Wild-type cells release only 5% of their MBP content during a Ca^{2+} treatment under standard conditions at 0° C. The finding that

MBP readily enters the periplasm of Ca^{2+}-treated *ΔmalE* cells (see above), whereas exit of MBP is low in Ca^{2+}-treated *malE*$^+$ cells, argues for the presence of binding sites for MBP in the periplasm. It is not clear at present whether the observed binding of MBP is a consequence of the presence of Ca^{2+} (Ca^{2+}-MBP crossbridges with other negatively charged compounds in the periplasm are conceivable), or if binding also takes place in the absence of Ca^{2+} (see also Chap. 10).

The ability of cells to take up MBP into the periplasm decreased at the end of the exponential growth phase. This decrease probably reflects the profound alterations in the cell envelope which occur at this stage of growth. Interestingly, an *lpo* mutant, which lacks lipoprotein and therefore the normal covalent linkage of the outer membrane with the rigid murein layer (HIROTA et al. 1977), could still take up MBP after being in stationary phase for 15 h (BUKAU 1986).

7.4 The Inner Membrane Is Not Significantly Permeabilized by Ca^{2+}

Under standard conditions for reconstitution (BRASS et al. 1983) the inner membrane apparently was not permeabilized to the same extent as the outer membrane. This can be concluded from the high viability of the cells subjected to the reconstitution procedure (80%). Ca^{2+}-treated cells which were transferred into new growth medium resumed growth without any lag, indicating that the high external Ca^{2+} concentration had not equilibrated with the cytoplasm. A dramatic disturbance of metabolism and strong effects of the proton motive force driven Ca^{2+}-export system (BREY and ROSEN 1979) on the membrane potential would otherwise have been expected.

The extent of inner membrane leakiness can be directly judged from experiments in which we treated cells with fluorescently labelled macromolecules (MBP, GBP, dextrans) in the presence of 300 m*M* Ca^{2+}. The ratio of uniformly fluorescent cells (cytoplasmic localization of MBP) to cells exhibiting only peripheral, periplasmic fluorescence was about 1/1000 (BRASS et al. unpublished; see also Chap. 10).

Conditions for transfer of low molecular weight proteins through the inner membrane have been described; they involve extreme plasmolysis with 1.6 *M* sucrose, and treatment with the polycationic dye spermidine (IRBE and OISHI 1980). Cells subjected to this treatment lose their viability unless supplied with small molecular weight solutes like amino acids, nucleotides, etc. This system should be useful for developing a bioassay for regulator proteins.

The outer membrane may be more sensitive than the inner membrane to Ca^{2+} treatment since it is linked covalently, via lipoprotein (BRAUN and REHN 1969), and noncovalently, via porins and OmpA protein, to the rigid murein layer (ROSENBUSCH 1974). Microlesions may occur because these bonds do not allow shrinkage of the membrane after binding of Ca^{2+}. Ca^{2+}-induced shrinkage of artificial membranes has been reported (DAWSON and HAUSER 1970). Some physicochemical characteristics, such as outer membrane-inner membrane junctions, a high phosphatidylethanolamine content, and the presence of LPS

bound to protein, may also contribute to a higher sensitivity of the outer membrane, and might favor nonbilayer phases (reviewed in LUGTENBERG and VAN ALPHEN 1983). The fact that parts of the LPS-fatty acid acyl chains carry hydroxyl groups may also destabilize the outer membrane bilayer.

7.5 Characteristics of Reconstituted Maltose Transport in Ca^{2+}-Treated Cells

Addition of exogenous MBP allows reconstitution of maltose transport in *malT*^{c} strains carrying the nonpolar deletion *ΔmalE444*, but not in the *malT*^{c} *ΔmalB112* control strain carrying a deletion covering four genes necessary for maltose uptake (see Fig. 12). Addition of sodium azide (20 m*M*) to the uptake mixture completely inhibits maltose uptake in reconstituted cells, showing that reconstituted transport is due to active transport (BRASS et al. 1983).

The apparent K_m of reconstituted maltose transport in *ΔmalE malT*^{c} cells was found to be identical to that of wild-type cells (2 μ*M*). V_{max} of reconstituted transport was 20%–40% that of wild-type cells (BRASS et al. 1983). This showed that the reconstitution procedure developed by us is a gentle method, allowing normal function of all components involved in maltose transport. We conclude that the different domains of the MBP molecule have a high stability and are not inactivated during the purification procedure, which employs different freeze drying steps. MBP denatured in 8 *M* urea and renatured by dialysis showed normal binding of substrate, but only about 10% of its original activity in reconstitution experiments (BRASS unpublished).

A *lamB malE* strain was also reconstituted; as expected, these cells required a much higher maltose concentration (in the 100 μ*M* range) to achieve good rates of maltose transport. Similarly, when MBP from a *malE* Dex$^-$ mutant was used for reconstitution, cells showed the same elevated K_m for maltose transport as the original mutant (BECK and BRASS unpublished). We are encouraged to believe that the transport properties of reconstituted cells reflect the properties of the strains and binding proteins employed rather than artifacts of the reconstitution technique.

7.6 Different Approaches for Measuring the Affinity of MBP for Its Protein Partners in Maltose Transport

The reconstitution system with Ca^{2+}-treated whole cells allows variation of the periplasmic concentration of MBP without changing the copy number of the other membrane bound maltose transport components (maltoporin and the complex formed by the MalF, G, and K proteins). We wanted to use reconstitution to study the interaction of MBP with its partners in transport by determining the affinity of MBP for these proteins. The concentration of MBP needed to achieve half-maximal reconstitution in *malT*^{c} *ΔmalE* cells was about 1 m*M* MBP (see Fig. 21); that of *malT*^{c} *malE lamB* cells was not much different (twofold lower; BECK and BRASS unpublished). These concentrations are similar

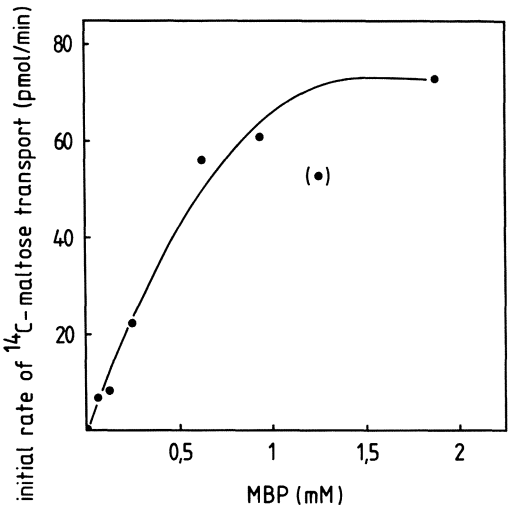

Fig. 21. Concentration optimum for purified MBP in reconstitution of maltose transport in a nonpolar *malE* mutant. Samples of 1×10^9 cells were incubated for 30 min at 0° C in a reconstitution mixture containing 100 m*M* Tris buffer, pH 7.2, 250 m*M* $CaCl_2$ and different concentrations of MBP (0–75 mg/ml). After wshing of the cells the efficiency of reconstitution was determined by measuring the initial rates of reconstituted maltose transport. (From BRASS et al. 1983, modified)

to the estimated MBP concentration in the periplasm of maltose-induced wild-type cells (DIETZEL et al. 1978). It is important to know if the dependence of reconstitution on MBP concentration is determined by the interactions of MBP with other proteins, or rather by some aspect of the process by which MBP passes through the Ca^{2+}-permeabilized outer membrane. This question must remain open, however, since we do not really know at present whether MBP equilibrates freely between the external medium and the periplasm, or how the periplasmic volume is affected by plasmolysis during the Ca^{2+} treatment. We are presently trying to solve these questions by measuring the concentration of MBP in the periplasm of reconstituted cells.

An independent approach to measure the affinity of MBP for its partners in transport (and chemotaxis) was performed by others (MANSON et al. 1985). They tested maltose transport and chemotaxis (see Sect. 9.4) in different *malE* signal sequence mutants (BEDOUELLE et al. 1980) with strongly reduced amounts of periplasmic MBP, but normal expression of *lamB*- and *malF,G,K*-genes. They found that strains with tenfold reduced MBP concentrations (about 100 μ*M*) still had transport rates about 60% those of wild-type cells. The discrepancy between the results of the reconstitution approach and the signal-sequence mutation approach might indicate that MBP cannot equilibrate freely between the external medium and the periplasm in calcium-treated cells. The signal-sequence approach thus appears to be more appropriate to determine the MBP dependence of maltose transport (see, however, Sect. 9.4).

7.7 General Applicability of the Ca^{2+}-Dependent Reconstitution Procedure

The Ca^{2+}-dependent reconstitution procedure is generally applicable. Besides maltose transport in *malE* mutants of *E. coli,* the binding-protein-dependent galactose transport of *E. coli* could be reconstituted by addition of shock fluid

Table 3. General applicability of the Ca^{2+}-dependent reconstitution procedure with whole cells. (From BRASS et al. 1983)

Strain	Relevant genotype	Treatment	Conc (mg/ml) of shock fluid (strain)	Initial rate of transport (pmol/min per 7.5×10^7 cells) of:		
				Maltose (6×10^{-6} *M*)	Galactose (5×10^{-7} *M*)	Histidine (3×10^{-8} *M*)
E. coli						
JB3018-2	*malE*$^+$	MMA	–	597		
		Ca^{2+}	–	423		
HS3018	*ΔmalE*	Ca^{2+}	–	0.22		
		Ca^{2+}	20 (pop1080)	94.8		
		Ca^{2+}	20 (LT2)	79.8		
LA5539	*mgl*$^+$	MMA	–		81.1	
		Ca^{2+}	–		6.7	
LA6021	*mgl*	Ca^{2+}	–		0.16	
		Ca^{2+}	40 [LA5709 (pHG4)]		17.5	
LA6022	*mgl*	Ca^{2+}	–		0.00	
		Ca^{2+}	40 [LA5709 (pHG4)]		20.4	
LA6028	*mgl*	Ca^{2+}	–		0.00	
		Ca^{2+}	40 [LA5709 (pHG4)]		0.02	
S. typhimurium						
TA271	*hisJ*$^+$ *dhuA*	Vogel-Bonner				0.80
		Ca^{2+}				0.56
TA1835	*hisJ hisP*	Ca^{2+}	–			0.014
		Ca^{2+}	40 (TA271)			0.00
TA2918	*hisJ*	Ca^{2+}	–			0.29
		Ca^{2+}	40 (TA271)			0.80

containing high amounts (90% of total protein) of galactose-binding protein (GBP). The method is fast and easy enough to be used as a screening procedure to distinguish mutations affecting only periplasmic components of a transport system from those affecting inner membrane transport proteins. Strains lacking only GBP (LA6021 and LA6022) can be easily distinguished from those also defective for the *mglA*, *mglC*, and *mglE* gene products (LA6028) on the basis of competence (or incompetence) for reconstitution of galactose transport (Table 3).

The procedue is also applicable to *S. typhimurium*. Histidine transport in *S. typhimurium* strain TR2918 (*hisJ*) lacking the histidine-binding protein could be restored by addition of shock fluid from wild-type strains. A deletion mutant covering the *hisP* gene in addition to *hisJ* (strain TA1835) could not be reconstituted (Table 3). The described procedure should also be applicable to a variety of other gram-negative bacteria such as *Aerobacter aerogenes* and *Klebsiella pneumoniae*. These bacteria are also capable of Ca^{2+}-induced uptake of DNA (TAKETO 1975).

8 Reconstitution Compared with Transformation

Bacterial transformation (uptake and expression of exogenous DNA) is an essential process in molecular cloning experiments. The efficiency of transformation depends on the efficiency of DNA uptake, the activity of the restriction systems of the recipient cell which degrade foreign DNA, and, for systems dependent on recombination of the introduced DNA into the host chromosome, the degree of homology of the introduced DNA sequences with the recipient DNA. Several bacterial species, such as *Bacillus subtilis* and *Hemophilus influenzae,* have developed the ability to transport DNA into the cell (SOLTYK et al. 1975; SOCCA et al. 1974). Very little is known about the mechanism of DNA uptake in bacteria such as *E. coli,* which have not developed a natural competence for transformation. Competence for uptake of DNA in *E. coli* can be induced with Ca^+ (MANDEL and HIGA 1970). The development of competence usually involves two stages: (a) incubation in Ca^{2+} and DNA at 0° C, and (b) a short temperature shock during which the temperature of the transformation mixture is increased up to 42° C. The temperature shock seems to be dispensable in some species of bacteria.

We investigated whether the Ca^{2+}-induced uptake of protein into the periplasm is mechanistically related to the Ca^{2+}-induced uptake of DNA during transformation. We compared the conditions required for transformation by plasmid DNA with the conditions necessary for reconstitution of maltose transport by exogenous MBP (BUKAU et al. 1985) (see Table 4). Under optimal conditions about 20% of the cells were fully competent for uptake of protein into the periplasm. This was seen by microscopic observation of single cells reconstituted in maltose chemotaxis (BRASS and MANSON 1984). The same conclusion could be drawn from microscopic observation of cells which were reconstituted with fluorescently labeled MBP. About 30% of the cells did take up the labeled protein and exhibited a high fluorescence, clearly localized peripherally in the periplasm (BRASS et al. 1986). The frequency of uniformly labeled cells (cytoplasmic localization of the fluorescent MBP) was much lower and in the range between 10^{-4} and 10^{-3}. Similarly, the fraction of cells competent for uptake of DNA into the cytoplasm is also very low. Under optimal conditions, using strains with high efficiency of transformation, uptake of plasmid DNA was seen in only about 0.1%–1% of the cells. With normal *E. coli* K12 strains (e.g., MC4100) this value is even lower. The much lower competence of the cells for uptake of DNA into the cytoplasm is probably due in part to the fact that the inner membrane is much less susceptible to Ca^{2+}-induced permeabilization than the outer membrane (see Sect. 7.4).

Plasmolysis of the Ca^{2+}-treated cells turned out to be an important prerequisite for optimal efficiency of transformation and reconstitution of transport. Sucrose strongly stimulated the efficiency of both processes at suboptimal Ca^{2+} concentrations (BUKAU et al. 1985). The stronger effect of plasmolysis on transformation compared with reconstitution (see Fig. 22) suggests that plasmolysis might also permeabilize the inner membrane. This is corroborated by the finding of others (IRBE and OISHI 1980) that cells subjected to extreme plasmolysis

Table 4. Comparison of transformation and reconstitution

	DNA-import in transformation	Binding-protein-import in reconstitution
I. Similarities		
Ca^{++}-concentration optimum	400 mM (BUKAU et al. 1985)	400 mM (BRASS et al. 1983)
Stimulation by plasmolysis	40-fold at 650 mOsM (BUKAU et al. 1985)	5-fold at 650 mOsM (BUKAU et al. 1985)
Temperature optimum		
(a) for growth of culture	37° C (TAKETO 1974)	37 °C (BUKAU 1986)
(b) for Ca^{++}-treatment	0 °C (TAKETO 1974)	0 °C (BRASS et al. 1983)
Growth phase dependence	Competent in exponential phase Incompetent in stationary phase (TAKETO 1974)	Competent in exponential phase Incompetent in stationary phase (BRASS et al. 1983)
pH-optimum	pH 7–8 (TAKETO 1974)	pH 7–8 (BRASS et al. 1983)
Ion specificity	$Ca^{++} \gg Ba^{++} > Sr^{++} > Mg^{++}$ (WESTON et al. 1981)	$Ca^{++} \gg Ba^{++} > Sr^{++} Mg^{++} = Ni^{++}$ (BUKAU et al. 1985)
II. Differences		
Phosphate dependence in pretreatment	3-fold increase at 0.1–5 mM, strong inhibition at phosphate-concentrations higher than 10 mM (BUKAU et al. 1985)	Optimum at 100 mM, absolute requirement of phosphate (BUKAU et al. 1985)
Heat shock	80 × increase (BUKAU et al. 1985; BERGMANS et al. 1981)	No increase (BUKAU et al. 1985)

take up low molecular weight proteins into the cytoplasm. These cells, however, are not viable.

An interesting difference between transformation and reconstitution became apparent when the role of the heat shock was examined. Proteins can pass through the outer membrane during Ca^{2+} treatment ot 0° C, whereas DNA can pass through the cell envelope only during or after a temperature shift of the Ca^{2+}-treated cells from 0° C to 42° C (BUKAU et al. 1985; MANDEL and HIGA 1970; BERGMANS et al. 1981). We examined the possibility that DNA might enter the periplasm during the Ca^{2+} treatment at 0° C. The finding that a high efficiency of transformation was seen only when exogenous DNA was still present during the heat pulse does not support this possibility. Rather, entry of DNA through the outer membrane likely occurs only during or after the heat pulse.

The conclusion that DNA might pass through the outer membrane during the heat step has also been reached by others (BERGMANS et al. 1981). The drastic effect of heat shock on the outer, and perhaps also on the inner membrane, is indicated by the significant increase in the release of periplasmic proteins from the heat-shocked cells (VAN DIE et al. 1983) and by their reduced viability compared to cells kept at 0 °C (BUKAU et al. 1985). A stronger perturba-

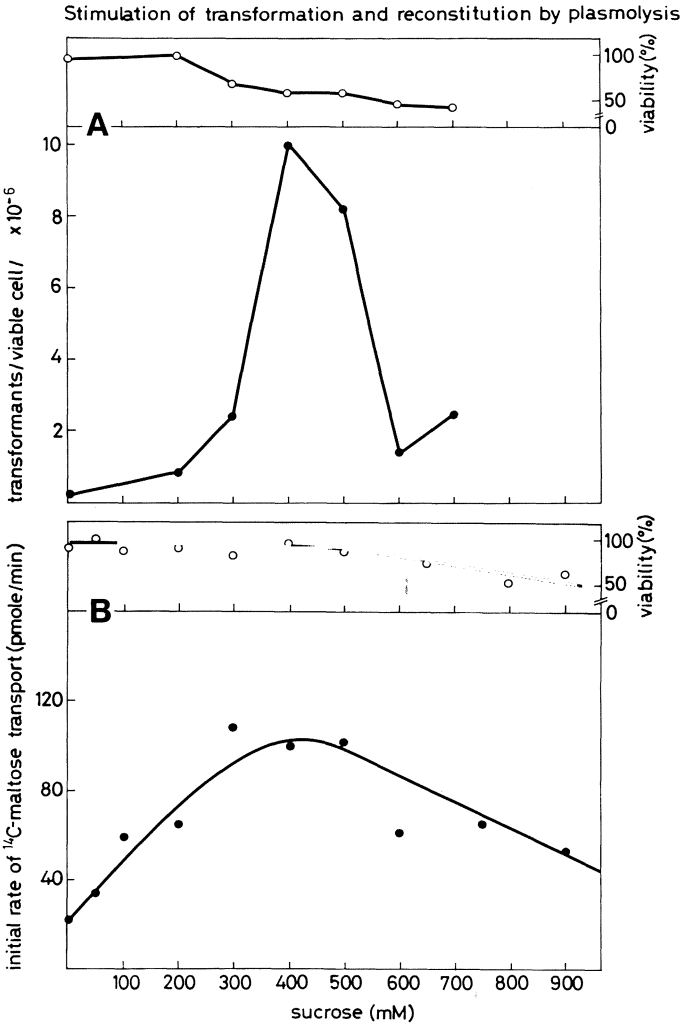

Fig. 22A, B. Plasmolysis stimulates transformation and reconstitution. *malE* cells were subjected to the standard procedure for transformation with plasmid pACYC184 and reconstitution of maltose transport with MBP (for details see BUKAU et al. 1985) except that the cells were treated with a suboptimal Ca^{2+} concentration (50 m*M*) and increasing concentrations of sucrose in the prewash and the transformation and reconstitution mixture. A five fold stimulation of reconstitution and a 40-fold stimulation of transformation by plasmolysis (400 m*M* sucrose) was observed. (Reprinted with permission of BUKAU et al. 1985)

tion of the membranes is obviously essential for uptake of large DNA molecules, whereas much smaller protein molecules can enter the periplasm during a Ca^{2+} treatment at 0° C. This hypothesis should be tested in future experiments with small DNA fragments.

As stated above (see Sect. 7.2), Ca^{2+} ions alone appear to be ineffective in permeabilization of the outer membrane for entry of proteins. A pretreatment

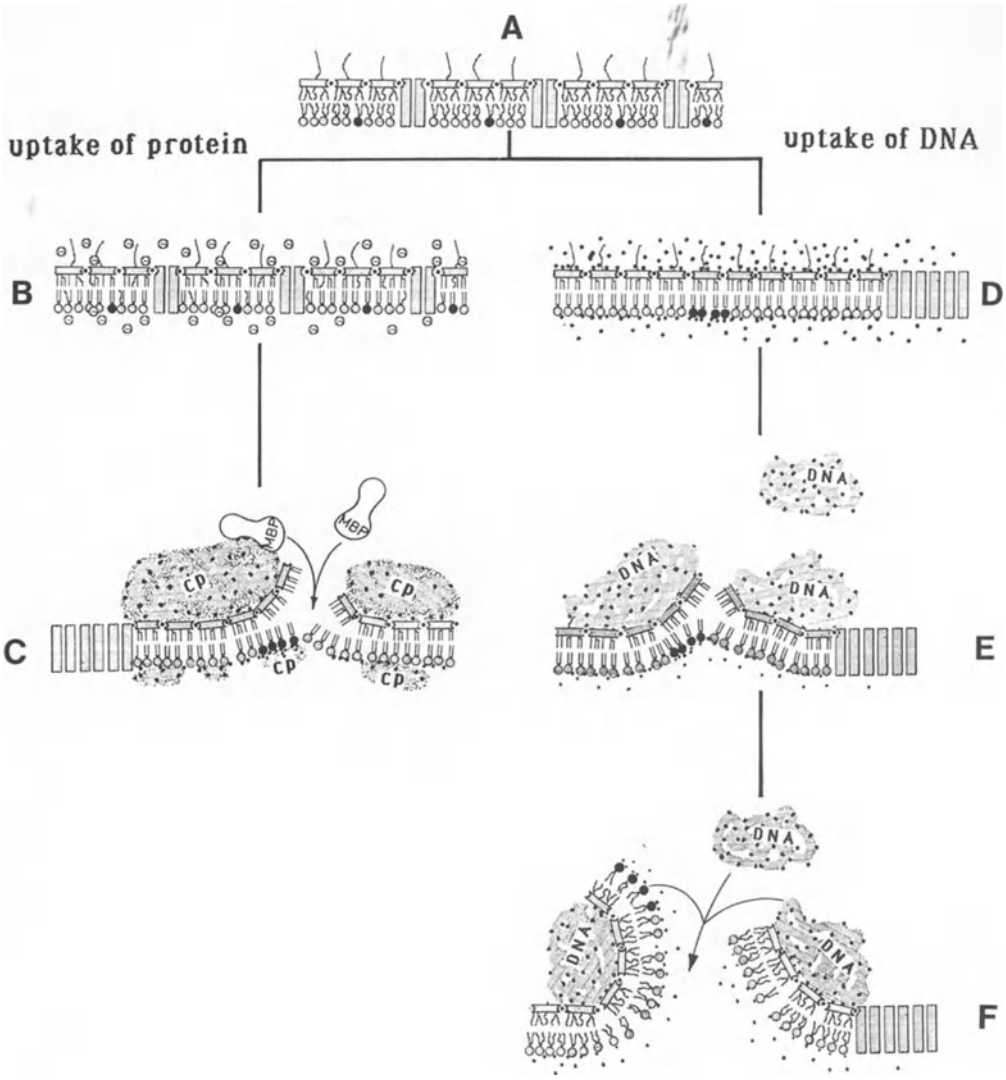

Fig. 23a–f. Possible mechanisms for Ca^{2+}-mediated uptake of protein and DNA across the outer membrane of *E. coli*.

(**A**) The outer membrane (OM) of *E. coli* is shown schematically with lipopolysaccharide molecules (LPS) in the outer leaflet facing the external medium and neutral or negatively charged phospholipids in the inner leaflet, facing the periplasmic space. LPS molecules are crossbridged with each other or with membrane proteins by Mg^{2+} or Ca^{2+}.

Uptake of protein. (**B**) Washing cells with inorganic phosphate (⊖) at 0° C causes an increase in the negative charge density of the OM and leads to a transition of membrane lipids from liquid-cristalline to gel phase. (**C**) Resuspension of phosphate pretreated cells in Ca^{2+} and protein (maltose-binding protein, *MBP*, e.g.) containing buffer at 0° C results in formation of calcium phosphate precipitates (*CP*, amorphous calcium phosphate, or octacalcium phosphate, or cristalline hydroxyapatite) bound to LPS and acidic phospholipids of the OM. This causes dehydration of the membrane and might also induce phase separation of OM-components into LPS-rich and protein-rich domains. As a consequence, microlesions occur in the OM which allow passage of free protein or of protein bound to calcium-phosphate complexes from the external medium into the periplasmic space.

of Tris-washed cells with phosphate prior to the addition of Ca^{2+} is essential for reconstitution competency. Calcium phosphate complexes are apparently the active principle for outer membrane permeabilization (see Fig. 23). Phosphate pretreatment, however, was not essential for transformation (although low concentrations of phosphate also stimulated this process). The fact that transformation, in contrast to reconstitution, also occurred in the absence of inorganic phosphate may indicate that DNA, complexed at its own phosphate groups with Ca^{2+}, catalyzes its own entry (see Fig. 23). A similar hypothesis has been published previously (GRINIUS 1980), in which it was suggested that binding of Ca^{2+} to DNA might covert it to a polycation which can interact with membranes in a manner similar to other polycations (see Sect. 6.3), resulting in a self-promoted uptake of DNA.

Our interpretation of the dual requirement of Ca^{2+} and phosphate for reconstitution, and the observed stimulation of transformation by pretreatment with low (10 m*M*) phosphate, is that calcium-phosphate complexes are formed in close contact with the cell envelope and interact with the highly negatively charged lipopolysaccaride molecules to permeabilize the outer membrane. The involvement of LPS in transformation is indicated by the finding that rough mutants of *E. coli* are more easily transformed than wild-type cells (TAKETO 1977).

Interestingly, a similar combined action of Ca^{2+} and phosphate at the plane of the membrane was seen in studies of fusion and leakage of phospholipid vesicles and erythrocyte membrenes (FRALEY et al. 1980; HOEKSTRA et al. 1983, 1985). It has been found that induction of leakiness and fusion are stimulated upon conversion of an amorphous calcium-phosphate complex to a solid crystalline phase. These complexes were shown to interact with the membrane and are believed to lead to local destabilization of membrane bilayers by dehydration, gel to liquid-crystalline phase transition, and phase separation of phospholipids. Multivalent calcium-phosphate complexes are more effective than free calcium in this respect, presumably because they cause extensive crosslinking (CAFFREY and FEIGENSON 1984; OHNISHI and ITO 1974). The possible biological relevance of calcium- and calcium-phosphate-induced membrane rearrangements has been discussed (FRALEY et al. 1980).

Divalent cations can induce nonbilayer lipid phase formation in vitro (VERKLEIJ 1984), which leads to the disruption of the membrane permeability barrier (MANDERSLOOT et al. 1981). The involvement of nonbilayer structures in DNA

Uptake of DNA. (**D**) Recipient cells are suspended in Ca^{2+} at 0° C. Even though this treatment induces similar effects on membranes as calcium-phosphate (dehydration, phase transition and phase separation), it is not sufficient to induce membrane disruptions that allow uptake of macromolecules across the OM. (**E**) Subsequently DNA is added to the cells. Binding of DNA, complexed with Ca^{2+} at its own phosphate groups, to LPS molecules leads to perturbations of the OM structure, similarly as binding of calcium phosphate complexes (see C). (**F**) The induced microlesions become enlarged during the subsequent heatshock, which now allows entry of DNA into the periplasm. Symbols: LPS  ; neutral () and negatively charged phospholipids (); membrane proteins (); divalent cations (•). (From B. BUKAU, Thesis University of Konstanz 1986)

uptake by E. coli during the heat shock in the presence of Ca^{2+} has recently been suggested. ^{31}P-NMR data were taken as indication for the formation of these structures (SABELNIKOV et al. 1985).

In summary (see Table 4), most conditions affecting reconstitution and transformation are similar, including the Ca^{2+} concentration and pH optima, the effect of other divalent cations, stimulation by plasmolysis and phosphate, the kinetics of entry of DNA and MBP, and the requirement for exponential phase cells. In addition, a mutual exclusion of DNA and MBP has been observed (BUKAU et al. 1985). Optimal conditions for reconstitution specifically reflect optimal permeabilization of the outer membrane. We conclude from the similar requirements for transformation that most aspects of the transformation protocol mainly influence the permeation of DNA through the outer membrane. Both macromolecules seem to gain access to the periplasm via a similar route.

9 Chemotactic Functions of the Cell Envelope

When *E. coli* moves up and down a gradient of attractive or noxious substances, temporal changes in the external concentrations of these substances can be sensed by the cell with the aid of chemoreceptors. The bacterial sensory system controls the behavior of the cells by changing the ratio of swimming [caused by counterclockwise (CCW) flagellar rotation] to tumbling [caused by clockwise (CW) flagellar rotation]; tumbling seves to reorient the cells. Chemotactic excitation results in a shift in the balance between CCW and CW rotation (LARSEN et al. 1974b). For example, addition of an attractant like maltose to a suspension of motile cells results in an immediate suppression of tumbling (MACNAB and KOSHLAND 1972). After some time the cells resume their original pattern of swimming and tumbling, even though maltose is still present (see Sect. 9.3 for discussion of adaptation). Addition of repellents results in a brief enhancement of tumbling. The chemotaxis machinery of *E. coli* comprises a network of signaling elements through which sensory information about the chemical environment is transmitted to the flagella. The complexity of this sensory system (about 50 genes are required for either motility or chemotaxis; reviewed in PARKINSON and HAZELBAUER 1983) indicates its great biological relevance.

9.1 Bacteria Swim with Rotating Flagella

E. coli cells swim using about 6 flagellar filaments that are inserted at random points in the cell envelope. The structure and function of the prokaryotic flagella are biologically unique and different from those of eukaryotic flagella. The bacterial flagellum consists of a long filament attached by a hook to a basal complex embedded in the cell envelope (DEPAMPHILIS and ADLER 1971). The filament is a moderately rigid left-handed helix of identical protein subunits, whereas the hook is thought to be more flexible. Each filament contains a rotary motor that turns the filament like a propeller (BERG and ANDERSON

1983). The motor is powered by proton flux (MANSON et al. 1977), not by ATP (LARSEN et al. 1974a). Rotating elements are unique in biology. When the motor turns CCW, the filaments work together in a bundle that drives the cell steadily forward (the cell swims smoothly); when the motor turns CW the bundle flies apart and the cell tumbles (LARSEN et al. 1974b). Slightly prolonged phases of smooth swimming up the gradient result in chemotaxis.

9.2 Chemoreceptors and Signal Transduction

The sugar and amino acid chemoreceptors thus far identified in *E. coli* fall into three categories. (a) Primary chemotactic signal transducers are essential inner membrane proteins and are called methyl-accepting chemotaxis proteins (or MCPs) because they are multiply methylated during adaptation to chemotactic stimuli (see Sect. 9.3). MCP I (Tsr) mediates taxis to L-serine and related amino acids (HEDBLOM and ADLER 1980). MCP II (Tar) mediates taxis to L-aspartic acid and to maltose and repellent taxis to Ni^{2+} and Co^{2+} (READER et al. 1979; WANG and KOSHLAND 1980). MCP III (Trg) is involved in chemotaxis to galactose and ribose (KONDOH et al. 1979; HAZELBAUER et al. 1981). A fourth transducer (MCP IV or Tap) was known and even sequenced (BOYD et al. 1981) but without any assigned function. Very recently it was found that Tap enables cells to respond chemotactically to dipeptides (MANSON et al. 1986). (b) Four soluble periplasmic binding proteins (for maltose, galactose, ribose, and dipeptides) function as chemoreceptors for these substrates (HAZELBAUER and ADLER 1971; HAZELBAUER 1975a; AKSAMIT and KOSHLAND 1972; HAZELBAUER and ADLER 1971; MANSON et al. 1986). Transduction of the chemotactic signal in these systems is believed to be triggered by the substrate-induced conformational shift observed in most binding proteins (BOOS 1972). The substrate-induced conformation of binding proteins is thought to allow interaction with the corresponding cytoplasmic membrane-bound MCPs (see Figs. 12 and 24). MBP was shown to interact with MCP II (Tar) (KOIWAI and HAYASHI 1979; RICHARME 1982a; MANSON and KOSSMANN 1986) and GBP and RBP are thought to bind to MCP III (Trg) (KONDOH et al. 1979). Amino acids bind directly to their espective signal transducers. (c) Yet another category of chemoreceptors is comprised by some components of the phosphotransferase sugar transport system (PTS). The cytoplasmic membrane-bound, substrate-specific enzyme II's function as chemoreceptors and signal transducers for certain PTS substrates (ADLER and EPSTEIN 1974; LENGELER et al. 1981) (see Fig. 24).

9.3 Adaptation

As explained lucidly in a recent review (HAZELBAUER and HARAYAMA 1983), sensory systems should produce a graded response in proportion to the intensity of the stimulus. The bacterial motility machinery, however, has an all or none-character in that the cell either tumbles or runs. The way in which bacteria grade their chemotactic response is by controlling the duration of tumble and run phases. This control requires the presence of an adaptation process. The

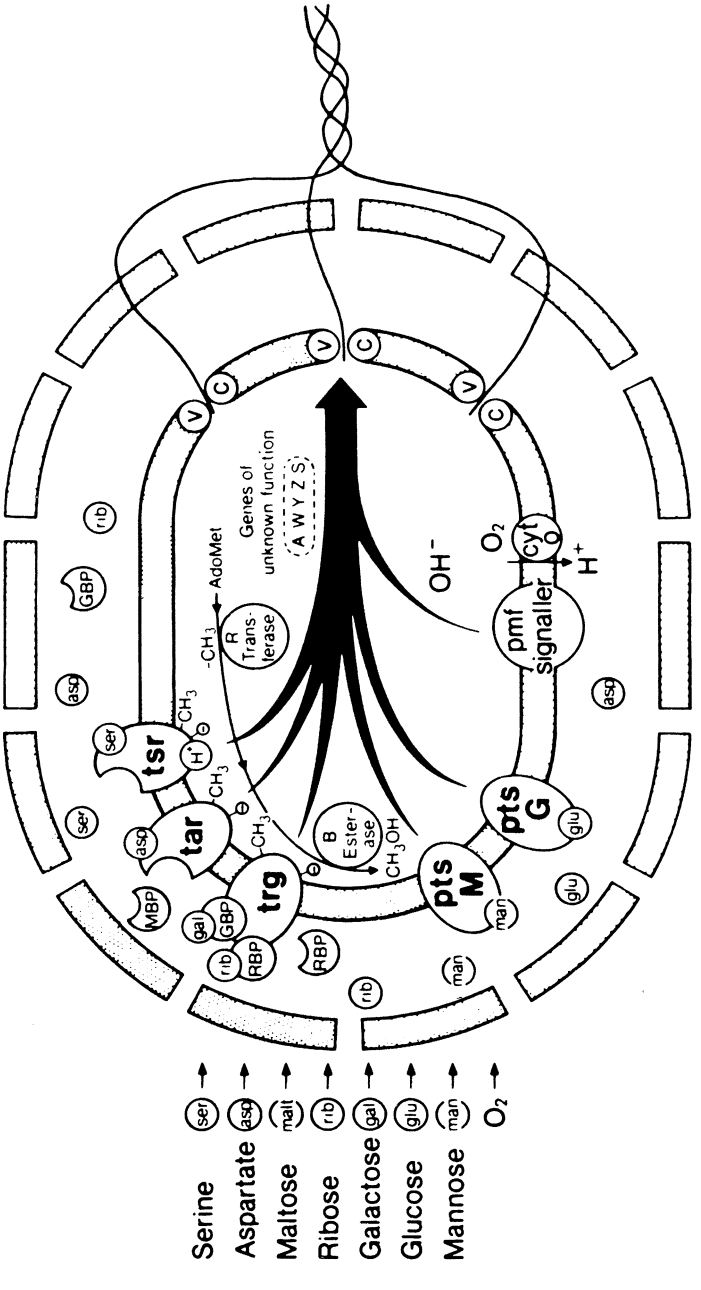

Fig. 24. Sensory transduction in *E. coli* and *S. typhimurium*. Attractants and repellents cross the outer membrane via porins and bind to soluble binding proteins in the periplasm (*MBP, GBP, RBP*) or to membrane bound signal transducer proteins (*Tsr, Tar*). Transduction of the chemotactic signal is thought to be triggered by binding of free substrate or substrate-loaded binding protein to the signal transducer (e.g., binding of aspartate or MBP-mal to Tar). Chemotactic signals arise from different signal transducers (*Tsr, Tar, Trg*, ptsM, ptsG, and an unknown proton motive force signaler). All sensory pathways converge prior to CheV and CheC, the "switch" proteins which are believed to regulate the rotational sense of the flagellar motor. Proteins CheA, CheW, CheY, CheZ, and CheS are chemotaxis gene products with unassigned function in signal processing. For signals processed through MCPs (TSR, TAR, TRG) adaptation is dependent on methylation by the methyltransferase (CheR) and on demethylation by the methylesterase (CheB). (Reprinted with permission of NIWANO and TAYLOR 1982)

duration of the chemotactic response initiated by the receptor-MCP interaction is proportional to the change in the receptor occupancy (BERG and TEDESCO 1975; SPUDICH and KOSHLAND 1975). The duration of the response to an attractant stimulus is limited by an adaptation process in which the MCP molecules are methylated at specific γ-carboxyl groups of glutamyl residues (KORT et al.

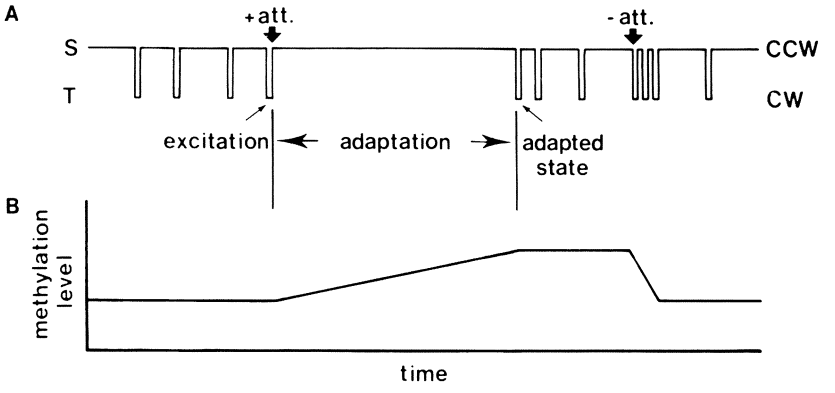

Fig. 25A, B. Schematic representation of chemotactic behavior. (**A**) Pattern of swims and tumbles. A cell alternates between swims (*S*) and tumbles (*T*) which result from counterclockwise (*CCW*) and clockwise (*CW*) rotation, respectively, of the flagellar motor. Addition of attractant (+*att*) results in immediate suppression of tumbles. After adaptation there is a short "overshoot" period during which periods of CW rotation are more frequent than before stimulation. Removal of attractant (−*att*) causes an increased frequency of tumbles. Adaptation to this negative stimulus occurs about ten fold more rapidly than adaptation to the equivalent positive stimulus. (**B**) Changes in methylation during tactic behavior. The level of carboxyl methylation of specific glutamyl residues of the relevant transducer molecules increases during adaptation to positive stimuli and decreases during adaptation to negative. (Reprinted with permission of HAZELBAUER and HARAYAMA 1983)

1975). Multiple methylation of the signal transducers is accomplished by the CheR protein, a methyltransferase, which uses S-adenosylmethionine as the methyl donor. Demethylation of the transducers after dissociation of the attractant (or the attractant-loaded binding protein), leading to down-adaption, is due to the methylesterase activity of the CheB protein (see Fig. 24). Strains with deletions of the *cheR* or *cheB* genes respond to chemotactic stimuli, but do not adapt normally (GOY et al. 1978). A graded response occurs because the larger the chemotactic stimulis, the greater the number of occupied signal transducers (MCPs) and the longer the time required for the methylation system to neutralize the activated transducers.

The adaptation mechanism (the adaptation process of PTS signal transducers is unknown) allows measurement of changes in the external attractant concentration, not of absolute concentration. The advantage of adaptation, besides grading, is that adapted cells can respond to further increases of the occupancy of the same signal transducer (if it is not yet saturated) and to occupancy of a different class of receptors. When the receptor occupancy and the level of MCP methylation are balanced, a cell swims and tubles in its unstimulated pattern. When they are not balanced, the cell shows a bias toward swimming (for attractants) or tumbling (for repellents) (see Fig. 25). Methylation is a relatively slow biochemical process; therefore, the extent of methylation of the MCPs is a reflection of the chemical environment in the immediate past. In contrast, receptor binding is a rapid process (an association rate constant of $2.3 \times 10^7\ M^{-1}\ s^{-1}$ has been measured for MBP and maltose; MILLER et al. 1983), and therefore reflects the instantaneous concentration of attractant or

repellent. The MCPs compare past and present. Bacteria, which thus have a rudimentary memory, respond to temporal change in solute concentration. Direct measurement of spatial gradients would not be possible because of the small size of bacteria. This is nicely documented by the ingenious experiment of BROWN and BERG (1974), who showed that temporal changes (local spatial changes were excluded) of the concentration of the attractant L-glutamic acid, accomplished by the addition of the enzyme alanine-aminotransferase to a mixture of alanine and 2-oxoglutaric acid, induced a chemotactic response in the bacteria.

The signal transducer proteins Tsr (MCP I), Tar (MCP II), Trg (MCP III) and Tap (MCP IV) are integral membrane proteins with molecular weights between 65000 and 58000. About 1000 copies of these proteins are present per cell (Trg only 100–200). They contain multiple glutamic acid residues (4–6) which can be reversibly methylated by the methyltransferase. Based on nucleotide sequence data for the *tsr, tar* and *tap* genes (KRIKOS et al. 1983; BOLLINGER et al. 1984), the proteins appear to have a transmembrane structure consisting of an periplasmic NH_2-terminal domain, two membrane spanning domains, and a cytoplasmic COOH-terminal domain (Fig. 26). The COOH-terminal domains of the MCPs possess highly homologous sequences and contain the sites for methylation, while the NH_2-terminal sequences are only distantly related. This is expected, since the NH_2-terminal regions carry out the highly specific receptor functions of the MCPs i.e. binding of small ligands or of ligand loaded binding proteins (aspartate and maltose loaded MBP in case of MCP II) (see Fig. 24). The high degree of homology in the cytoplasmic domains indicate that the MCP genes have evolved from a single ancestral gene.

A high degree of similarity of the cytoplasmic domain of the different signal transducers is expected because of their identical functions (signaling and adaptation). The different signaling pathways converge at this point. Even though the nature of the chemotactic signal is unknown (see below), only one unified signal is assumed to exist and only one set of enzymes (*cheB* and *cheR* gene products) is known to be involved in methylation and demethylation of the different signal transducers (see Fig. 24).

Recently, the proposed periplasmic and cytoplasmic domains of one signal transducer (MCP II; aspartate receptor) was separated by biochemical means. Two proteolytic fragments of MCP II from *S. typhimurium* have been purified which are generated during preparation of this protein by an endogenous protease or by trypsin incubation of the intact protein (MOWBRAY et al. 1985). Proteolytic fragment 1 (PF1; amino acids 1-259) represents the periplasmic domain of MCP II and contains, in addition, the two membrane spanning helices. This fragment was separated from the rest of the protein but still retained the high affinity binding aspartate binding function ($K_D = 1\ \mu M$). Fragment 2 (PF2; amino acids 260–552) representing the cytoplasmic domain contained the normal sites of reversible methylation. It could be methylated in vitro although at a much slower rate.

The primary effect of methylation may be charge neutralization. This could modulate an interaction between transducers and the cytoplasmic membrane or other protein components of the chemotaxis system. Interestingly, aggrega-

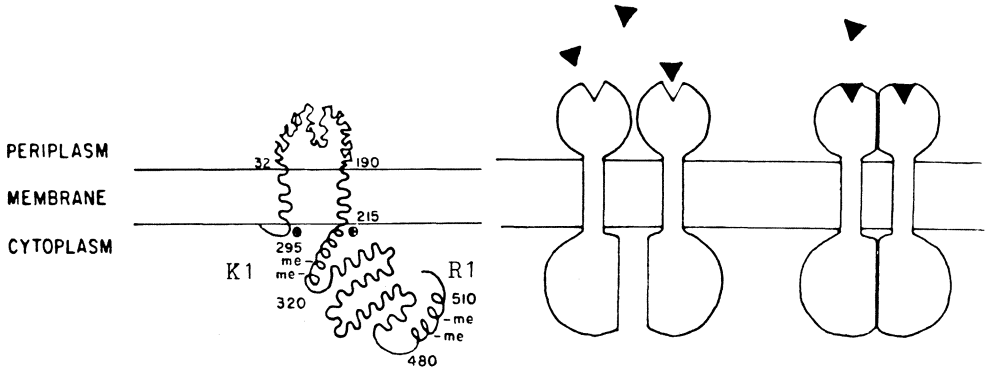

Fig. 26. A model of the transmembrane disposition of MCPs and for their potential function in signal transduction. The Tsr-MCP is shown spanning the membrane. The sites of methylation (*me*) at the cytoplasmic domain of the protein are clustered in two α-helical regions. The same overall structure is envisaged for the other MCPs. Upon binding of a ligand the transducer may undergo a conformational change (multimerization) which results in signaling activity. (Reprinted with permission of KRIKOS et al. 1983; © M.I.T. Press)

tion of purified, detergent solubilized MCP II and of its proteolytic fragments (PF1 and PF2) to tetramers was observed. Aggregation of PF1 remained unchanged after addition of aspartate, alteration of the pH of the buffer, or use of ionic or nonionic detergents (MOWEBRAY et al. 1985).

9.4 What is the Chemotactic Signal?

The signal transducers produce an unknown signal which travels to the switch of the flagellar motor (consisting at least, in part, of the proteins CheC and CheV; see Fig. 24). The switch most likely controls the rotational sense of the motor. The latency between an iontophoretically applied stimulus (α-methyl aspartate) and the observed change in swimming behavior was 0.2 s. This was observed in a flow cell with single cells tethered to glass with antibodies directed against flagellar filaments, so that rotation of the cell body could be used to monitor the rotation of a flagellar motor (SEGALL et al. 1982).

It is clear that signaling does not require uptake of substrate (this holds true for all MCP-mediated signals, but not for PTS-mediated signals) and does not require protein synthesis or growth. It has been proposed that the signal could be a change in the membrane potential (SZMELCMAN and ADLER 1976) or other transmembrane ion fluxes (KOSOWER 1983), and it has been suggested that free calcium ions (ORDAL 1977) or cyclic GMP could regulate the chemoc behavior (BLACK et al. 1980).

Recently, experiments with long, filamentous cells indicated that an internal signal of short range exists. This was concluded from experiments in which the filaments were stimulated with α-methylaspartate using an iontophoretic pipette placed at different positions along the filament (see Fig. 27). Motors near the pipette responded, whereas those sufficiently far away from the motor

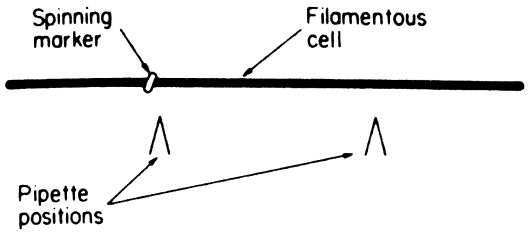

Fig. 27. The chemotactic signal has a short range. Long filamentous *E. coli* cells carrying a spinning flagellar marker were stimulated iontophoretically with a micropipette at different positions close to the cell surface either near the marker or far away. Stimulation was either a short (10 s) or long (60 s) pulse of aspartate. *Scale,* the *marker* is 20 μm from the left end of the cell. (Reprinted with permission of SEGALL et al. 1985)

did not, even when the pipette was near the cell surface. The results suggest that the signal is generated at the transducers and destroyed in the cytoplasm. The space constant for the signal substance, which reaches the flagellar motor by diffusion, was 2 μm in a *cheR cheB* deletion and 6 μm in a *cheZ* mutant (SEGALL et al. 1985). This short range of the signal rules out signaling by changes of the membrane potential, which should have a much longer range (about 100 μm), at least for strong stimuli. Based on the calculated lateral diffusion coefficient (D_L) of the chemotactic signal ($D_L = 10^{-7}$ cm^2 s^{-1}) the authors suggest that the signal could be a diffusible protein, possibly a *che* gene product, that is reversibly modified by other *che* gene products. SEGALL et al. (1985) argue that simple release or binding of small molecules like free calcium by the signal transducer in response to chemoeffector binding also cannot be the chemotactic signal since in this case the calcium ions should diffuse away from the point of release and the initial change in rotational bias of nearby motors would decline. This was not observed even with long lasting stimuli (30 s) in *cheRB* filaments, which do not adapt. We think, however, that the observed signal characteristics (short range of the signal but long lasting rotational bias of nearby motors) could also be explained by a model in which local stimulation of signal transducers allows long lasting entry of exogenous calcium ions into the cytoplasm. The apparent low D_L of the signal and its short range could be due to binding of Ca^{2+} to phospholipids or proteins and subsequent expulsion of Ca^{2+} by the active Ca^{2+} export machinery. The fact that mutants defective in the genes responsible for this process showed only subtile variations in chemotactic response (BREY and ROSEN 1979) does not rule out this possibility, since the described mutants exhibited a strongly leaky phenotype and still exhibited a residual export activity.

9.5 Reconstitution of Maltose Chemotaxis in *malE* Mutants by Import of MBP into the Periplasm

Maltose chemoreception in *E. coli* requires the product of the *malE* gene which codes for the periplasmic MBP (HAZELBAUER and ADLER 1971; HAZELBAUER

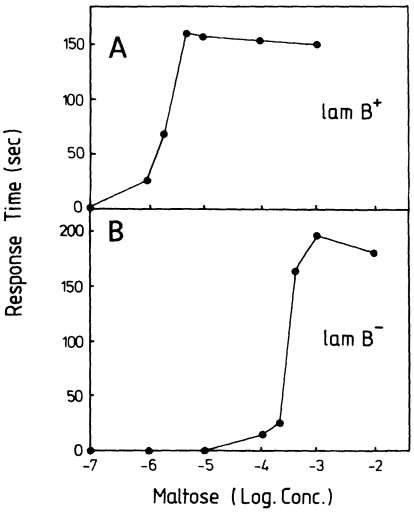

Fig. 28. Maltoporin extends the sensitivity of the maltose chemotaxis system 100-fold. The maltose response of wild-type and *lamB* mutants was compared with tethered cells in the flow chamber. Cells carrying only one flagellum were bound to the coverslip of the chamber via antibodies directed against the flagellum. Spinning cells were viewed in the microscope and their response to a flow of buffer containing different maltose concentrations (10^{-7}–10^{-2} M) was measured as the length of the initial period of uninterrupted CCW rotation (see also Fig. 23). (Reprinted with permission of BRASS and MANSON 1984)

1975a). Maltose binds indirectly in the form of maltose-loaded MBP to the maltose/aspartate signal transducer MCP II (KOIWAI and HAYASHI 1979). The product of the *lamB* gene, the outer membrane maltoporin which increases the permeation of maltose through the outer membrane (SZMELCMAN et al. 1976) is also required for chemotaxis of maltose and longer dextrins (HAZELBAUER 1975b). Maltoporin increases the sensitivity range of cells to respond to maltose stimuli 100-fold from 10^{-4} *M* to 10^{-6} *M* maltose (BRASS and MANSON 1984; see Fig. 28).

Soon after the identification of periplasmic binding proteins as chemoreceptors in chemotaxis, attempts were made to study the first steps of signal transduction in vivo by reconstitution experiments with purified binding proteins. Reconstitution of galactose chemotaxis by addition of GBP to osmotically shocked wild-type cells after EDTA treatment had been reported (HAZELBAUER and ADLER 1971). A limitation of these experiments, however, was that mutants devoid of GBP could not be reconstituted. We have successfully adapted our procedure for reconstitution of transport (see Fig. 19; BRASS et al. 1981, 1983) to reconstitute maltose chemotaxis in *malE* mutants (BRASS and MANSON 1984). Addition of purified maltose chemoreceptor (MBP) to MBP-free *malE* cells during a pretreatment with Ca^{2+} resulted in a restoration of maltose chemotaxis. We assayed the competence of the reconstituted cells to respond to maltose stimuli both in a flow chamber with tethered bacteria as described by BERG and TEDESCO (1975) and in the capillary assay as described by ADLER (1973). About 20% of the MBP-pretreated *malE* cells tethered in a flow cell responded to stimuli of 10^{-4} *M* maltose, with uninterrupted rotation of the cells in the run direction (CCW) for about 100 s (Fig. 29). This response time is about half that of wild-type cells. The rest of the population did not respond to maltose stimuli, but responded perfectly well (with wild-type response times) to aspartate stimuli. The chemotactic signals for maltose and aspartate are processed by the same signal transducer (MCP II) (SPRINGER et al. 1977). Thus,

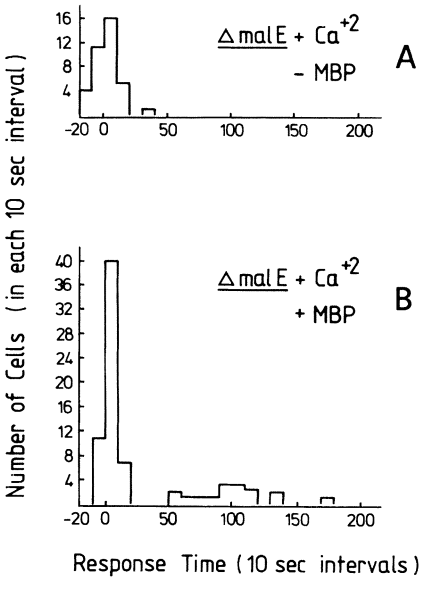

Fig. 29 A, B. Reconstitution of maltose chemotaxis in *malE* cells. Samples of 10^9 $\Delta malE$ cells were pretreated with $CaCl_2$ in the presence or absence of 0.5 m*M* MBP. Response times of individual cells to 10^{-3} *M* aspartate (data not shown) and 10^{-4} *M* maltose were determined in the flow cell. Data from several microscopic fields were pooled and are shown in histogram form. (**A**) Strain MM129 (*malT*^{c}*-1* $\Delta malE444$ *lamB*$^+$) with Ca^{2+}-treatment, no MBP; (**B**) strain MM129 with Ca^{2+} treatment, plus MBP. All the cells of the population, including those which did not respond to maltose (80%), showed normal response times toward aspartate (mean response times of between 4 and 6 min). (Reprinted with permission of BRASS and MANSON 1984)

the observation of individual tethered bacteria offers an unique opportunity to determine the fraction of cells competent for uptake of MBP. The ability of individual reconstituted cells to respond to maltose in the flow cell persisted for more than 2 h, indicating good retention of MBP in the periplasm.

The maltose response of reconstituted *malE* cells measured with the capillary assay (accumulation of cells in a maltose containing capillary) was unexpectedly high. Accumulation of *malE* cells pretreated with Ca^{2+} and saturating MBP concentrations (0.5 mM MBP) was similar to that with *malE*$^+$ cells pretreated with $CaCl_2$. Possible reasons for this higher sensitivity of the capillary test for cells reconstituted in maltose chemotaxis were discussed by BRASS and MANSON (1984). (i) Underestimation of the response times of cells tethered in the flow cell or (ii) a selection of a subpopulation of cells in the capillary test in which a correlation between good motility and disproportionally good reconstitution exists could explain the different results seen in the flow cell and the capillary assay.

Maltose transport and metabolism are not prerequisites for maltose chemotaxis in *E. coli* (HAZELBAUER 1975a). There could be, however, genes within the *mal* regulon whose products, although not needed for growth on maltose, are involved in maltose chemotaxis. This possibility had not been tested up to new because of the strict *malT* dependence of *malE* gene expression (HATFIELD et al. 1969). By Ca^{2+} treatment, we could introduce MBP into strains lacking a functional MalT protein. Analysis of tethered cells showed that both a *malT*::Tn*10* mutant carrying in addition the deletion *ΔmalB112,* which removes both promoters of the *malB* region (Fig. 12), can be reconstituted (Fig. 30). These results clearly proved that MBP (besides maltoporin, see above) is the only component of the *mal* regulon essential for maltose chemotaxis.

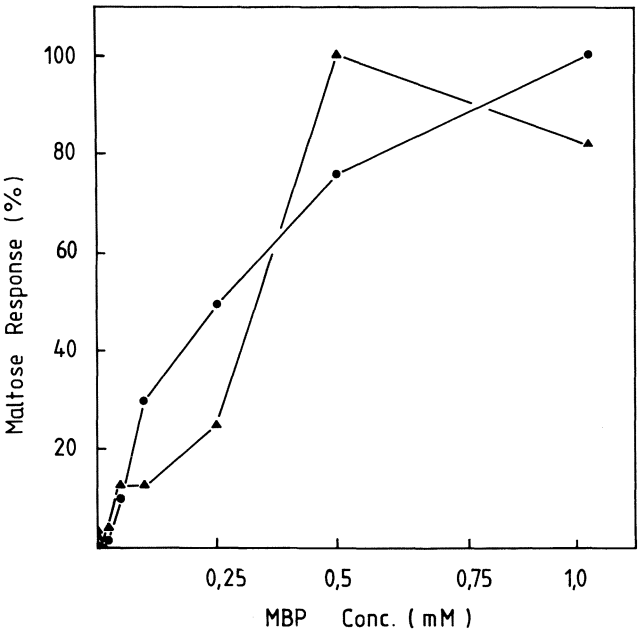

Fig. 30. MBP concentration dependence for reconstitution of maltose chemotaxis in *malE* cells. Ca^{2+}-treated cells of a *malE* strain (●) and a *malB malT* strain (▲) were incubated with different concentrations of purified MBP. After washing, the reconstituted ability of the cells to respond to maltose was measured in the capillary assay. At saturating MBP concentrations the reconstituted maltose response is similar to that of $malE^+$ wild-type cells. (Adapted from BRASS and MANSON 1984)

9.6 Attempts to Measure the Affinity of MBP for the Maltose/Aspartate Signal Transducer (MCP II)

The concentration of MBP necessary for half-optimal reconstitution of maltose chemotaxis as measured in the capillary assay was around 0.25 m*M* for a *ΔmalE* strain. A *ΔmalB malT* strain showed a similar MBP dependence of reconstitution (see Fig. 30). As discussed in Sect. 7.6, the periplasmic concentration of MBP in reconstituted cells is not known. An argument in support of the assumption that external MBP equilibrates freely within the periplasm during reconstitution can be taken from the data of others (MANSON et al. 1985). They found that in signal sequence mutations with strongly reduced periplasmic MBP concentrations a half-maximal chemotactic response occurred at around 250 μ*M* MBP, a value close to that which can be calculated from our reconstitution data (Fig. 30). These two different approaches show that MBP has a low affinity for the maltose/aspartate signal transducer (MCP II). The astonishing high affinity for MBP and MCP II which was measured in vitro by affinity chromatography (10^{-7} *M* MBP; RICHARME 1982a) appears to have been an artifact.

9.7 Cross Species Reconstitution of Maltose Chemotaxis and Evolution of the Tar-MBP Interaction

The reconstitution technique using Ca^{2+}-treated whole cells has recently been used to study the evolutionary divergence of binding proteins and signal transducers by introduction of binding proteins from one species into another (DAHL and MANSON 1985). MBP is produced by *Salmonella typhimurium, Klebsiella pneumoniae, Enterobacter aerogenes,* and *Serratia marcescens.* MBP from all of these species cross-reacted with antibody against the *E. coli* protein and had a similar molecular weight (about 40000). The *Shigella flexneri* and *Proteus mirabilis* strains examined did not synthesize MBP. The isoelectric points of MBP from different species varied from the acid extreme of *E. coli* (4.8) to the basic extreme of *E. aerogenes* (8.9). All species with MBP transported maltose with high affinity. Maltose chemotaxis was observed only in *E. coli* and *E. aerogenes.* Yet, MBP isolated from all five species could be used to reconstitute maltose transport and taxis in a *ΔmalE* strain of *E. coli* after permeabilization of the outer membrane with calcium (DAHL and MANSON 1985).

The result that *S. typhimurium* MBP was able to reconstitute maltose chemotaxis in *E. coli* was unexpected since LT2 does not respond to maltose in the capillary assay or on swarm plates, but responds strongly to aspartate. The aspartate response is dependent on the presence of a functional Tar protein. These results show that the chemotactic machinery of LT2 functions well, but the interaction of Tar with MBP is defective. MIZUNO et al. (1986), using a different approach, have independently reached a similar conclusion. The results of the reconstitution experiments using *K. pneumoniae* were also unexpected, since this is a nonmotile species, and yet its MBP functions perfectly well in the periplasm of *E. coli* as chemoreceptor in maltose chemotaxis (DAHL and MANSON 1985).

Based on the observation that the genes coding for sugar binding proteins do not map in or near the *che* gene clusters and are regulated differently than *che* genes, HAZELBAUER suggested that a pre-existing amino acid sensory system was secondarily adapted to interact with the sugar binding proteins (HAZELBAUER and HARAYAMA 1983). Thus, Tar possibly evolved in such a way that interaction with MBP eventually created a chemosensory pathway for maltose (the same may be true for Trg and its interaction with GBP, and RBP). In this context one may speculate that maltose chemotaxis has arisen independently in the genera *Escherichia* and *Enterobacter* very late in bacterial evolution. An alternative possibility is, that maltose chemotaxis is a trait that arose early in bacterial evolution of the *Enterobacteriaceae* but Tar of *S. typhimurium* has lost this information secondarily. This does not seem very likely since the ability to perform chemotaxis towards maltose and maltodextrins is of great selective advantage to organisms that can transport and metabolize these substrates. The finding that Tar from *E. coli* can interact with MBPs from different species in which the Tar-MBP interaction is defective might indicate that Tar from *E. coli* is an evolutionarily progressive protein which recognizes conservative domains of different MBP molecules. These domains might be quite stable because of the transport-optimized structure of MBP.

9.8 Reconstitution of Chemotaxis in Cell Envelopes Lacking Cytoplasmic Components of the Chemotaxis Machinery

The reconstitution system (Ca^{2+}-treated cells) described above rendered the periplasmic space accessible to experimental manipulation. Recently, a subcellular system consisting of cell envelopes devoid of cytoplasm, but containing functional flagella, has been described for *E. coli* and *S. typhimurium* (EISENBACH and ADLER 1981). The envelopes are osmotically sensitive, having an intact cytoplasmic membrane and at least part of the cell wall. This system maintains a functional flagellar apparatus and leaves the cytoplasmic side of the inner membrane accessible to experimental manipulation. Cell envelopes, even those prepared from *cheB* mutant cells defective in methylesterase activity and possessing a strong CW bias, acquired an exclusively CCW sense of rotation upon the release of their cytoplasm. The observed CCW rotation is not the consequence of mechanical damage or reduced proton motive force (the envelopes were energized by addition of lactate as electron donor); it is probably due to loss of the cytoplasmic protein which is required for expression of CW rotation (or repression of CCW rotation). This component, the "CW facilitator," could be the product of one of the *che* genes with unknown function (e.g., *cheA, cheW,* or *cheY;* see Fig. 25) in which mutations lead to CCW rotation. It appears that in the absence of the CW-facilitator the wild-type motor may be mechanically unable to rotate other than CCW (run direction). These results might indicate that the motor itself is not a reversible unit. However, the complex between the switch motor unit (*cheV* and *cheC* gene products) and the CW facilitator may possibly be reversible. This hypothesis can now be tested by insertion of the purified products of the *cheA, cheY, cheA,* and *cheZ* genes, which have been cloned and sequenced (MUTOH and SIMON 1986), into the cell envelope system. The results of the first reconstitution experiments of this type were reported recently. Addition of purified CheY protein to the envelopes showed that this protein is probably involved in excitatory signaling by direct interaction with the switch motor complex. The flagella of envelopes containing purified CheY regained the ability to rotate in CW direction and the fraction of envelopes which rotated CW was dependent on the concentration of inserted protein (100% at 48 μM CheY protein) (EISENBACH et al. 1985). In addition to interaction with the flagellar structure, CheY can probably interact with the CheR methyltransferase and can negatively regulate the methylation of MCPs. Thus, CheY is a bridge between the generation of the chemotaxis signal by the MCPs and the effector organelle, the flagellum (MATSUMURA et al. 1985).

10 Determination of the Lateral Diffusion Coefficients of Bacterial Cell Envelope Components

A crucial element of the fluid mosaic phospholipid bilayer model is the assumption that lipids and proteins can move laterally in the plane of the membrane.

This view is confirmed by a variety of biophysical measurements such as NMR, ESR, and in particular by the fluorescence recovery after photobleaching (FRAP) of fluorescently labeled macromolecules (CHERRY 1979). The knowledge of the diffusion coefficients of protein complexes is, in many cases, essential for understanding the organization and functioning of such membranes. What are the diffusion coefficients of protein complexes in prokaryotic membranes? Is their diffusion fast and free or slow and directed?

Lateral diffusion coefficients (D_L) of phospholipids measured in situ in membranes are about 10^{-8} cm^2 s^{-1}; D_L for membrane proteins (not retarted by extramembranal constraints) is about 1×10^{-10} cm^2 s^{-1}, while soluble proteins of about 15000 daltons exhibit D_L values of around 1×10^{-7} cm^2 s^{-1} in aqueous solution.

As stated recently (KELL 1984), the small size of bacteria should allow complete randomization of membrane proteins during the time necessary for a cell to divide (20 min), even if one envisages slow protein mobility with a D_L of 10^{-11} cm^2 s^{-1}. This follows from the inverse square relationship between the radius of spherical bacteria (r) and D_L in Eq. 1 describing the relaxation time for protein randomization.

$$\tau = \frac{r^2}{2D}$$

A number of observations, however, indicate that randomization is not at all the rule for prokaryotic membranes. Examples for nonrandomly organized membranes are provided by (a) the cytoplasmic and intracytoplasmic membranes of purple nonsulfur photosynthetic bacteria (Rhodospirillaceae), where both membranes form a continuous membrane system but are clearly different in function and composition, while their protein constituents apparently do not mix; (b) The outer and inner membrane of gram-negative bacteria are connected by several (up to 200) sites of adhesion (BAYER 1979). If proteins of both membranes would mix and outer membrane porins would integrate into the cytoplasmic membrane, membrane potential and ion grandients would collapse in the same way they do after integration of pore-forming cytotoxic colicins into the cytoplasmic membrane (e.g., colicin K and I); in addition to the Bayer bridges, at sites of cell division, extended murein-membrane attachment sites, periseptal annuli, surround the cell. The annuli might seal off the division site and might ensure that membrane- and periplasmic components might remain in a specialized compartment (see Sect. 4.3). Indications that these annuli function indeed as permeability barriers for lateral mobility of periplasmic macromolecules are described below.

Thus, the distributions of the components of outer and inner membranes of bacteria are significantly nonrandom. Possible explanations for this phenomenon are anchorage of membrane proteins to cytoplasmic and/or cell envelope components, and domain formation by localized or global lipid crystallization (MURPHY and WOODROW 1983). Anchorage of outer membrane proteins by covalent bonds and salt bridges to the peptidoglycan layer is well known (BRAUN and REHN 1969; ROSENBUSCH 1974). This correlates with a marked immobility of outer membrane porins (SMIT and NIKAIDO 1978). The low rate of diffusion

of porins in vivo is in marked contrast to the relatively rapid D_L observed for LPS in vivo ($D_L = 2 \times 10^{-10}$ cm^2 s^{-1}; SCHINDLER et al. 1980). Since LPS is only found in the outer leaflet of the outer membrane, some sorting mechanism must be postulated to prevent its entering the inner leaflet during LPS export into the outer membrane. Membrane-cytoskeleton interactions, or retardation by a matrix protein as described for eukaryotic cytoplasmic membranes, are, however, not known for bacterial inner membranes.

The above-discussed aspects of protein mobility focused on cell differentiation by membrane compartmentalization. Besides this aspect, knowledge of D_L values of specific components of multimeric enzyme complexes or of transport and chemosensory systems can be crucial for deciding between competing working models. One example of this was already provided in Sect. 9.4 concerning the chemotactic signal. Knowledge of the high lateral mobility of the chemotactic signal ($D_L = 10^{-7}$ cm^2 s^{-1}; SEGALL et al. 1985) will strongly influence any theory about the still unknown nature of this signal. Another example of this is provided by recent studies of the interaction of proteins involved in electron transport in the inner membrane of mitochondria. The lateral mobility of several mitochondrial membrane proteins was measured. The data allowed the conclusion that electron transport is coupled to the lateral diffusion of all redox components in the mitochondrial membrane. It does not require ordered chains, assemblies, or aggregates of redox components (GUPTE et al. 1984).

We measured the lateral mobility of different periplasmic binding proteins, such as MBP, in situ in the periplasmic space. As discussed above, binding proteins are essential components of many transport systems and can also serve as chemotactic receptors. The precise role of these proteins in transport and chemotaxis remains obscure, partly because little is known about the nature of the periplasm and the precise structural relationship between the inner and outer membrane. A recent report (HOBOT et al. 1984) suggests that the entire periplasm may be filled with a viscous peptidoglycan gel. Even though no precise values are available for the pore size of the peptidoglycan network (see Sect. 4.3), the cell wall is thought not to impede movement of binding protein. Mobility of binding proteins in the periplasm has been considered to be a relatively simple diffusion of water-soluble proteins in the aqueous bulk phase. However, it is obvious that diffusion of binding proteins which have elipsoidal dimensions of about $40 \times 40 \times 70$ Å (SAPER and QUIOCHO 1983) could be restricted mechanically be the inner and outer membrane which are at the most 140 Å apart (in nonplasmolyzed cells). Diffusion of proteins between apposed membranes has never been measured before.

We have developed a procedure to investigate the movement of proteins within the periplasm of *E. coli* by FRAP (BRASS et al. 1986). We have introduced biologically active, fluorescently labeled MBP into the periplasmic space of cephalexin-induced filaments of *E. coli* cells, and measured its lateral diffusion coefficient by the fluorescence photobleaching recovery method. Maltose transport of cephalexin-treated wild-type cells was not affected by filamentation. Diffusion of the fluorescent protein in the periplasm was found to be surprisingly slow ($D_L = 2.7 \times 10^{-10}$ cm^2 s^{-1}), about 1000-fold less than would be expected for diffusion in aqueous medium (see Fig. 31).

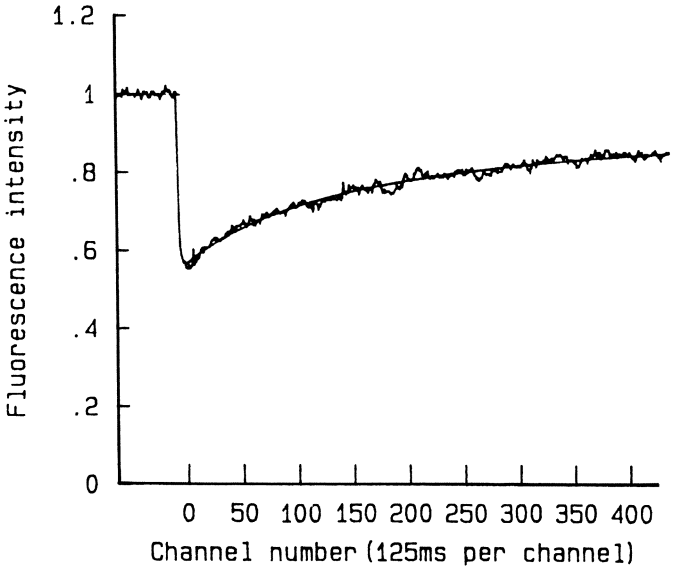

Fig. 31. Measurement of the periplasmic mobility of MBP by fluorescence photobleaching recovery. Rhodamine-labeled MBP was incorporated into the periplasm of *E. coli* JB200 *malE*. The *noisy curve* is the observed fluorescence intensity, normalized to a prebleach level of 1.0 arbitrary units. The *smooth curve* is the nonlinear least squares fit for a single diffusing component. The *time axis units* are channel numbers; channel 1 is that immediately after completion of the bleaching pulse. Each channel is 0.125 s wide. An unexpected low rate of lateral diffusion of periplasmic MBP ($D_L = 2 \times 10^{-10}$ cm^2 s^{-1}) can be calculated from these data, about 1000-fold less than would be expected for diffusion in aqueous medium. Fluorescence recovery also allows to calculate the fraction of mobile MBP molecules (R value), which is 95% in this experiment. (Reprinted with permission of BRASS et al. 1986)

As discussed in BRASS et al. (1986) there are several mechanisms by which the periplasmic mobility of proteins could be reduced. One obvious possibility is that MBP interacts with the known specific membrane proteins involved in maltose transport and chemotactic functions: maltoporin, the MalF-MalG-MalK transport components, and the chemotactic signal transducer MCP II. Thus, we determined the lateral diffusion coefficient for MBP in a strain deleted for the genes encoding all membrane proteins possibly interacting with MBP. The coefficient for MBP in this strain was identical to that observed in the parental strain, both in the presence and in the absence of the substrate maltose. Thus, the retardation in MBP mobility in the periplasm is not due to interactions with known specific transport or chemotaxis proteins.

Retardation of binding proteins in the periplasmic space might be due to ionic interactions of binding proteins with the peptidoglycan matrix and/or hydrophobic interactions with the outer and inner membrane surfaces. Indeed, a strong tendency of GBP and MBP to bind to hydrophobic surfaces was observed by in vitro FRAP experiments using siliconized and phospholipid coated glass (BRASS et al. unpublished). Thus, binding proteins could perhaps function as peripheral inner membrane proteins, exhibiting two-dimensional

diffusion on the plane of the membrane and directing the substrate to the relatively few inner membrane transport proteins. As discussed in detail by ADAM and DELBRÜCK (1968) and MCCLOSKEY and POO (1984), a two-dimensional diffusive search can increase the efficiency of finding a target (in our case the inner membrane MalFGK complexes, Tar, or the outer membrane maltoporin trimer), and can increase the efficiency of the collisions of the proteins by alignment of reactive groups through restricted rotation. If this possibility is true, one should expect that only periplasmic proteins exhibit the unexpected low lateral diffusion coefficient, whereas other macromolecules should exhibit a higher mobility. We found, however, a similar lateral mobility for a fluorescently labeled dextran molecule of 40000 molecular weight compared to that of periplasmic binding proteins (BRASS et al. unpublished). This indicates that specific properties of the introduced periplasmic proteins, such as hydrophobic or ionic interactions with other periplasmic components and/or reversible binding protein multimerization, which has been claimed as a mechanism for controlling the activity of binding proteins (ANTONOV et al. 1976), are probably not the cause for the extremely low mobility of macromolecules in the periplasm. A more likely explanation could be that the periplasm has an extremely high apparent viscosity caused by ordering of water molecules around periplasmic macromolecules. Indeed, it has recently been suggested that the periplasm might consist of a gel which could confer viscosity (HOBOT et al. 1984). Still another reason for the strong retardation of macromolecules could be that the periplasm contains physical barriers to protein movement. It is well known that the periplasm contains many structural components which might exert a sieving effect, including the peptidoglycan and the periplasmic domains of the outer membrane proteins which interact with peptidoglycan.

In recent experiments (ROTHFIELD et al., manuscript in preparation) we addressed the question if periseptal annuli which completely surround the cell at sites of cell division (see Fig. 9) function as tight barriers to long-distance movement of proteins in the periplasm. In FRAP experiments we measured the lateral mobility (D_L) and the fraction of mobile molecules (R value) of fluorescent binding proteins in the periplasm at different locations of *ftsA* filaments, at pole caps, at potential division sites, and at sites free of periseptal annuli. We found similar D_L values for the binding proteins all over the cell but a strikingly lower fraction of mobile molecules at annuli containing regions compared to annuli-free regions of the cell filaments (R values of $35\% \pm 9$ and $62\% \pm 12$, respectively). Upon repeated bleaching of pole caps, fluorescence intensity was bleached to background levels and hardly recovered, even though at distant areas of the same filament fluorescence intensity was as high as the initial value before any bleaches. These experiments indicate that periseptal annuli divide the periplasm into different separate compartments.

The procedures described by us (BRASS et al. 1986) demonstrate the possibility of incorporating a variety of proteins, and also other molecules, into the periplasm, and should be applicable to a variety of studies of both the structure and the function of this poorly characterized cellular compartment. It is clear from our data that the interactions of substrate-binding proteins in the periplasm are very much more complex than has generally been supposed. The possible

mechanism by which binding proteins function in transport and chemotaxis must be reassessed in light of these data.

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Clostridial Neurotoxins: Handling and Action at the Cellular and Molecular Level

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1 Introduction

Tetanus and botulism have fascinated mankind since they were first described by Hippocrates (cited by MAJOR 1965) and KERNER (1817) respectively. Following the ascent of bacteriology at the end of the past century, the flow of research has been uninterrupted, and it may be safely stated that no other group of toxins has aroused as much interest as the clostridial neurotoxins. The pertinent literature presents not only a historical account of their handling by the scientific community, but also a cross-section of the development of science and the time-dependent ways of thinking and experimenting. The progress in understanding started with the detection of the causative bacteria and their toxins at the end of the 19th century, the period dominated by microbiology. A stage of macroscopic physiology ensued, resulting in the assignment of the intoxication to the spinal cord in tetanus and to the peripheral nerve endings in botulism. In parallel, the ascent of immunology led to the development of toxoids and antibodies and paved the way for treatment and prophylaxis of the diseases.

During the past 20 years, interest has moved to the elementary processes underlying the synthesis, the pharmacokinetics, the pharmacodynamics, and the immunological properties of the toxins. Biochemistry is now the theoretical and methodological centerpiece of research, assisted by cell culture and electrophysiology. The ultimate aim is to understand tetanus and botulism at the molecular level and to regard them as molecular diseases. In contrast to the earlier stages of research, the trend is now away from medical application of conventional knowledge. Of course, all present researchers, like their ancestors, hope that their efforts will sooner or later benefit people who succumb to these terrible toxic infections.

The field of tetanus and botulism is well covered by reviews which mirror the state of art at the time of their publication as well as the preferences of their authors. There was a plethora of them between 1978 and 1982. Tables 1 and 2 compile the comprehensive surveys on tetanus and botulism. The present review may be regarded as a continuation of two previous publications. For tetanus toxin, it sets forth the lines drawn by WELLHÖNER (1982), and for botulinum toxins SIMPSON's review (SIMPSON 1981), with special emphasis on botulinum A toxin.

Evidently, tetanus toxin attracts considerably more interest than the botulinum toxins do. Comparison of the tables also shows that the last synoptic, voluminous review covering both toxins is now 31 years old (WRIGHT 1955). A short comparison has been given recently (MELLANBY 1984).

Table 1. Reviews on tetanus

Year	Reference	Form of presentation	Topic
1955	PRÉVOT	B	Mainly bacteriology, immunology, and treatment
1955	WRIGHT	J	Mainly pharmacology; combines studies on tetanus and botulism
1959	TURPIN and RAYNAUD	J	
1963	LAURENCE and WEBSTER	J	Mainly clinical aspects
1966	KRYZHANOVSKY	B	The most comprehensive one-author compilation, including author's data; unfortunately not translated from the Russian
1971	VAN HEYNINGEN and MELLANBY	B	Accent on biochemical mode of action
1975	Anonymous (ed)	S	Many contributions on all aspects of tetanus
1977	BIZZINI	B	Biochemistry of the toxin
1978	HABERMANN	H	Epidemiological and clinical aspects
1979	BIZZINI	J	Biochemistry and biochemical mode of action
1980	VAN HEYNINGEN	J	Biochemistry of the toxin; interaction with binding sites
1981	MELLANBY and GREEN	J	Mode of action
1981	VERONESI (ed)	B	The most comprehensive multi-author publication covering all aspects of tetanus
1982	WELLHÖNER	H	Basic research on tetanus toxin and its effects
1984b	BIZZINI	H	Vaccines, epidemiology
1985	NISTICÓ et al.	S	Many contributions on all aspects of tetanus
1985	HABERMANN and GORETZKI	H	Monoclonal antibodies

B, Book, or part of one; *H*, contribution to a handbook; *J*, contribution to a journal; *S*, collection of contributions to a symposium

The present contribution does not attempt to furnish another updated review of what has already been so well presented by others. Instead, it tries to uncover the elementary processes as far as they can be approached today, and to define the empty spaces that might be filled by the work of tomorrow. It searches for common links, and thereby also for differences between the clostridial neurotoxins. It will show that, although the manifestations of tetanus and botulism fundamentally differ in the physician's view, the causative toxins, their biosyn-

Table 2. Reviews on botulism

Year	Reference	Form of presentation	Topic
1955	PRÉVOT	B	Mainly bacteriology, immunology, and treatment
1955	WRIGHT	J	Mainly pharmacology
1971	BOROFF and DAS GUPTA	B	Mainly nonclinical aspects
1977	SMITH	B	Mainly bacteriology and disease
1980	SUGIYAMA	J	Biochemistry and pharmacology
1980	GUNDERSEN	J	Pharmacology
1981	LEWIS (ed)	S	Numerous contributions on all aspects of botulism
1981	SIMPSON	J	Biochemistry and neuromuscular effects
1983	SAKAGUCHI	J	Biochemistry

B, Book, or part of one; *J*, contribution to a journal; *S*, collection of contributions to a symposium

thesis, and their actions are often very similar in terms of basic biochemistry and pharmacology.

2 Biosynthesis

Like defense mechanisms against antibiotics, the aggressive weapons of bacteria, i.e., toxins, are not seldom encoded outside the chromosomes (see, for instance, ALOUF et al. 1984). Synthesis of clostridial neurotoxins, despite their many similarities in structure and function, may be governed by plasmid, bacteriophage, or chromosomal DNA. So far, it is not certain for every toxin where the information on its structure is stored. Moreover, all publications to date deal with the generation of the peptide chain. Nothing is known about the gene expression of proteases that are required (see below) to tailor the final toxin.

The biosynthesis of the hemagglutinins and other proteins which accompany, or even complex with, the botulinal neurotoxins (see the review by SAKAGUCHI 1983) is also still in the dark. The plasmid DNA of *Clostridium tetani* and the phage DNA of *Clostridium botulinum* are large enough to carry, in addition to information concerning the structure of the toxins, information about their processing enzymes or their complexing admixtures.

2.1 Plasmids

Tetanus toxin is the only representative whose coding by a plasmid is not doubted. The possible relevance of this extrachromosomal structure in *C. botu-*

linum strains is unknown, although they may harbor plasmids, too (see SUGIYAMA 1980). The lack of correlation between toxigenicity and the other general properties of *C. tetani* has been known for a long time, as loss of toxigenicity was a nuisance for people interested in toxin production. NISHIDA et al. (1969) attempted to identify nontoxigenic bacteria from soil and from a laboratory strain. The first comprehensive work on the genetic basis of tetanus toxin production stems from HARA et al. (1977). They treated a toxigenic strain derived from Harvard A47 with acridine orange, *N*-methyl-*N'*-nitrosoguanidine, rifampicin, or ultraviolet light. The recovery of nontoxigenic clones was 0.8%–3.2%, far above the rate expected for mutation. As compared with those of the toxigenic parent strain, the culture filtrates of the nontoxigenic derivatives contained similar quantities of all the antigenic components except tetanus toxin. HARA et al. (1977) also observed a bacteriophage, as had PRESCOTT and ALTENBERN (1967a, b) earlier. However, none of the nontoxigenic derivatives was "cured" of its prophage. Although all toxigenic strains so far investigated also contained prophages, it is improbable, for various reasons, that bacteriophages carry the genetic information for tetanus toxin. For instance, direct transfer of toxigenicity in mixed cultures was not achieved. Moreover, the rate of loss of toxigenicity was high, and the loss was stable.

In contrast to the role of bacteriophages, the role of a plasmid is clear. Plasmid content paralleled tetanus toxin production in 52 strains, with one notable exception, however. In this case a nontoxigenic strain harbored a large plasmid (LAIRD et al. 1980). Other observations also point toward a diversity among the plasmids. For instance, strains selected randomly as to origin contained plasmids that differed in molecular weight between 25 Kb and 70 Kb (Kilobases). Those probably derived from the high-yield Harvard strain each harbored a plasmid of approximately 49 Kb, with an identical restriction endonuclease pattern (LAIRD et al. 1981). FINN et al. (1984) observed the existence of plasmids in numerous nontoxigenic strains and found that spontaneous deletions of 22 Kb from the original 75 Kb plasmid rendered the harboring bacteria nontoxigenic.

A pool of synthetic oligonucleotides based on the reported N-terminal sequence of tetanus toxin (ROBINSON and HASH (1982); see also Fig. 4) was used to screen various toxigenic and nontoxigenic plasmids and to map the gene on the plasmid. The putative location of the gene on a 16.5 Kb *Eco*RI fragment is shown in Fig. 1a. It is interesting to note that the deleted plasmid still hybridized with the oligonucleotide probe, indicating that the DNA encoding the amino terminus was retained.

Part of the amino acid sequence of tetanus toxin was established at the DNA level. One group (FAIRWEATHER et al. 1986) started from a total DNA preparation. They sequenced the first 30 amino acids of a highly purified fragment C (see Fig. 4), and synthesized a mixed oligonucleotide coding for amino acids 6–11 of the fragment C sequence as probe which hybridized with a 2 Kb fragment of *C. tetani* DNA. A sequence of 1.8 Kb was determined which encoded the entire fragment C and a larger part of the N-terminal half of the heavy chain. Various DNA fragments encoding all or part of fragment C were transferred into *E. coli* using an expression vector, and fragments of different

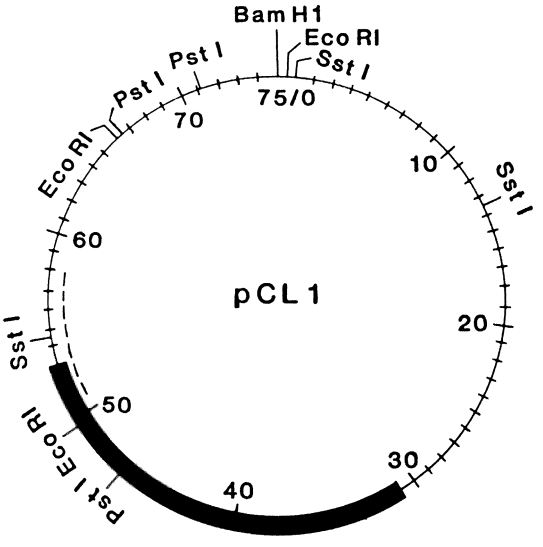

Fig. 1a. Mapping of the tetanus toxin gene on a toxigenic plasmid pCL1 (from FINN et al. 1984, with permission). The exact localization of the toxin gene is still to be established

sizes above fragment C were expressed. They contained an antigenic determinant since they reacted with a monoclonal antibody against fragment C (Fig. 1 b).

The relevance of the gene technological work on tetanus toxin is twofold. It furnishes the primary structure whose knowledge is a precondition for the assignement of the various properties of tetanus toxin, for instance ganglioside binding, internalization, toxicity and immunogenicity, to the amino acid sequence. Moreover, it opens the way to minimal vaccines, as shown by the expression experiments.

The apparent chemical similarities between tetanus toxin and botulinum A, B, and E toxins (see Fig. 4) are astonishing, since the former is encoded by a plasmid, whereas the genes for the latter are present on the chromosome. One wonders whether the sequences of C_1 and D botulinum toxin, which are presumably encoded by bacteriophages (see Sect. 2.2), are similar to the other sequences. If so, a common ancestor DNA must have taken many different phylogenic paths.

2.2 Bacteriophages

In contrast to tetanus toxin, some botulinum toxins are encoded by bacteriophages. Eight antigenically distinct toxins are known: A, B,[1] C_1, C_2, D, E, F, G. Despite their structural similarities, the genetic information is stored differently. By combining the observations of VINÉT and FREDETTE (1968), INOUE and IIDA (1968), and EKLUND et al. (1969), one can conclude that every type of *C. botulinum* harbors phages. The authors distinguish different groups according to morphology and lysogeny. A summary is given in Table 3.

[1] The term "B toxin" refers to an immunologically heterogeneous group, originating from proteolytic or nonproteolytic strains (DAS GUPTA and WOODY 1984).

Table 3. Synopsis of clostridial strains and their toxins (Adapted from SMITH 1977)

Group	Type	Property	Encoded by		Toxin(s) synthesized
			Phages	Plasmid	
Clostridium botulinum					
I	A	Proteolytic	Questionable	Questionable	A
	A_F	Proteolytic	Questionable	Questionable	A
	B	Proteolytic	Questionable	Questionable	B[c]
	F	Proteolytic	Questionable	Questionable	F
II	B	Nonproteolytic	Questionable	Questionable	B[c]
	E	Nonproteolytic	Questionable	Questionable	E
	F	Nonproteolytic	Questionable	Questionable	F
III	C	Nonproteolytic but gelatinolytic	Established[a]	Absent	$\underline{C_1}$, C_2[b,d], D[b]
	D	Nonproteolytic but gelatinolytic	Established[a]	Absent	C_1[b], C_2[b], $\underline{D}$
IV	G	Weakly proteolytic	Questionable	Questionable	G
Clostridium tetani					
		Weakly proteolytic	No	Established	Tetanus toxin

[a] For C_1 and D but not for C_2

[b] Minor toxins, not produced by all strains. The major toxins are underlined

[c] The B toxins from proteolytic and nonproteolytic strains are immunologically different, whereas the product of each strain is immunologically homogeneous (for pertinent citations see DAS GUPTA and WOODY 1984)

[d] Some strains produce C_2 only

So far, the genetic relevance of the phages is established for toxins C_1 and D of the respective clostridia, but not for toxin C_2. In "double" producers encoding occurs in the chromosomal (C_2) and the phage (C_1) DNA. Bacteriophages are apparently *not* involved in toxin production by the remaining types. The role of plasmids in the various types has not yet been satisfactorily assessed. The similarities between the (phage-dependent) toxins C_1 and D are pronounced. Their amino acid compositions resemble each other. One monoclonal antibody (out of seven) reacts with the heavy chains, and another with both the light and heavy chains of C_1 and D toxins, indicating that both toxins have common antigenic sites on their heavy and light chains (MURAYAMA et al. 1984).

The work on bacteriophages has been summarized by EKLUND and POYSKY (1981) and IIDA and OGUMA (1981). Briefly, treatment of a toxigenic strain of *C. botulinum* C with mitomycin C led to lysis. However, when the toxigenic strain was grown in the presence of acridine orange, stable nontoxigenic cultures (3%–9%) occurred that were nonlysogenic. Treatment of a nontoxigenic strain with the induced lysate from the toxigenic strain restored the toxigenicity. Except for toxigenicity, the converted culture showed no differences from the nontoxigenic parent strain. A nontoxigenic but lysogenic strain was also obtained by treatment with *N*-methyl-*N*-nitrosoguanidine.

```
                                                  30                                                  60
AGA TCT TTA GAA TAT CAA GTA GAT GCA ATA AAA AAA ATA ATA GAC TAT GAA TAT AAA ATA
Arg Ser Leu Glu Tyr Gln Val Asp Ala Ile Lys Lys Ile Ile Asp Tyr Glu Tyr Lys Ile

                                                  90                                                 120
TAT TCA GGA CCT GAT AAG GAA CAA ATT GCC GAC GAA ATT AAT AAT CTG AAA AAC AAA CTT
Tyr Ser Gly Pro Asp Lys Glu Gln Ile Ala Asp Glu Ile Asn Asn Leu Lys Asn Lys Leu

                                                 150                                                 180
GAA GAA AAG GCT AAT AAA GCA ATG ATA AAC ATA AAT ATA TTT ATG AGG GAA AGT TCT AGA
Glu Glu Lys Ala Asn Lys Ala Met Ile Asn Ile Asn Ile Phe Met Arg Glu Ser Ser Arg

                                                 210                                                 240
TCA TTT TTA GTT AAT CAA ATG ATT AAC GAA GCT AAA AAG CAG TTA TTA GAG TTT GAT ACT
Ser Phe Leu Val Asn Gln Met Ile Asn Glu Ala Lys Lys Gln Leu Leu Glu Phe Asp Thr

                                                 270                                                 300
CAA AGC AAA AAT ATT TTA ATG CAG TAT ATA AAA GCA AAT TCT AAA TTT ATA GGT ATA ACT
Gln Ser Lys Asn Ile Leu Met Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr

                                                 330                                                 360
GAA CTA AAA AAA TTA GAA TCA AAA ATA AAC AAA GTT TTT TCA ACA CCA ATT CCA TTT TCT
Glu Leu Lys Lys Leu Glu Ser Lys Ile Asn Lys Val Phe Ser Thr Pro Ile Pro Phe Ser

      B | C                                           390                                                 420
TAT TCT | AAA AAT CTG GAT TGT TGG GTT GAT AAT GAA GAA GAT ATA GAT GTT ATA TTA AAA
Tyr Ser | Lys Asn Leu Asp Cys Trp Val Asp Asn Glu Glu Asp Ile Asp Val Ile Leu Lys

                                                 450                                                 480
AAG AGT ACA ATT TTA AAT TTA GAT ATT AAT AAT GAT ATT ATA TCA GAT ATA TCT GGG TTT
Lys Ser Thr Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile Ser Asp Ile Ser Gly Phe

                                                 510                                                 540
AAT TCA TCT GTA ATA ACA TAT CCA GAT GCT CAA TTG GTG CCC GGA ATA AAT GGC AAA GCA
Asn Ser Ser Val Ile Thr Tyr Pro Asp Ala Gln Leu Val Pro Gly Ile Asn Gly Lys Ala

                                                 570                                                 600
ATA CAT TTA GTA AAC AAT GAA TCT TCT GAA GTT ATA GTG CAT AAA GCT ATG GAT ATT GAA
Ile His Leu Val Asn Asn Glu Ser Ser Glu Val Ile Val His Lys Ala Met Asp Ile Glu

                                                 630                                                 660
TAT AAT GAT ATG TTT AAT AAT TTT ACC GTT AGC TTT TGG TTG AGG GTT CCT AAA GTA TCT
Tyr Asn Asp Met Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser

                                                 690                                                 720
GCT AGT CAT TTA GAA CAA TAT GGC ACA AAT GAG TAT TCA ATA ATT AGC TCT ATG AAA AAA
Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile Ile Ser Ser Met Lys Lys

                                                 750                                                 780
CAT AGT CTA TCA ATA GGA TCT GGT TGG AGT GTA TCA CTT AAA GGT AAT AAC TTA ATA TGG
His Ser Leu Ser Ile Gly Ser Gly Trp Ser Val Ser Leu Lys Gly Asn Asn Leu Ile Trp

                                                 810                                                 840
ACT TTA AAA GAT TCC GCG GGA GAA GTT AGA CAA ATA ACT TTT AGG GAT TTA CCT GAT AAA
Thr Leu Lys Asp Ser Ala Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro Asp Lys

                                                 870                                                 900
TTT AAT GCT TAT TTA GCA AAT AAA TGG GTT TTT ATA ACT ATT ACT AAT GAT AGA TTA TCT
Phe Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr Asn Asp Arg Leu Ser

                                                 930                                                 960
TCT GCT AAT TTG TAT ATA AAT GGA GTA CTT ATG GGA AGT GCA GAA ATT ACT GGA TTA GGA
Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met Gly Ser Ala Glu Ile Thr Gly Leu Gly

                                                 990                                                1020
GCT ATT AGA GAG GAT AAT AAT ATA ACA TTA AAA CTA GAT AGA TGT AAT AAT AAT AAT CAA
Ala Ile Arg Glu Asp Asn Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn Asn Asn Asn Gln
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                                                1070                                                1080
TAC GTT TCT ATT GAT AAA TTT CGG ATA TTT TGC AAA GCA TTA AAT CCA AAA GAG ATT GAA
Tyr Val Ser Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala Leu Asn Pro Lys Glu Ile Glu

                                                1110                                                1140
AAA TTA TAC ACA AGT TAT TTA TCT ATA ACC TTT TTA AGA GAC TTC TGG GGA AAC CCT TTA
Lys Leu Tyr Thr Ser Tyr Leu Ser Ile Thr Phe Leu Arg Asp Phe Trp Gly Asn Pro Leu

                                                1170                                                1200
CGA TAT GAT ACA GAA TAT TAT TTA ATA CCT GTA GCT TCT AGT TCT AAA GAT GTT CAA TTG
Arg Tyr Asp Thr Glu Tyr Tyr Leu Ile Pro Val Ala Ser Ser Ser Lys Asp Val Gln Leu

                                                1230                                                1260
AAA AAT ATA ACA GAT TAT ATG TAT TTG ACA AAT GCG CCA TCG TAT ACT AAC GGA AAA TTG
Lys Asn Ile Thr Asp Tyr Met Tyr Leu Thr Asn Ala Pro Ser Tyr Thr Asn Gly Lys Leu

                                                1290                                                1320
AAT ATA TAT TAT AGA AGG TTA TAT AAT GGA CTA AAA TTT ATT ATA AAA AGA TAT ACA CCT
Asn Ile Tyr Tyr Arg Arg Leu Tyr Asn Gly Leu Lys Phe Ile Ile Lys Arg Tyr Thr Pro

                                                1350                                                1380
AAT AAT GAA ATA GAT TCT TTT GTT AAA TCA GGT GAT TTT ATT AAA TTA TAT GTA TCA TAT
Asn Asn Glu Ile Asp Ser Phe Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val Ser Tyr

                                                1410                                                1440
AAC AAT AAT GAG CAC ATT GTA GGT TAT CCG AAA GAT GGA AAT GCC TTT AAT AAT CTT GAT
Asn Asn Asn Glu His Ile Val Gly Tyr Pro Lys Asp Gly Asn Ala Phe Asn Asn Leu Asp

                                                1470                                                1500
AGA ATT CTA AGA GTA GGT TAT AAT GCC CCA GGT ATC CCT CTT TAT AAA AAA ATG GAA GCA
Arg Ile Leu Arg Val Gly Tyr Asn Ala Pro Gly Ile Pro Leu Tyr Lys Lys Met Glu Ala

                                                1530                                                1560
GTA AAA TTG CGT GAT TTA AAA ACC TAT TCT GTA CAA CTT AAA TTA TAT GAT GAT AAA AAT
Val Lys Leu Arg Asp Leu Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp Lys Asn

                                                1590                                                1620
GCA TCT TTA GGA CTA GTA GGT ACC CAT AAT GGT CAA ATA GGC AAC GAT CCA AAT AGG GAT
Ala Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile Gly Asn Asp Pro Asn Arg Asp

                                                1650                                                1680
ATA TTA ATT GCA AGC AAC TGG TAC TTT AAT CAT TTA AAA GAT AAA ATT TTA GGA TGT GAT
Ile Leu Ile Ala Ser Asn Trp Tyr Phe Asn His Leu Lys Asp Lys Ile Leu Gly Cys Asp

                                                1710
TGG TAC TTT GTA CCT ACA GAT GAA GGA TGG ACA AAT GAT
Trp Tyr Phe Val Pro Thr Asp Glu Gly Trp Thr Asn Asp
```

Fig. 1b. Sequence of part (see Fig. 3a) of the heavy chain (FAIRWEATHER et al. 1986). Coding of the C-fragment begins with nucleotide 367. Note that the C-fragment contains four half-cystines. Two of them are linked by a disulfide bridge in a still unknown position (see Fig. 3a), and the fragment contains two free SH groups (see BIZZINI et al. 1977)

A series of conversion experiments led to the conclusion that there are more than one type-C toxigenic phage, in addition to the nontoxigenic phage mentioned above. Moreover, there is strong evidence that the same phage can promote the simultaneous production of C_1 and D toxins. In contrast, production of C_2 toxin was apparently not mediated by a phage. Cross-infection experiments were successful. For instance, a nontoxigenic strain type D was not converted by the induced lysate of its toxigenic D parent, but by that of a toxigenic

C. Botulinum		
C_1	→	D
D	→	C_1
C. novyi type A	→	*C. botulinum* type C_1

Fig. 2. Three conversions that have been established. *Arrow* denotes which phage (*left*) may replace the original (*right*)

strain C. As expected, it produced type C_1 toxin only. Conversely, nontoxigenic strain C bacteria could be converted to producers of type D toxin by appropriate phages. Thus, the toxin produced is not determined by the host bacterium but by the harbored phage.

Not seldom, strains C and D lose their toxigenicity through repeated passages. In some cases, loss of toxigenicity was enhanced by antiphage serum, indicating a "cure" from the phage infections. In most cases, toxigenicity was lost without antiserum, and could be restored by filtrates of the supernatants from toxigenic strains. In other cases, toxigenicity was also lost in the absence of antiserum, but some of the resulting nontoxigenic strains neither lysed nor converted to toxigenicity when exposed to the original phage. As at least one nonconverting phage was observed, it was assumed that some bacteria remained nonproducers, because their original converting phage had undergone a mutation to a nonconverting phage. In this way, they became resistant to infection by other converting phages.

Interstrain conversion has been reported so far for *C. botulinum* types C and D only. Even more astonishing was the observation that *Clostridium novyi* may also serve as a donor for phages able to infect *C. botulinum* C. For instance, a phage-free strain of *C. botulinum* type C could be infected with *C. novyi* type-A phage and thus became a producer of the dominant type-A novyi toxin, while it became a producer of botulinum C_1 toxin or D toxin when the appropriate phages were applied. Fortunately, no report on the reverse conversion exists, i.e., that *C. novyi* might carry phages encoding a botulinum toxin. Figure 2 summarizes the three conversions so far established.

3 Biochemistry of the Toxins as a Prerequisite to Understanding Their Action

The field has been amply reviewed (see Table 1 for tetanus toxin and Table 2 for botulinum toxins). This chapter will discuss the structural data (Fig. 3–6, Table 4) with respect to production and function.

3.1 Post-translational Handling

All clostridial neurotoxins possess similar structures (Fig. 3b, Fig. 4), although there is no immunological cross-reactivity between any except botulinum C_1 and D toxins (MURAGAMA et al. 1984) and E and F toxins (see SUGIYAMA 1980).

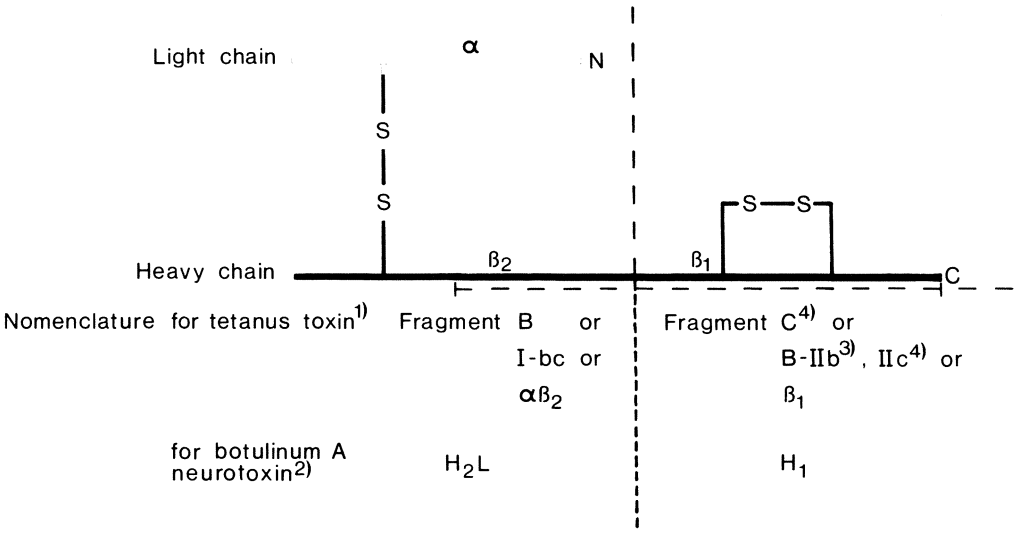

Fig. 3a. General structure of clostridial neurotoxins after nicking, and nomenclature of their fragments. *1*, According to BIZZINI (1979); *2*, according to SHONE et al. (1985); *3*, produced from tetanus toxin by intrinsic protease(s); *4*, produced from tetanus toxin by papain. The broken line indicates the sequence known from DNA analysis (see Fig. 1b)

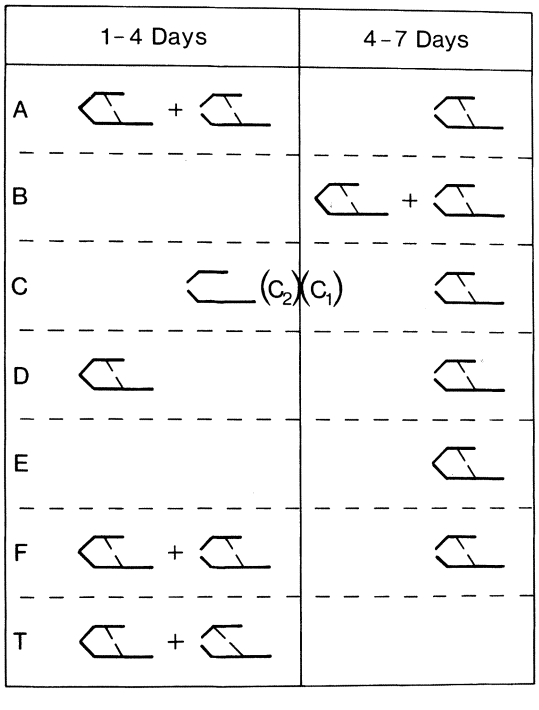

Fig. 3b. Structure of individual clostridial neurotoxins derived from bacterial cultures of varying age (from DASGUPTA 1981, with permission). Tetanus toxin (*T*) is added for comparison

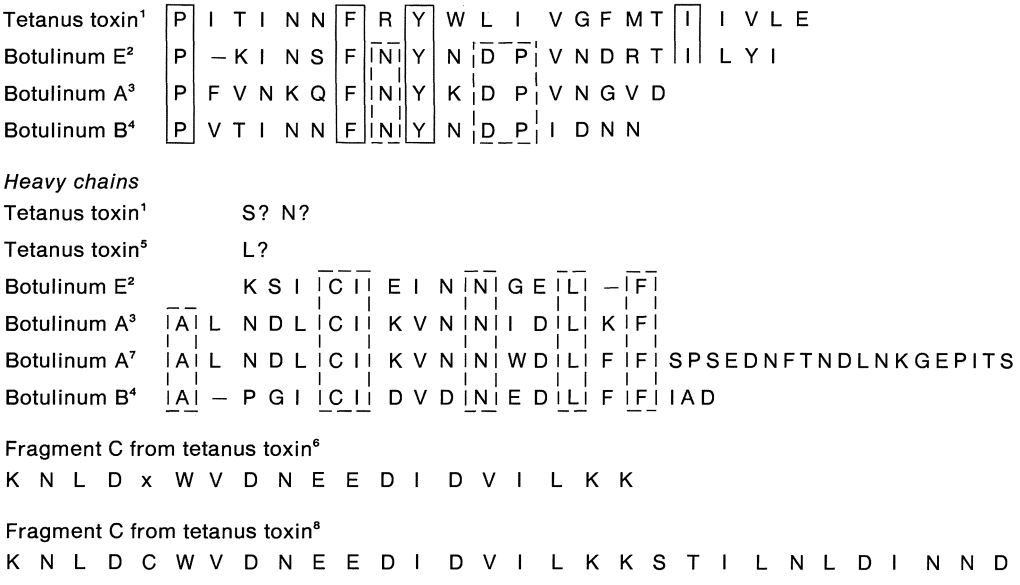

Fig. 4. Partial N-terminal amino acid sequences of the heavy and light chains of tetanus and botulinum A, B, and E neurotoxins and of fragment C of tetanus toxin. Amino acids common to all listed toxins are framed by *solid lines*, those common to the botulinum toxins by *broken lines*. *1*, ROBINSON and HASH (1982), pos. 3 and 4 were corrected (HENSCHEN, unpublished); *2*, SATHYAMOORTHY and DASGUPTA (1985a, b), SCHMIDT et al. (1985); *3*, SCHMIDT et al. (1984, 1985), SATHYAMOORTHY and DASGUPTA (1985b); *4*, SCHMIDT et al. (1985), SATHYAMOORTHY and DASGUPTA (1985b); *5*, NEUBAUER and HELTING (1979); *6*, A. HENSCHEN, unpublished data – *x* is to be replaced by C, according to the DNA sequencing data; *7*, SHONE et al. (1985) – note the differences from SCHMIDT et al. (1985) at positions 12 and 15; *8*, FAIRWEATHER et al. (1986)

Independent of the gene carrier, the first products identified are polypeptides with molecular weights of between 140000 and 160000. Recently, the proposal was made that the gene for tetanus toxin arose through duplication events (TAYLOR et al. 1983). The assumption is based on similarities of amino acid composition between the heavy and light chains and on identities in peptide patterns following limited cleavage by cyanogen bromide or V_8-protease from *Staphylococcus aureus*. It remains to be shown whether this hypothesis holds for botulinum toxins and can be confirmed by sequencing of tetanus toxin. At least the 20 N-terminal amino acids of the light chain and the C-fragment do not correlate well in tetanus toxin (see Fig. 4).

Why the neurotoxins have to be this large is still a mystery. Large size might be required for their multiple attachment to the target membrane; it might serve a structural background for more than one function, or just protect against degrading enzymes. On the other hand, large size prevents passage through the functional barriers of the central nervous system in situ, thus restricting the access of the neurotoxins to their possible target.

Once produced, *tetanus toxin* stays within the bacterial cell. Immunogold staining renders it visible throughout the cytoplasm, without preferential local-

ization close to an organelle (ROSENBERG and HABERMANN, unpublished). Spores were absent in this preparation.

Botulinum A toxin has been made visible by immunoferritin in spheroblasts. In ultrathin sections the labeled antibody was arranged around the outer spore coats and on the surface of the exosporium (DUDA and SLACK 1969). It is not certain wheter the distribution of botulinum A toxin is the same in bacteria. Nothing is known about the distribution of other botulinum toxins in bacteria and their spores. It should also be kept in mind that at the dawn of botulinum research the hemagglutinins could not be distinguished from the neurotoxin proper.

Subsequent to production, the toxins are subjected to post-translational processing. It cannot be stated when, where, and how this starts. As the extracellular fluids contain bacterial proteases, susceptible bonds of the primary gene product might be hydrolyzed there. At least in the case of tetanus toxin, the primary products are probably not compartmented in granules, and their release from the cells requires many days of autolysis (see, for instance, HELTING et al. 1979). Protoxins should be sought that eventually become hydrolyzed in the course of preparation of intracellular tetanus and botulinum neurotoxins.

More light has been shed on the later events. With the exception of botulinum E toxin (and some B toxin; see Fig. 3b), all other toxins are nicked by enzymes in the culture broth. Nicking preferentially occurs in the course of autolysis. However, nicking within the bacterial cell cannot be excluded, as exemplified by tetanus toxin. The extracted tetanus toxin may consist up to 20% (HABIG et al. 1983) or 50% (HELTING et al. 1979) of two-chain forms.

Is nicking required for the expression of toxicity? This is often supposed for some *botulinum toxins*. For instance, the toxicity of botulinum E toxin increased about 50-fold upon trypsin treatment (DUFF et al. 1956). In other strains, nicking and increase of toxicity depended largely on the protease content of the cultures and the time of contact between protease and toxin. Un-nicked and nicked molecules may coexist in varying proportions (DAS GUPTA 1981; see also Fig. 3b). Figure 3b shows that nicking by itself does not necessarily lead to separation of the two chains. With the exception of C_2 toxin (see below), they are held together by at least one disulfide bridge. In addition, hydrophobic interactions and/or hydrogen bridges are to be considered. Dissociation and reassociation of the chains of botulinum neurotoxins can be achieved under mild conditions (type A neurotoxin, KOZAKI et al. 1981; type B neurotoxin, KOZAKI et al. 1977), even in the absence of a denaturant (C neurotoxin, SYUTO and KUBO 1981). This might indicate a low-affinity, noncovalent interaction between the two chains of various botulinum toxins once the disulfide bonds have been split. In order to restore toxicity, it is not sufficient just to remix the chains. Time and appropriate ionic environments are required to bring them into the proper arrangement, which regenerates up to about 70% of the original toxicity (SUGIYAMA 1980; SYUTO and KUBO 1981). One possible exception to the disulfide concept is the botulinum C_2 toxin (see Fig. 3b), whose two parts are not linked at all. This toxin is encoded not by a bacteriophage (like C_1 or D toxins) but probably by the bacterial chromosome. Curiously enough, only C_2 toxin is not a true neurotoxin (see Sect. 5.1.1).

Reduction of nicked *tetanus toxin* does not allow separation of the chains. Additional denaturation, for instance by SDS or urea, will be needed. The problems are discussed in the reviews of ROBINSON and HASH (1982) and HELTING and HABIG (1984). To restore its toxicity, oxidative closure of the disulfide bridge(s) is required (MATSUDA and YONEDA 1976) between the two chains (MATSUDA and YONEDA 1974, 1975) of the toxin. The question of whether nicking is required for, or raises the toxic potency of tetanus toxin has not yet been settled. So far, the preparations of intracellular toxin available are mixtures of un-nicked and nicked toxin and may with age shift to the latter. Radiolabeled un-nicked toxin enters the nerve for axonal transport (HABIG et al. 1983). The toxin is apparently not nicked within the poisoned mammalian cell (see p. 126). If there is any, the gain in toxicity by nicking is small. HABIG et al. (1983) report the toxicity of nicked and un-nicked to be the same. Others found trypsin treatment to raise the toxicity of autolysates of young tetanus bacilli (SEKI et al. 1954, cited by MATSUDA and YONEDA 1974) and the toxicity of intracellular toxin, the latter about threefold (MATSUDA and YONEDA 1974). Perhaps other proteolytic events in addition to nicking favor toxicity (see Sect. 3.2).

All tetanus toxin preparations used in our laboratory, whether "extracellular" from the *Behringwerke*, Marburg, or "intracellular" from the *Institut Pasteur*, Paris, contain intrinsic protease activity. This activity becomes detrimental to the toxin molecule itself when denaturing agents (urea or SDS) are added to concentrated solutions (GORETZKI and HABERMANN 1985). The intrinsic nicking protease for tetanus toxin (HELTING et al. 1979) is a small protein (mol. wt. about 27000). It is markedly inhibited by phenylmethylsulfonyl fluoride, an inactivator of esterase-proteases, and also by the α_2-macroglobulin trypsin inhibitor. Nothing is said about its sensitivity to reducing agents. It is also not known whether the intrinsic proteases are specific for the proteins produced by the respective clostridial strains, or whether they can attack all the clostridial toxins. Tetanus toxin itself is apparently devoid of proteolytic activity (unpublished data).

Different extrinsic enzymes may nick tetanus and botulinum toxins. Trypsin has already been mentioned for botulinum E toxin, and it also seems appropriate for tetanus toxin (MATSUDA and YONEDA 1974). Papain nicks the single-chain tetanus toxin (NEUBAUER and HELTING 1981).

The multitude of enzymes (intrinsic protease, papain, trypsin) able to nick the toxins indicates that the position of the break is not specific. Accordingly, the freshly exposed N-terminal of tetanus toxin is not homogeneous (see below, and Figs. 4 and 5).

In the foregoing we have tacitly assumed that nicking is the only proteolytic process involved in activating botulinum neurotoxins. However, at least in this group, a second point of attack is to be considered; it is located tentatively at the C-terminal end of the heavy chain. The hypothesis dates back to 1972 (for review, see DAS GUPTA 1981).

The intrinsic enzyme responsible for this type of hydrolysis of botulinum A, B, and F toxins resembles clostripain from *C. histolyticum*. Like trypsin, it splits at the arginine and lysine bonds, but it does not nick. It is resistant to the usual trypsin inhibitors and belongs to the SH-proteases, as it is only active

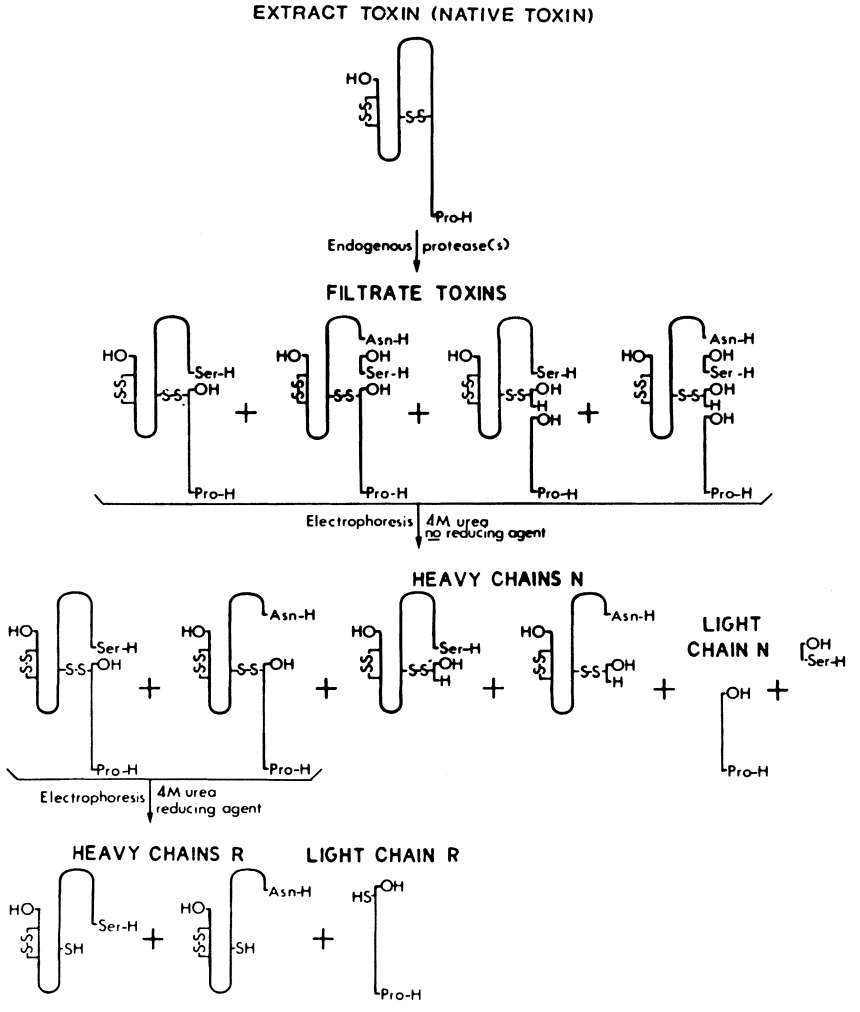

Fig. 5. Molecular model of tetanus toxin. *N*, nonreduced toxin; *R*, reduced toxin, as sources for the chains (ROBINSON and HASH 1982, with permission)

when reduced (DAS GUPTA and SUGIYAMA 1972). OHISHI and SAKAGUCHI (1977) achieved activation before nicking of botulinum B toxin by the appropriate choice of pH and enzyme concentrations. They gave evidence for a similar dissociation of activation and nicking for A and F toxins. The most convincing observation in this direction was made by OHISHI et al. (1980a). As shown in Fig. 3b, C_2 toxin consists of two parts that are not linked by a disulfide bridge(s). Nevertheless, it is further activated by trypsin. The lack of disulfide bonds allows the parts to be exposed separately to trypsin, and then to be mixed for toxicity measurement. It turned out that tryptic activation proceeded by cleavage of the heavy moiety. So far it is not known which of the putative pathways correctly depicts the events occurring with the other botulinum toxins (Fig. 6).

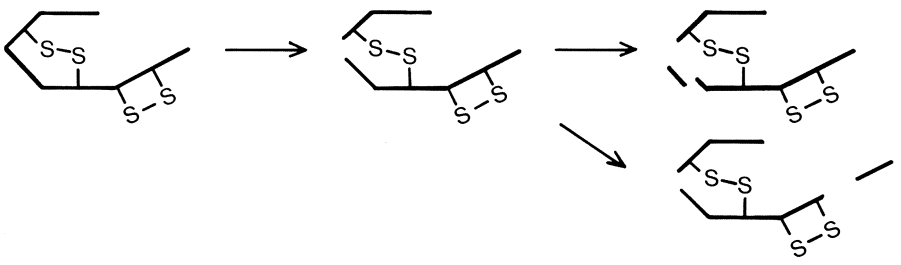

Fig. 6. Hypothetical ways of post-translational processing of neurotoxins (from DASGUPTA 1981, modified)

No similar non-nicking activation has been demonstrated in the post-translational processing of tetanus toxin. However, analysis of the N-terminal amino acid residues of the heavy chain (see Fig. 5) indicates nonhomogeneities that can be explained best by multiple cleavage. ROBINSON and HASH (1982) assume that following nicking, additional hydrolysis can take place at one bond in the heavy and one in the light chain. Thus, two types of tetanus toxin, appear to exist depending on whether the processed chains are still linked by the disulfide bridge or not. Their model also predicts the existence of two heavy and two light chains. However, we found no appreciable amounts of toxin which had been dissociated by mere denaturation with SDS, and no heterogeneity of heavy and light chains as to their molecular weights. Robinson's scheme may help to explain why more than one amino acid has been reported as the N-terminal for the heavy chain. The N-terminal of native tetanus toxin appears to survive the post-translational processing unaltered, because both extracellular and intracellular toxins as well as the light chain have proline as their first amino acid (NEUBAUER and HELTING 1979, 1981; DIMARI et al. 1982b). The same statement also applies for the botulinum toxins investigated (see Fig. 4). Neither do we know if nicking is relevant for the biological effects of tetanus toxin, nor do we know the role of the additional cuts. To summarize: (a) Proteolytic activation by intrinsic or extrinsic proteases is important for all botulinum toxins so far investigated, whereas the data for tetanus toxin are ambiguous. (b) Nicking is possible with all clostridial neurotoxins; however, its role in raising toxicity is not clear. Our lack of knowledge about nicking weakens the hypothesis (see p. 126) that the two toxin chains have to be separated to become biologically active, for instance as A (acting) or B (binding) moieties.

3.2 Chain Fragmentation

Besides nicking, proteases can split *tetanus toxin* at additional positions, resulting in loss of toxicity. So far, no fragment with a toxin-like action has been described. With some of the proteases employed, a fragment has been obtained which retains at least one property of tetanus toxin, i.e., binding to gangliosides. Originally, this fragment was observed by BIZZINI and his group upon freezing crude toxin (BIZZINI et al. 1977; for review, see BIZZINI 1979). Freezing appears

to prepare the toxin for an intrinsic protease, as has also been shown for other denaturing manipulations (see p. 106). This still unidentified intrinsic protease can be replaced by papain (HELTING and ZWISLER 1974, 1977). The split product (for nomenclature see Fig. 3a) was nontoxic, had a molecular weight of about 45000, had lysine as the N-terminal amino acid (NEUBAUER and HELTING 1981), and was endowed with a disulfide loop (BIZZINI et al. 1977). Its N-terminal moiety has been sequenced (see Fig. 4). MATSUDA and YONEDA (1977) presented evidence that the fragment occupies the C-terminal of the heavy chain, and this was confirmed by NEUBAUER and HELTING (1981). Some preparations of this fragment C contain a derivative which is hydrolyzed within the disulfide loop. It yields two smaller pieces upon reduction (unpublished data from this laboratory). The split products resulting from the intrinsic (BIIb), tryptic, or papain (fragment C) proteolysis of tetanus toxin are very similar, albeit not strictly identical. They may be taken as equal for most purposes (BIZZINI et al. 1981). The reason why proteolytic treatment of whole toxin destroys toxicity might be the preferential sensitivity of the heavy chain to proteases, for instance, to chymotrypsin or papain (GORETZKI and HABERMANN 1985).

Cultures may contain still other pieces of tetanus toxin. Single chains have been reported by DIMARI et al. (1982a). A fragment with a molecular weight of 90000 has been isolated by TAYLOR and HABERMANN (unpublished data) and called 90K polypeptide. It yields a 40000 mol. wt. fragment when exposed to trypsin. Fragments C, BIIb and 90K stem from the heavy chain, as confirmed by the use of monoclonal antibodies. Still another, larger piece of heavy chain is obtained when intrinsic proteases of tetanus toxin are activated by urea (GORETZKI and HABERMANN 1985).

Recent data strongly argue for chemical similarities between tetanus toxin and *botulinum toxins* beyond their common size and two-chain structure. As in tetanus toxin, a disulfide loop exists at the C-terminal part of the heavy chains of A and B toxins (DASGUPTA 1981; SHONE et al. 1985). Data on the amino acid sequences of the various clostridial neurotoxins are given in Fig. 4. They show many sequential homologies between the three botulinum toxins assessed, and also between them and tetanus toxin, although the latter is encoded in a plasmid whereas the botulinum A, B, and E toxins are in the bacterial chromosome. One looks forward to the structures of the botulinum toxins C_1 and D which are encoded by bacteriophages.

Botulinum E toxin has been obtained unnicked. The N-terminal of the single chain is identical with that of the light chain. Therefore, the site of nicking of the former is about one-third of the distance from its N-terminal end. So far, there are several pieces of evidence for the fact that this nicking is not linked with raising toxicity. The postulated second, "activating" hydrolysis has not yet been located, but it is certainly far away from the N-terminal of intact type-E neurotoxin (SATHYAMOORTHY and DASGUPTA 1985a).

Reconstitution experiments with moieties other than heavy and light chains have been tried with tetanus toxin only. Fragments B and C, when coupled by a disulfide bridge, were nontoxic, although they bound to synaptic membranes and gangliosides and ascended to the spinal cord (BIZZINI et al. 1980a, b). Fragment B was also bound to thiolated B-chains of ricin or wheat germ

agglutinin. A slight, typical toxicity (lethal dose about 2.5 mg/kg or 0.5 mg/kg respectively) was achieved, which is, however, about 10^6 times lower than that of genuine toxin (BIZZINI 1984a).

3.3 Structure-Activity Relationships

3.3.1 Tetanus Toxin

The main intention of this review is to link structure and biological action. The latter may be defined as the ability to bind to biological targets and/or to exert pharmacological effects. Denaturation of whole tetanus toxin, for instance by treatment with formaldehyde, chloramine-T, or iodine, quickly destroys the neurotoxicity, whereas binding ability is less sensitive and immunogenicity is retained best (HABERMANN 1973a, 1978). This hierarchy is the basis of toxoiding. It was tempting to assume that tetanus toxin contains three distinct sites of biological relevance: one mediating binding, a second responsible for the ensuing pharmacological actions, and a third for its immunological properties (for review, see HABERMANN 1978; WELLHÖNER 1982). In the light of the multiplicity of monoclonal antibodies, the last-mentioned properties can no longer be ascribed to a single site (for review, see HABERMANN et al. 1984). The number of immunogenic sites detected is proportional to the assiduousness of the people making monoclonal antibodies (for review, see HABERMANN and GORETZKI 1985). We can therefore restrict ourselves to the question of whether separate individual sites are responsible for binding and action.

As to binding, the heavy chain is an appropriate candidate for two reasons. First, the heavy chain of tetanus toxin yields fragment C, which shares binding sites with whole toxin on brain (MORRIS et al. 1980; GOLDBERG et al. 1981), thyroid (MORRIS et al. 1980), and kidney (HABERMANN and ALBUS 1986) preparations. The 90K polypeptide is also a derivative of the heavy chain and competes with tetanus toxin for binding to brain membranes (TAYLOR and HABERMANN, unpublished). The pharmacologically inert fragment C delays the neuromuscular paralysis of the phrenic nerve hemidiaphragm when given concurrently with tetanus toxin (SIMPSON 1984a, b) apparently in a competitive manner. Despite major differences in binding kinetics between tetanus toxin and its fragment C (see HABERMANN and ALBUS 1986, and Table 10), the assumption of a common receptor appears to be justified.

Second, the heavy chain itself may be used under appropriate conditions for binding studies and may be compared with the light chain. As expected, the former, but not the latter is adsorbed to ganglioside-cerebroside liposomes (VAN HEYNINGEN 1976). Toxicity of heavy and light chains is minimal (see Table 4) and is now assumed to be due to contamination with the parent toxin (HELTING and ZWISLER 1977). An N-terminally shortened heavy chain such as the 90K polypeptide found in our laboratory is also nontoxic.

The complementary fragment B (see Fig. 3a) contains that part of the toxin which remains after subtraction of fragment C (HELTING and ZWISLER 1977). It competes minimally, if at all, with tetanus toxin binding (MORRIS et al. 1980).

Table 4. Toxicity of tetanus toxin and subunits

Polypeptide	MLD/mg protein	Relative toxicity	Reference
Tetanus toxin	30000000	GP > Ms > Rat	[1]
Heavy chain	≧2200		[2]
Light chain	<250		[2]
Fragment B	5	GP > Ms > Rat	[3, 4]
Fragment C	≪0.1		[1]

GP, Guinea pig; *Ms*, mouse; 1. HELTING and ZWISLER 1977; 2. MATSUDA and YONEDA 1975; 3. HELTING et al. 1978; 4. The very low toxicity is further decreased by a factor of at least 10 when fragment B is run over a column with insolubilized anti-fragment C monoclonal antibody (unpublished experiments)

The low paralytic potency of fragment B on the mouse diaphragm (SIMPSON 1984a) was sensitive to monoclonal antibodies against fragment C and was also inhibited by fragment C in a presumably competitive manner (SIMPSON and HOCH 1985), indicating a trace contamination with tetanus toxin. Residual toxicity was removed when fragment B was run over a solid phase coupled with monoclonal antibody directed against fragment C (unpublished data).

Is there a physicochemical basis for toxicity? The heavy chain displays, in contrast to tetanus toxin and the light chain, some hydrophobicity in charge-shift electrophoresis (WARD et al. 1981). Tetanus toxin binds [^{3}H] triton X 100 at pH values below pH 4. As the light chain and fragment C do not behave in this way, it is assumed by exclusion that hydrophobicity rests in the heavy chain (BOQUET and DUFLOT 1982; BOQUET et al. 1984). Admittedly, the extreme pH values used make the extrapolation into the physiological range risky. On the basis of hydrophobicity in vitro, and of toxicity in vivo, the following hypothesis nevertheless appears to be debatable.

The first step might consist in binding to neuronal membranes through the nontoxic fragment C, i.e., the C-terminal half of the heavy chain (β_1). This would facilitate the second step (internalization?), which may require the mentioned hydrophobicity of the N-terminal (β_2) part of the heavy chain. The light chain might hide the hydrophobic domains of the heavy chain and simultaneously improve the solubility. The various parts of the molecule complement each other, as has been described for other toxins (HABERMANN and BREITHAUPT 1978). The existence of a single, solely responsible toxic site, resembling a prosthetic group, appears extremely unlikely. Toxicity, internalization, and ascent are promoted by the cooperation of all parts of the molecule (WELLER et al. 1986).

Recently, some confusion arose owing to the assumption of a fully toxic tetanus toxin which fails to bind to a solid-phase ganglioside column (LAZAROVICI et al. 1984). However, this claim not only contradicted the results obtained by affinity chromatography on synaptosome columns (HABERMANN 1976), but also could not be reproduced with the same type of solid-phase ganglioside (HABERMANN and TAYOT 1985). The discrepancy is explainable by the presence of biologically inactive or less active components in every toxin sample so far studied, which behave like tetanus toxin in disc gel electrophoresis but have a lower affinity for the solid-phase column or for brain membranes. Microheter-

ogeneities of tetanus toxin appear to occur by autolysis of purified toxin (GORETZKI and HABERMANN 1985) and are also evident in the analysis of ^{125}I-toxin (AN DER LAN et al. 1980).

In summary, binding to membranes is possible with nontoxic material, as exemplified by fragment C and by mildly toxoided toxins (HABERMANN 1973a; HABERMANN and TAYOT 1985), but toxicity always requires binding.

Much research has been devoted to the question of whether individual amino acid residues are responsible for toxicity or binding of tetanus toxin (for details, see WELLHÖNER et al. 1982). The wealth of data indicates that every modification so far assessed lowers the toxicity of the original molecule, and that this process is not an all-or-none reaction but occurs step by step with the severity of the physical or chemical influences. There is no parallelism between decrease in binding and ascent on the one hand and loss of toxicity on the other (HABERMANN 1973a).

3.3.2 Botulinum Toxins

Much less is known about structure-activity relationships of botulinum neurotoxins and their implications for their mode of action. However, what is known reveals many similarities with tetanus toxin. An equivalent of fragment C has been prepared from the heavy chain of botulinum B toxin (KOZAKI et al. 1978) and from botulinum A toxin (SHONE et al. 1985). Binding of neurotoxin type A to synaptosomes rests on the heavy chain (WILLIAMS et al. 1983). It can be abolished by trypsin treatment, which destroys the C-terminal half of the heavy chain (called H_1), but leaves the remainder of the toxin (nontoxic, called H_2L) apparently intact. A particularly trypsin-sensitive link, whose lysis leads to loss of toxicity and binding, appears to be located close to the C-terminal of the heavy chain (SHONE et al. 1985). The binding sites on synaptosomes are certainly not identical with those of tetanus toxin, since the latter only moderately (WILLIAMS et al. 1983) or hardly (HABERMANN 1974) prevents the binding of type-A ^{125}I-neurotoxin, and conversely this toxin does not antagonize ^{125}I-tetanus toxin binding to brain matter. Nevertheless, some functional correlate seems to exist. Fragment C from tetanus toxin delays not only the effect of the parent toxin at the neuromuscular junction (SIMPSON 1984a, b, 1985), but also the action of some botulinum toxins (SIMPSON 1984c). It was preventive against C and E neurotoxins but not against those of the A, B, D, F, and G strains. Antagonism of fragment C with the toxins C and E occurred at the level of binding. As compared with the potency of the toxins, very high concentrations of fragment C (10^{-6} mol/l) were required to produce significant protection.

4 Pharmacokinetics at the Molecular or Membranal Level

Like the preceding ones, this chapter is restricted to the elementary processes. They will be classified into (a) binding and membranal events, (b) internalization, and (c) axonal transport; (c) might be regarded as an extension of (b).

4.1 Binding

4.1.1 General Aspects

Binding of drugs or toxins has both pharmacodynamic and pharmacokinetic implications. If it occurs at a true receptor, it triggers a signal chain leading to a biological event. This classical concept implies (a) chemical specificity of the drug-receptor interaction, (b) amplification, and (c) specificity of the effect. In contrast, a functionally silent binding site may be regarded as a compartment in the pharmacokinetic sense, without pharmacodynamic relevance. With toxins, a pharmacokinetic interaction is not seldom the prerequisite for a pharmacodynamic interaction. For instance, diphtheria or cholera toxins first bind to the outside of target cells by way of a pharmacokinetic receptor. Then a toxic constituent enters the cells – either the intact toxin or a toxic moiety of it – and triggers the pharmacodynamic response. Evidently, the receptors of entrance-prone toxins no longer require extreme specificity, because selectivity is linked to the toxic action within the cell. The pharmacokinetic binding sites merely have to concentrate the toxins for the subsequent internalization. Unlike the receptors for drugs of low molecular weight, they are not necessarily proteins. In case of clostridial neurotoxins, long-chain gangliosides still confer some organotropy on the central nervous system. GM_1, the pharmacokinetic receptor for cholera toxin, is a widely distributed membrane glycoprotein. Diphtheria toxin uses the phosphate residues of widely distributed lipids as receptors, particularly phosphatidylinositol (ALVING et al. 1980) and probably also a glycoprotein (PROIA et al. 1979). Abrin and ricin react in a lectin-like manner with nonreducing terminal galactose residues. It is a matter of convenience that pharmacokinetic binding sites should be termed "receptors" at all.

Internalization may occur directly through the membrane or by adsorptive pinocytosis. The latter process may carry the toxin into lysosomes, where it is exposed to low pH and proteases. Some toxin, either modified or native, might be released from the organelles into the cytoplasm and reach its pharmacodynamic target. The biological advantage of the two-step process is obvious. In the first step, the toxin is concentrated on the surface of the target cells, depending on the affinity and the number of the pharmacokinetic binding sites. The second step may represent another amplification mechanism, for instance, if the final agent is an enzyme. In extreme cases, a few toxin molecules may be sufficient to destroy target cells. These mechanisms have been well documented for cholera, diphtheria, and pertussis toxins. They have in common a complex structure, with subunits specific for binding and internalization (B-moiety, from binding), and for action (A-moiety; for review, see NEVILLE and CHANG 1978).

Clostridial neurotoxins resemble the toxins mentioned. They are extremely potent, and they are internalized, as is evident from their subsequent axonal transport. They consist of two chains that might possess different functions, in particular binding (B) or action (A), and might be dissociated during contact with the cell or during internalization. However, the analogy stops here. Neither has any epicellular dissociation or intracellular processing been shown for clostridial neurotoxins, nor has internalization been defined as a prerequisite for

action, nor has a specific intracellular target been identified. Therefore, this chapter is restricted to established pharmacokinetic binding sites and transport phenomena, and is salted with some speculations concerning pharmacodynamic receptors.

4.1.2 Tetanus Toxin

Tetanus toxin was the first toxin whose selective binding to its target organ was established (WASSERMANN and TAKAKI 1898; MARIE 1898). The now classical work of van Heyningen (for review, see VAN HEYNINGEN and MELLANBY 1971) located tetanus toxin binding to *gangliosides*, in particular to those of the G_{1b} series. The pertinent data are best summarized by two tables. One of them gives the moles of tetanus or cholera toxin fixed per mole of the respective gangliosides (Table 5). The fixation of the former was measured by the decrease in general toxicity, whereas the fixation of cholera toxin was demonstrated by loss of its local toxicity, as measured by blueing of rabbit skin.

The table shows that, whereas cholera toxin interacts only with GM_1 ganglioside, tetanus toxin "sees" many gangliosides – however, with different affinities. This toxin reacts preferentially with those gangliosides having two neuraminic acid residues at their lactose residue. Accordingly, tetanus toxin also interacts with GQ-type gangliosides (see, for instance, HOLMGREN et al. 1980). Van Heyningen's group made a second, not less important observation: the toxin-fixing capacity depends not only on the nature of the gangliosides, but also on their endowment with other lipids (Table 6).

The relevance of the carriers also makes it understandable why the data from different laboratories concerning tetanus toxin binding differ to some degree, in particular with respect to the gangliosides of lower affinities, for instance GM_1 and GD_{1a}. Whereas HOLMGREN et al. (1980) still observed some toxin binding to these gangliosides in a solid-phase assay, HABERMANN and ALBUS (1986) found them practically negative in an overlay technique. It appears to be very important how the gangliosides are presented. HOLMGREN et al. (1980) used polystyrene surfaces, while HABERMANN and ALBUS (1986) used silica gel plates, which had to be treated with a quenching solution in order to prevent nonspecific binding of radiolabeled tetanus toxin. This may have abolished some low-affinity binding. Moreover, binding to gangliosides depends heavily on the ionic environment. Whereas GM_1 and GD_{1a} did not measurably bind tetanus toxin in a physiological medium, they did so when Tris buffer was used instead (HABERMANN et al. 1985). The position of gangliosides is also important in brain membranes. Even their mild heating to 56° C largely depressed binding of labeled tetanus toxin (HABERMANN and ALBUS 1986).

Is there any binding site in brain tissue other than gangliosides? To answer this question, brain membranes were solubilized with SDS and subjected to electroblotting. No band was "stained" with tetanus toxin other than the front where the gangliosides migrated, which rules out any SDS-resistant protein. The participation of an SDS-sensitive and/or heat-labile (see above) protein is nevertheless possible (HABERMANN and ALBUS 1986). CRITCHLEY et al. (1985) came to similarly negative results with cell cultures.

Table 5. Moles of toxin fixed per mole of ganglioside (Adapted from VAN HEYNINGEN 1974)

	Tetanus toxin	Cholera toxin
GM_2	<0.01	<0.0005
GM_1	0.08	0.5
GD_{1a}	0.08	<0.0005
GD_{1b}	0.55	<0.0005
GT_{1b}	0.55	<0.0005

Table 6. A comparison of the toxin-fixing capacity of complexes (containing 25% ganglioside) of ganglioside with different lipids (From VAN HEYNINGEN and MELLANBY 1968)

Second lipid	Complex required to fix 10 LD_{50} (mg)
Cerebroside	<0.07
Sphingomyelin	2.0
Lecithin	5.4
Cholesterol	8
Tripalmitin	8

Gangliosides differ by their carbohydrate moieties. Provided that tetanus toxin reacts exclusively with them in a lectin-like manner, the potency range of gangliosides should be reflected by the binding potency of the respective sugars. This was, however, not the case. The ceramide-free oligosaccharides had always a lower affinity to the toxin than the parent gangliosides. In addition, they were about equipotent, so the toxin could no longer distinguish between the oligosaccharide from GM_1 and that from GD_{1b} and GT_{1b}. Thus, not only the environment but also the lipid moiety of the gangliosides contributes to their affinity for tetanus toxin (HABERMANN et al. 1985). Gangliosides in an aqueous environment always exist as micelles, probably held together by hydrophobic interactions between the ceramides. It is possible that the differences in toxin binding are due to the ordered arrangement of the gangliosides as compared with the free movement of the isolated oligosaccharides. The thermosensitivity mentioned with regard to the binding sites fits in with this view, since mild denaturation of membranes may lower the accessibility or affinity of the thermostable gangliosides to or for the toxin. A hydrophilic lipid-lipid interaction between gangliosides and phosphatidylcholine bilayers has been described as existing preferentially with long-chain (GQ_1) as compared with GM_1 gangliosides (OLLMANN and GALLA 1985). LEE and GRANT (1980) redistributed gangliosides in lecithin bilayers with the lectin wheat germ agglutinin. Dynamic interactions between structurally versatile ganglioside clusters and toxins should be considered with respect to specificity and sequelae of the binding process.

The gangliosides of the D, T, and Q series are predominantly located in neurons (for a comprehensive review, see ANDO 1983). This explains the *neurono-*

tropy of the toxin, which is a precondition for its ascent, for its action, and also for its use as a neuronal marker. As no other binding site for the toxin in nervous tissue is known, in particular no sialoglycoprotein, gangliosides should be regarded as at least the pharmacokinetic binding sites. Their large capacity renders tetanus toxin a suitable neuronal marker, as shown for mammalian cell cultures (DIMPFEL et al. 1975, 1977; MIRSKY et al. 1978; BRENNEMAN and NELSON 1985). However, a few cultured neurons are not stained by tetanus toxin (RAJU and DAHL 1982). The question of whether these cells were primarily toxin negative or merely lost the binding sites under culture conditions is still not settled. Amphibian neurons are also labeled by tetanus toxin, provided they have become morphologically differentiated (VULLIAMY and MESSENGER 1981). Glial cells are generally toxin negative. In very rare cases, astrocytes express tetanus toxin receptors (SCHNITZER and SCHACHNER 1981a; RAFF et al. 1983; SCHNITZER et al. 1984). The latter authors have described immature tetanus-positive cells which share an antigen (S-1) with glial cells. Since tetanus toxin receptors belong to the earliest markers expressed during development, it is difficult to say whether these cells are precursors of neurons or of glia, or whether they are multipotent. There is strong evidence that in cerebellar cultures, at least, binding sites for tetanus toxin are absent from maturing glial cells (SCHNITZER and SCHACHNER 1981a, b). NAGATA et al. (1986) have assessed the expression of cell surface markers in cultured astrocytes from early postnatal mouse cerebellum. These cells do not react with tetanus toxin. However, removal of neurons leads to the expression of toxin binding, and readdition of purified neurons suppresses it. Thus, toxin binding is not an invariable property of these cells. Which surface component is expressed and which is not depends on the communication between the cells.

Attempts to use tetanus toxin for fluorescent staining of neurons in slices have been made only occasionally (see, for instance, SCHNITZER and SCHACHNER 1981a; SCHNITZER et al. 1984). Immunohistochemical localization of tetanus toxin binding at the motor end-plates of the frog and the mouse (ZIMMERMANN et al. 1981) was not unexpected, as the toxin paralyzes the neuromuscular transmission (see Sect. 5.3.2) and can be located there by autoradiography (WERNIG et al. 1977).

In other fields of pharmacology, the number of binding sites per cell is small, e.g., a few thousand. In contrast, the number of long-chain gangliosides per neuronal cell is extremely high. Calculations by MELLANBY and GREEN (1981) give a ratio of 10^5 GD_{1b} molecules in brain membrane preparations per tetanus toxin molecule bound, and the ratio for botulinum toxins may be on the same order. LEDEEN (1985) estimates that gangliosides, defined as sialic acid-containing glycolipids, represent 5%–10% of total lipid in the neuronal plasma membrane (which is at least tenfold more than in any other membrane), and at least 12% in nerve endings. Fluorescence microscopy demonstrates that the toxin decorates the whole surface of cultured nerve cells, with occasional patching. So the gangliosides are largely accessible from the outside of the cell membrane, not only to tetanus toxin but also to neuraminidase, as will be discussed later in this chapter. The extreme excess of binding sites may contribute to the tremendous potency of tetanus toxin, as it increases the chance for single molecules to meet a binding site once the toxin has reached

a nerve cell (receptor excess). It may be questioned whether all these binding sites are functionally equivalent and whether they display the same affinities and specificities. The very broad concentration-binding diagram seen with radioactive toxin (see, for instance, HABERMANN et al. 1985) indicates their heterogeneity.

Quantitative aspects of toxin binding have been assessed repeatedly since ^{125}I-tetanus toxin became available in 1970. The tracer bound not only to brain membranes but also to liposomes made from ganglioside-cerebroside mixtures which van Heyningen had used for his toxin neutralization assays (Table 6). ^{125}I-toxin preferentially interacted with synaptosomes as compared with the other ultracentrifugal fractions (HABERMANN 1973b). The same was true for genuine toxin (MELLANBY and WHITTAKER 1968). ^{125}I-toxin binding was prevented and reversed by antitoxin. The binding sites were preferentially located in the gray matter, as shown by autoradiography (HABERMANN 1973b). However, at this point a series of anomalies arose which distinguished tetanus toxin from other ligands and made some calculations inapplicable. Even today the seemingly simple interaction between tetanus toxin and pure gangliosides has not yet been settled in quantitative terms, i.e., expressed a dissociation constants and number of binding sites.

For instance, binding of ^{125}I-tetanus toxin to ganglioside-cerebroside complexes is hardly competitive with unlabeled tetanus toxin. This indicates more a kind of "solution" of the toxin in the lipid mixture than a ligand-"receptor" interaction obeying the law of mass action (HABERMANN, unpublished). A quantitative comparison between binding to various pure gangliosides or ganglioside-cerebroside complexes is also hampered by the fact that gangliosides in aqueous "solution" form large micelles with micellar weights of around 10^6. The situation is different with isolated membranes, particulate brain, and intact cells. Here, binding studies might become more complicated for another reason. At least with nerve cells, internalization is to be expected, carrying the ligand, and perhaps also the binding site, into a separate compartment. Again, calculations based on the law of mass action are to be regarded with caution.

The cell possesses various instruments to handle the gangliosides. A cytoplasmatic protein can transfer various glycolipids from membrane to membrane (GAMMON and LEDEEN 1985). Provided it transports them together with bound tetanus toxin, it might assist trans-synaptic transport. Retrograde axonal transport of gangliosides has also been reported (AQUINO et al. 1985), the gangliosides possibly serving as vehicles for the toxins. Finally, the gangliosides may not be distributed statistically but as clusters, which may interact (a) between themselves, (b) with the toxin, or (c) with their support. Each of these reactions may modulate (and be modulated by) the binding process itself and the properties of the membranes carrying the binding sites.

The binding data depend on variables, which differ among the various laboratories. *Substrate*: We have already discussed the structural specificity of gangliosides and their oligosaccharides concerning tetanus toxin binding (see p. 115). *Lipid environment*: When the ganglioside is embedded in a membrane, its binding properties may change. It has already been mentioned that it is very difficult to saturate liposomes made from gangliosides and cerebrosides, even by adding an excessive amount of unlabeled tetanus toxin together with labeled toxin.

As the toxin can be removed from the liposomes by antitoxin (HABERMANN 1973b), a kind of reversible internalization may be assumed, that sets free the ganglioside head group for further toxin. Clearly, the distinction between "saturable" and "nonsaturable" binding is problematic, as is the distinction between "specific" and "nonspecific" binding. A second example stems from GOLDBERG et al. (1981) who worked with membrane concentrations as low as 20 ng/ml. When they raised the concentration to 500 ng/ml, the K_D for tetanus toxin rose about tenfold.

Another problem is the massive influence of *pH and ionic strength*. Normally, the fixation of radiolabeled toxin is measured at very low ionic strength (0.025 or 0.05 mol/l Tris acetate) and low pH (mostly pH 6.0) buffer (GOLDBERG et al. 1981; ROGERS and SNYDER 1981). This system has yielded the best binding data (LEE et al. 1979; MORRIS et al. 1980), albeit far from physiological. Raising pH and ionic strength invariably decreases toxin binding. Thus, the K_D values reported (1.2 nmol/l, ROGERS and SNYDER 1981; 2–12 nmol/l, GOLDBERG et al. 1981) should be regarded with caution. The same applies for the extremely large and variable numbers of binding sites reported (about 0.70 pmol/mg protein by ROGERS and SNYDER 1981; 1.5–9 nmol/mg by GOLDBERG et al. 1981). We (WELLER et al. 1986) looked for binding of ^{125}I-tetanus toxin in physiological solution and found both a high- (K_D 125 pmol/l, B_{max} 1.3 pmol/mg) and a low-affinity (K_D 2.22 nmol/l, B_{max} 22 pmol/mg) binding site. MELLANBY and POPE (1976) reported a high-affinity binding site in the 10-pmol/l range. High-affinity binding sites are not unexpected, as even femtomolar concentrations of tetanus toxin block the synaptic transmission in primary nerve cell cultures (see Sect. 5.3.1.1).

Degradation of gangliosides: Treatment of brain membranes with *Vibrio cholerae* neuraminidase converts the gangliosides of the GQ_1, GT_1, and GD_1 series to GM_1. Nevertheless, there is some residual binding (HABERMANN 1973b; HABERMANN et al. 1981; ROGERS and SNYDER 1981). Depending on its physical state, GM_1 itself retains some toxin binding, albeit much less than that of GD_{1b} or GT_1 (VAN HEYNINGEN 1974; MORRIS et al. 1980; HOLMGREN et al. 1980). The K_i values (nmol/l) given for toxin binding to brain membranes by ROGERS and SNYDER (1981) were GM_1 450, GD_{1a} 95, GD_{1b} 10, and GT_{1b} 6. If gangliosides of the G_{1b} type are involved in the action of tetanus toxin, their complete degradation by neuraminidase should prevent the toxin effects. However, in previous studies the biological activity of tetanus toxin was not changed by pretreatment of its substrate with neuraminidase, regardless of what test is used (choline uptake, HABERMANN et al. 1981; neuromuscular junction, HABERMANN et al. 1980; acetylcholine release from particulate brain, BIGALKE et al. 1981a; electrical activity of mouse spinal cord neurons in culture BIGALKE et al. 1986). It was proposed that a large "receptor" excess waits for toxin binding, so that even the binding potency remaining after treatment with neuraminidase is sufficient to trigger the toxin effects. The resistance of the toxin effects to neuraminidase should not be taken as an indicator of a nonganglioside receptor site mediating the toxin effects (see p. 145), the more as recently the toxin effect on hippocampal slices was prevented by enzyme treatment (WIERASZKO 1985).

Table 7. Comparison of the fixation of tetanus toxin with its effects on transmitter release, choline uptake, and neuromuscular transmission (Adapted from HABERMANN et al. 1981)

	Inhibition of		Fixation
	Transmitter release and choline uptake	Neuromuscular transmission (HABERMANN et al. 1980)	
Neuraminidase sensitivity	Not found	Not found	Partial
Temperature dependence	Strong positive correlation	Strong positive correlation	Negligible
Time course (time to onset)	Slow (30–90 min)	Slow (30–90 min)	Fast (less than 10 min)

A comparison between binding and action further supports the assumption that binding is a prerequisite of the toxin effects, but only as the first step in a reaction chain. For instance, binding to neuronal structures proceeds quickly to its equilibrium, i.e., within about 10 min. It is largely reversible (GOLDBERG et al. 1981; HABERMANN et al. 1981) and over a wide range independent of temperature (LEE et al. 1979; HABERMANN et al. 1981). In contrast, in vivo the symptoms of tetanus proceed slowly; this can be explained by the time required for axonal transport.

In vitro, binding precedes the toxin's action on choline uptake (HABERMANN et al. 1981). Inhibition by tetanus toxin of neuromuscular transmission (HABERMANN et al. 1980; SCHMITT et al. 1981), choline uptake (HABERMANN et al. 1981), release of noradrenaline (HABERMANN 1981b), of GABA (ALBUS and HABERMANN 1983), and of enkephalin-like material (JANICKI and HABERMANN 1983) all increase with time for about 2 h at 37° C, and rise strongly with temperature. Taken altogether, a model requiring at least two steps – binding and effect – is strongly supported (see Table 7).

In this context, cooperation between ganglioside and other binding sites at the neuronal membrane should be considered. The work of Boquet's group (see p. 138) suggests the existence of two distinct sites on the toxin molecule, one of them located on the β_2-moiety of the heavy chain and perhaps involved in internalization. The other would represent the classical ganglioside binding site on the fragment C (WELLER et al. 1986). Possible candidates for nonganglioside "receptors" would be the phosphatidylinositols. Vesicles made from this functionally important (see BERRIDGE and IRVINE 1984) group react with tetanus toxin at low pH, resulting in fusion and aggregation (CABIAUX et al. 1985).

Nervous tissue was initially thought to be the only target of tetanus toxin, with respect to both binding and action. Now the number of reports on *non-neural binding sites* is increasing, although no direct extraneuronal action of the toxin has been reported so far. In many cases, long-chain gangliosides are the putative binding sites for the toxin. Thyroid plasma membranes contain small amounts of apparently long-chain gangliosides; this is quite unusual for

nonneuronal organs (MULLIN et al. 1976). The gangliosides bind not only thyrotropin but also tetanus toxin, which leads to striking similarities in the binding behavior of the two ligands. As calculated, tetanus toxin binds about 40 times better to brain than to thyroid membranes, whereas thyrotropin binds about four times better to thyroid than to brain. Optimal binding conditions of both ligands resemble each other closely; i.e., low pH, low salt, and low temperature are required (LEDLEY et al. 1977; LEE et al. 1979). ^{125}I-TSH binding to thyroid membranes can be inhibited not only by TSH but also by tetanus toxin and its fragment C (MORRIS et al. 1980). ^{125}I-TSH binding to brain membranes can be competed with by TSH and by tetanus toxin, whereas ^{125}I-tetanus toxin binding to brain membranes is depressed by tetanus toxin and promoted by TSH (LEE et al. 1979). However, so far no functional consequences of a direct interaction between tetanus toxin and its thyroid binding sites have been observed. An increased function of thyroid in tetanus (HABIG et al. 1978) may have many origins other than common receptors, and has not been observed by BÓBR et al. (1959). In addition to gangliosides, a glycoprotein is involved in thyrotropin binding (MELDOLESI et al. 1977). Some other organs too possess binding sites for tetanus toxin, for instance human and rodent thymic epithelium (HAYNES et al. 1983) and Langerhans' islets (EISENBARTH et al. 1982). Thymic epithelium and islet cells also react with a monoclonal antibody that recognizes ganglioside GQ_{1c} (KASAI and YU 1983), an obvious candidate for toxin binding. Why tetanus toxin reacts with a subset of rat adrenal cells (LIETZKE and UNSICKER 1983) is still not known. Rabbit kidney microsomes bind the toxin and its fragment C by their content in long-chain gangliosides which differ from those in brain (HABERMANN and ALBUS 1986). The number of binding sites in the kidney is small compared with those in brain membranes. Some binding can be detected in lipid extracts of rat kidneys. Long-chain gangliosides (GT_{1b}, GD_{1b}), among others, have been found in rat kidneys (TOMONO et al. 1984).

4.1.3 Botulinum Toxins

4.1.3.1 Structural Requirements of Toxin Binding

As with tetanus toxin (VAN HEYNINGEN 1976), the heavy chain from botulinum B toxin contains the binding site. In contrast to the light chain it competes with B neurotoxin for binding to synaptosomes (KOZAKI 1979). The respective heavy chain is also responsible for binding of C_1 neurotoxin (AGUI et al. 1983, 1985) and A neurotoxin (WILLIAMS et al. 1983). As compared with C_1 neurotoxin, the K_D values of the C_1 heavy chain were much higher, perhaps because the chain had been carboxymethylated during preparation (AGUI et al. 1985). Specificity is pronounced, as type-A or -E toxins were hardly able to compete with type-B radiolabeled toxin. Binding of radiolabeled A toxin was depressed by unlabeled A toxin and partially by E toxin, but not by B toxin. Competition for radiolabeled type-E toxin occurred with type-E toxin and partially with type-A toxin, but not with B toxin (KOZAKI 1979). Still closer similarities between tetanus and botulinum B neurotoxin were evident after treatment of the

Table 8. Cross-competition between various botulinum toxins (Data from KOZAKI 1979 and MURAYAMA et al. 1984)

		Labeled toxins			
		A	B	D	E
Native toxins	A	+	0[b]		(+)
	B	0[a]	+		0
	C_1			+	
	D			+	
	E	(+)	0		+

[a] Confirmed by WILLIAMS et al. (1983)
[b] EVANS et al. (1986) found this competition minimal but reproducible

heavy chain with trypsin. The resulting subfragment C bound to synaptosomes with approximately the same affinity as the parent heavy chain did (KOZAKI et al. 1978).

C_1 and D toxins appear to be closely related in binding behavior, which reminds us that they are both encoded by bacteriophage (see p. 99). Radiolabeled ^{125}I-D toxin can be competitively displaced from its synaptosomal (rat brain) binding site by both C_1 and D toxin, and also by the heavy chains of both toxins, but not by the light chain of D toxin (MURAYAMA et al. 1984). It is tempting to assume that C_1 and D toxin form a subgroup among the botulinum toxins.

Table 8 summarizes the data and underlines the specificity of botulinum B toxin. One wonders if the scheme also reflects the fundamental differences (see p. 157) in electrophysiological behavior between A and B toxins.

The fixation of radiolabeled type-A neurotoxin to synaptosomes is nearly completely prevented by antitoxin, largely by an excess of unlabeled toxin, and by pretreatment of the synaptosomes with *V. cholerae* neuraminidase (HABERMANN 1974). Accordingly, synaptosomes lost about 70% of their binding capacity for type-A neurotoxin when pretreated with neuraminidases from *C. perfringens* or *Arthrobacter ureafaciens* (WILLIAMS et al. 1983). Botulinum A neurotoxin does not compete with ^{125}I-tetanus toxin binding (HABERMANN 1976), whereas tetanus toxin competes slightly (HABERMANN 1976) or partially (WILLIAMS et al. 1983) with binding of ^{125}I-botulinum A neurotoxin.

4.1.3.2 Quantitative Aspects

Binding of type-A neurotoxin on synaptosomes in physiological medium is saturable with a high-affinity ($K_D = 0.6$ nmol/l, $B_{max} = 60$ fmol/mg) and a low-affinity ($K_D > 25$ nmol/l) binding site (WILLIAMS et al. 1983). Binding is reversible (HABERMANN 1974). For type B neurotoxin see note added in proof, p. 166.

Botulinum type-C_1 neurotoxin binds to synaptosomes independent of temperature. Equilibrium was reached within 10 min; thus, it resembles tetanus

toxin. Two binding sites were suggested, one with a K_D of 100 pmol/l and a B_{max} of 525 fmole per mg synaptosomal protein, and the other with a K_D of 2 nmol/l and a B_{max} of 5 pmol/mg protein. Only a very small amount (0.4%) of toxin was not removable from the synaptosomes after up to 1 h at 37° C (AGUI et al. 1983) and could be regarded as "internalized". Slightly higher K_D values were reported later by the same group (AGUI et al. 1985).

Botulinum toxins can be tested for binding in the digested, i.e., highly toxic, and the genuine, i.e., barely toxic, form. This provides the unique opportunity to correlate binding and effect, for instance with type-E neurotoxin on mouse brain synaptosomes. Although the digested toxin was about 30 times more poisonous, the number of binding sites (22 vs 19 pmol/mg) and K_D (apparently 100 pmol/l, not 100 nmol/l, as given in the publication) were not much different. The data indicate that the binding site of clostridium E neurotoxin is not altered by trypsinization. It is concluded that binding can occur without pharmacodynamic consequences. The subsequent inhibition of acetylcholine release apparently requires an additional step, occurring with trypsinized toxin only (KOZAKI and SAKAGUCHI 1982).

Binding assays with radiolabeled toxins require confirmation by functional tests. HABERMANN and HELLER (1975) have measured the loss of toxicity of botulinum A toxin due to binding, and so confirmed the radioactivity data. In these assays fixation of the toxin was much less stable against ionic forces than that of tetanus toxin. Although the ganglioside-rich gray substance binds this toxin much better than the white substance does, neither gangliosides nor ganglioside-cerebroside mixture nor brain extracts could replace the synaptosomes, this in accordance with the findings of VAN HEYNINGEN and MELLANBY (1973).

Do *gangliosides* mediate the binding of botulinum toxin to neuronal membranes, or are other components involved? The question has not yet been settled. WILLIAMS et al. (1983) found a weak prevention by gangliosides of ^{125}I-neurotoxin (type A) binding to synaptosomes. Remarkably, they had worked with physiological solution, where binding is weaker than in a low ionic strength, low pH environment. The considerable differences between type-A neurotoxin and tetanus toxin became apparent on ganglioside columns, too (HABERMANN et al. 1985). Here, ^{125}I-neurotoxin type A was eluted at lower pH and ionic strength than tetanus toxin. Again unlike tetanus toxin but in confirmation of HABERMANN and HELLER (1975), gangliosides barely prevented the binding of ^{125}I-neurotoxin type A to brain membranes when the assay was run in physiological solution. Further binding data for botulinum toxins should be scrutinized with respect to the molarity and pH of the buffer, and to various other conditions as emphasized for tetanus toxin (see p. 118). For instance, the preferential neuraminidase-sensitive binding of ^{125}I-neurotoxin type A to synaptosomes and their membranes has been tested in sucrose, which does not tell much about the in vivo relevance (KITAMURA 1976). Again in contrast to tetanus toxin, ^{125}I-neurotoxin type A requires a low-salt solution to interact with gangliosides, and it does not interact at all with their oligosaccharides (HABERMANN et al. 1985). Thus, one is not surprised that initial arguments for gangliosides as binding sites (SIMPSON and RAPPORT 1971) were rejected by VAN HEYNINGEN

and MELLANBY (1973), since toxin neutralization was absent in the presence of their 0.2% gelatin buffer.

Nevertheless, some data point to the participation of at least a material containing sialic acids, putatively gangliosides, in toxin binding. The role of ionic strength may explain the puzzling differences in the binding data obtained. Working with ^{125}I-neurotoxin type A, KITAMURA et al. (1980) found that only GT_{1b} partially prevented its binding to synaptosomes, while other gangliosides were not effective. In the prevention of toxicity, the molar ratios between gangliosides and toxin required to detoxify 50% of toxin were GM_3 6098, GM_2 2439, GM_1 2073, GT_{1a} 829, GD_{1a} 610, GD_{1b} 488, GQ_{1b} 27, and GT_{1b} 6 (KITAMURA et al. 1980). This series largely resembles that observed with tetanus toxin, with the exception of a much larger difference between GD_{1a} and GD_{1b} with the latter toxin. Among the gangliosides investigated by HABERMANN et al. (1985), GD_{1a} turned out to be the most suitable, the series of decreasing potency being $GD_{1a} > GD_{1b} \sim GM_1$, in contrast to that for tetanus toxin.

The ganglioside sensitivity of various botulinum toxins appears to differ. It has been mentioned that ganglioside GT_1 inactivated botulinum A toxin fully, but B and E toxins only partially (SAKAGUCHI 1983). As there are many interpretations at hand, this line of research requires further attention.

We have already discussed (see p. 119) the involvement of more than one site in the binding of tetanus toxin. AGUI et al. (1985) hold a similar view with respect to binding of botulinum C_1 neurotoxin to rat brain synaptosomes. As with tetanus toxin (see p. 118), two binding affinities (K_D 79 and 35000 pmol/l respectively) were found. Although unlabeled heavy chain inhibited the binding of ^{125}I-C_1 neurotoxin to both ganglioside GT_{1b} and synaptosomes, the authors distinguished two different binding sites of the toxin molecule with the aid of specific monoclonal antibodies, one directed against the GT_{1b} binding, the other against the binding to the synaptosomal "receptor". Notwithstanding the interesting approach, two problems have to be solved before general acceptance of the hypothesis is attained. Carboxymethylated heavy chain with low affinity was used. Therefore, both an enhancement of binding to the respective sites by the light chain and a depression of binding by carboxymethylation are to be considered. Moreover, the interaction with the monoclonal antibodies might also be influenced by the chemical modification. Further work is obviously required.

In summary, there is no doubt that specific binding sites do exist for botulinum neurotoxins, and probably even for the individual types with a varying degree of cross-reactivity. However, only a very small percentage of total binding sites are expected to display sufficient affinity, which is a prerequisite for the extreme potency of this toxin in vivo and on isolated organs. Perhaps a specific arrangement of intramembranal gangliosides is required for a receptor function (as in tetanus toxin), and it is destroyed upon membrane preparation. Nonganglioside binding sites must still be considered.

In order to distinguish between silent binding sites and true receptors, we have tried to prevent the action of botulinum A toxin by pretreatment of its substrates with *neuraminidase*. The results were ambiguous. The enzyme-treated neuromuscular junction of the mouse phrenic nerve-hemidiaphragm remained

fully sensitive to the toxin (HABERMANN et al. 1980). However, the enzyme diminished the action of the toxin on the electrical activity of spinal cord neurons in culture (BIGALKE et al. 1986). It also slightly diminished – at the borderline of statistical significance – the efficacy of botulinum A toxin on particulate brain preparations with respect to inhibition of acetylcholine (BIGALKE et al. 1981a) or noradrenaline (HABERMANN 1981b) release. Thus, the best evidence for a receptor function of sialic acid-containing membrane constituents, be they gangliosides or glycoproteins or both, stems from work on spinal cord cultures. The relevance for the in vivo situation is open.

The *topography* of binding of botulinum A toxin has been assessed with a large synaptosome fraction from rat brain, using ferritin-conjugated antirabbit-IgG (HIROKAWA and KITAMURA 1979). By electron microscopy, the toxin was localized on presynaptic but not on postsynaptic membranes, apparently as patches. Its distribution on the neuromuscular junction will be discussed later (p. 127, see DOLLY et al. 1984a, b and HIROKAWA and KITAMURA 1975). BIGALKE et al. (1986) have pretreated primary spinal cord cultures with neuraminidase and assessed binding, localization, and effect of radiolabeled tetanus toxin and botulinum A neurotoxin. They found, as did HABERMANN et al. (1985) on ganglioside preparations, that botulinum A toxin binds to neurons much better in a low-salt than in a physiological solution. Neuraminidase reduced tetanus toxin binding by more than 50% (see also DIMPFEL and HABERMANN 1977; YAVIN et al. 1983), but it nearly abolished binding of ^{125}I-botulinum A toxin, even in low-salt solution. It was then assessed whether the effect of botulinum toxin was altered by neuraminidase. Although under the experimental conditions required (physiological salt solution) only a slight binding of botulinum A toxin was visible, the enzyme nevertheless nearly abolished the effect of this toxin. The action of tetanus toxin was not affected. The experiment indicates that caution is required in the interpretation of morphological or biochemical binding studies which are performed without a functional control.

4.2 Internalization[2]

4.2.1 General Aspects: The Three-Step Model

The most advanced hypothesis concerning the handling of tetanus (SCHMITT et al. 1981) and botulinum A toxin (SIMPSON 1980, 1981) by nerve endings presumes three steps: In the *first*, toxin is *bound*. This has been described in the preceding paragraphs, and it has already been stated that binding per se is insufficient to elicit an action. A *second* step, called *translocation* or *internalization*, has to be sandwiched between the binding and the *action* of the clostridial neurotoxins. Otherwise, neither the axonal transport, nor the time course, nor the resistance of the toxin effects to antibodies are understandable. The term does not necessarily imply that the toxin molecules, once internalized, move

[2] "Translocation" would be a more appropriate term than "internalization"; both terms will be used interchangeably in this review.

freely within the cytoplasm. They may be included into lysosomes, or into the endoplasmatic reticulum, or they may even just be integrated completely or partially into the cell membrane as extrinsic membrane proteins. As long as we do not know the target(s) of the toxins beyond their binding sites, it is debatable whether internalization is required to proceed to the cytosol.

We have also to distinguish between the pharmacokinetic aspects of internalization, which are uncontested, and its pharmacodynamic aspects. Transport into the intracellular space is a conditio sine qua non for the ascent of tetanus toxin to the central nervous system and for the ensuing local tetanus. However, for the actions of tetanus and botulinum toxins at the neuromuscular junction, their mere entry into the membrane may be sufficient, and internalization into the cytosol might be just a pharmacokinetic phenomenon, irrelevant even for local paralysis. Once within the membranes the toxins could lead to pores, as seen in black lipid membranes (see p. 137), or to other physicochemical changes sufficient to disturb transmitter release.

The *third* step, the paralytic *action*, cannot yet be located within a chain of biochemical events. Clearly, the concept of the third step is borrowed from the ADP-ribosylating toxins such as diphtheria or cholera toxin which have their final targets within the cell. Unlike these toxins, clostridial neurotoxins lack known enzymatic properties.

Internalization is not peculiar to clostridial neurotoxins but is a general feature of proteins that form intimate contacts with nerve cells. It is easily demonstrated by subsequent axonal transport (see following chapter). Internalization may be measured in three ways: in vitro (see below), in vivo by retrograde axonal transport (see Sect. 4.3), or in vivo by pharmacodynamic studies (see Chap. 5).

In vitro, for instance on the isolated phrenic nerve-hemidiaphragm, on cell cultures, or on particulate material, noninternalized material may be removed by washing, with or without antibodies or detergents. That moiety may be defined as internalized which firmly adheres to its target. Otherwise, those molecules might be taken as internalized which can no longer be recognized by antibodies. If the toxin is labeled with ^{125}I and the antitoxin with ^{131}I, the quotient between the two isotopes may serve as an indicator of accessibility. Most internalization studies deal with radiolabeled material. It is not certain whether ^{125}I-toxin is handled by the cell exactly as is unlabeled toxin. This must be doubted if radiolabeling is linked with toxoiding. Commercial tetanus toxoid is no longer internalized; this is evidenced by its failure to ascend into the spinal cord when injected into the gastrocnemius muscle of rats (HABERMANN 1972). The more rigorous the conditions are for toxoiding, the more both binding and ascent are depressed (HABERMANN 1973a; WELLER et al. 1986). Other pertinent experiments were performed with tetanus toxin coated on gold, for instance, on a strain of rat liver cells (MONTESANO et al. 1982), or on the rat iris in situ (SCHWAB and THOENEN 1978). Again, it is dubious whether such large, crude particles can mimic the small, elaborate toxin molecule. It is tempting to assume that tetanus toxin is internalized as a complex with constituents of cell membranes, in particular gangliosides. Turnover studies suggest both orthograde and retrograde axonal transport of gangliosides. Functionally, the

persistence of tetanus symptoms might be correlated with the unusually long (34–39 days) half-life of gangliosides (see ANDO 1983).

The morphological basis of tetanus toxin internalization has not been elucidated. Internalization into the motor nerve endings is often assumed to occur by way of presynaptic pinocytosis, although convincing figures are lacking. In the nerve endings within the iris no participation of coated pits in the uptake of gold-labeled tetanus toxin was observed by SCHWAB and THOENEN (1978). On the liver cell line mentioned, noncoated pits have been shown to be involved preferentially in the uptake of gold-labeled tetanus and cholera toxins (MONTESANO et al. 1982). At least the experiments with tetanus toxin should be regarded with caution, because so far no binding sites for the toxins have been reported in rat liver.

What is the fate of internalized toxin? Tetanus toxin is surprisingly stable against enzymes; it would not otherwise have survived the various proteases in its culture fluid (HELTING et al. 1979). Brain homogenate does not measurably degrade tetanus toxin at pH 7.5 or 5.5, whereas it does so at pH 3.5 (HABERMANN 1973b). Even after its ascent into the spinal cord only a minor part appeared to be hydrolyzed (HABERMANN et al. 1977). In primary nerve cell cultures, ^{125}I-tetanus toxin persists for days (DIMPFEL and HABERMANN 1977) and even weeks (HABIG et al. 1986) without visible separation into its chains, and only traces of fragmentation in polyacrylamide gel electrophoresis. The half-life of the chains is practically the same (about 5 days; HABIG et al. 1986); this argues against a separate internalization of a chain or another toxin fragment. In this respect tetanus toxin is quite unlike other two-chain toxins, which are known to send their active moieties into the cells. For instance, the binding (B) chain of diphtheria toxin has a half-life of 2 h, whereas the A chain persists much longer (YAMAIZUMI et al. 1982). The binding part of cholera toxin lives for about 5 days, whereas its active part (A_1) has a half-life of only 1 day (MOSS et al. 1980). The lack of visible processing of tetanus toxin indicates that both its pharmacodynamic and pharmacokinetic properties rest on the genuine toxin molecule. When properly labeled toxin has been taken up into the cells and transported to the central nervous system, the label is suited for pharmacokinetic studies. Both in vivo (HABERMANN et al. 1977) and in cell culture (HABIG et al. 1986) intact toxin is present for the duration of the symptoms of tetanus toxin poisoning. It is tempting to assume that the persistence is not merely coincidental but rather necessary, and perhaps even sufficient to elicit the changes in cell function. However, final proof is required.

The metabolic stability of botulinum neurotoxins has not yet been assessed to a comparable degree.

Ideally, binding and internalization should be distinguished by the aid of electron microscopy. Surprisingly, few successful attempts have been reported. The resolving power is usually insufficient and some studies are beset with technical problems. For instance, highly purified neurotoxins must be used in order to avoid artifacts. The accompanying hemagglutinins must be removed from botulinum toxins; otherwise they might mediate neurotoxin binding through their own receptors.

4.2.2 Studies on the Neuromuscular Junction and on Synaptosomes

Light-microscopic autoradiographs were made from the *neuromuscular junction* of the mouse diaphragm poisoned in vitro with *botulinum A neurotoxin*. Radioactivity was concentrated over the end-plates, without evidence of ascent (HIROKAWA and KITAMURA 1975). DOLLY et al. (1984a, b) also exposed the mouse hemidiaphragm to radiolabeled botulinum A neurotoxin. Quantitative electron-microscopic autoradiography showed saturable binding restricted to the nerve terminal membrane with a density of about 150 per μm^2. There was a partial antagonism by tetanus toxin, and a total inhibition of binding by botulinum A heavy chain. "Hot spots" were absent, and the radioactivity was distributed not only over the presynaptic membrane but over the entire unmyelinated area. As with latrotoxin (MELDOLESI 1984), toxin binding exceeded the area involved in transmitter release. Binding was followed by energy-dependent internalization, which was prevented by dinitrophenol or sodium azide, whereas neither chloroquine, an inhibitor of the lysosomal pathway, nor Ca^{2+} influenced internalization. The existence of internalization was supported by the observation that small amounts of radiolabeled material were found within the axon. This is reminiscent of the (minor as compared with tetanus toxin) ascent into the spinal cord (HABERMANN 1974). The relevance of the binding sites was evident, since they were present on all cholinergic but not on adrenergic nerve endings. The following points still need clarification: (a) Is internalization required for the actions of the toxin? (b) Is the receptor a pharmacokinetic mediator for internalization, or a pharmacodynamic mediator of the toxin effect, or both? (c) Is there an intracellular processing of the toxin to active fragments? (d) What is the function of the abundant ($B_{max} = 60$ fmol/mg) and very affine ($K_D = 6 \times 10^{-10}$ mol/l) (WILLIAMS et al. 1983) synaptosomal binding sites of botulinum A toxin where internalization is negligible (DOLLY et al. 1984b) and pharmacological potency is less than with tetanus toxin (see HABERMANN 1981a)?

Comparable studies with *tetanus toxin* are less advanced. Mouse skeletal muscle was poisoned with ^{125}I-tetanus toxin in vivo and subjected to light-microscopic autoradiography (WERNIG et al. 1977). Radioactivity was pronounced in the end-plates and well visible in their supporting nerve bundles, indicating internalization and the start of axonal transport. However, the resolving power did not allow the distinction between epimembranal, intramembranal, and intracellular localization. Electron-microscopic autoradiography with tetanus toxin on neuromuscular junctions (PRICE et al. 1977) did not provide additional insights.

Synaptosomes. Electron-microscopic work with large synaptosomes located botulinum A neurotoxin to their presynaptic site without a pronounced affinity to the postsynaptic areas (HIROKAWA and KITAMURA 1979). There has been no comprehensive morphological study on binding and internalization of tetanus toxin in synaptosomes. Only a few authors have tried to apply the three-step scheme to synaptosomes. Using a double labeling procedure, Habermann and Albus (unpublished) found a minor (if any) entry of tetanus toxin into large brain synaptosomes.

Similarly, only a very minor amount (0.4%) of C_1-botulinum toxin bound to synaptosomes was not removed by washing (AGUI et al. 1983). Thus, it might be suspected that the three-step scheme does not apply at all to work with isolated synaptosomes. So far only a fast-binding step has been distinguished from a slow, long-lasting phase of irreversible action (see p. 145). However, we do not know the minimum amount of tetanus or botulinum toxin that must be internalized in order to inhibit transmitter release.

4.2.3 Studies with Surviving Cells

Isolated cells have been used to study the uptake of tetanus toxin. Evidence rests mainly on work with radiolabeled toxins whose fixation kinetics have been arranged, and sometimes even forced into a three-step scheme of binding, sequestration, and internalization. A study linking the kinetics with action, for instance at the electrophysiological level, would be difficult and is still lacking. It should be stressed that the data from the mouse hemidiaphragm preparations (see p. 154) have served for the construction of quite another three-step scheme, where binding, internalization, and action are distinguished by their functional (and not by their fixation) characteristics. The latter approach will be dealt with in Chap. 5 of this review.

Binding of tetanus toxin to *primary nerve cell cultures* has been studied (DIMPFEL and HABERMANN 1977), with the outcome that cell cultures do not behave basically differently from synaptosomes. Recent work referring to the above-mentioned three-step model stems mainly from Yavin's studies with tetanus toxin (YAVIN 1984; YAVIN and HABIG 1984; YAVIN et al. 1981, 1982, 1983); botulinum toxins have not yet earned much attention. Yavin's group used primary nerve cell cultures in general, and extended their work later to somatic neural hybrid lines which lack long-chain (GD_{1b}, GT_{1b}) gangliosides (YAVIN and HABIG 1984; YAVIN 1984), and recently to erythrocytes (LAZAROVICI and YAVIN 1985a, b). Briefly, the following qualities have been used to distinguish binding, sequestration, and internalization: dependence of binding on temperature and ionic strength; resistance against neuraminidase; resistance against detergent extraction; changes of binding characteristics with duration and temperature of incubation. The results are compiled in Table 9.

The distinction between reversibly and irreversibly fixed toxin appears quite clear. However, without loss of important information step 2 could be omitted and the process regarded as the transition from an accessible (step 1) to a no longer accessible (step 3) compartment. One must also consider that internalization simply means seclusion from the outside, and this may also be achieved by the cell layer if it just covers the toxin without internalizing it. Thus, a two-step model would result, fitting into the scheme deduced from the work on end-plates.

CRITCHLEY et al. (1985) tried to distinguish bound and internalized toxin in primary nerve cell cultures which were not depleted from other cells, the latter in contrast to Yavin's experiments. Toxin was fixed at 0° C but largely left the cell surface when the cells were brought up to 37° C. A substantial

Table 9. Distinction between binding, sequestration, and internalization (Compiled from the work of YARIN and colleagues, see text)

Step	Characterization
1. Binding	The toxin is loosely fixed to the cell surface. Fixation is enhanced by low ionic strength and low temperature, and is partially reversed when these are raised. Neuraminidase inhibits partially.
2. Sequestration	The toxin is in a stable association with the cell, i.e., after exposure to high-salt medium at 0°–4° C or addition of GD_{1b} ganglioside. Neuraminidase inhibits partially.
3. Internalization	Toxin is now in a compartment that is stable against salt, gangliosides, protease, and neuraminidase, and partially resistant to detergent extraction. This toxin is still poisonous in mice. Internalization depends on temperature.

amount of it dissociated into the medium. Some radioactivity disappeared into a compartment which could be reopened with 0.1% triton and therefore was regarded as internalized. The features of the compartment were morphologically discrete; it was thought not to be identical with lysosomes, as the half-life of the cell-associated toxin was long. The functional significance of the putative internalization has not yet been established.

In order to gain better insight into the individual steps, various somatic neural *hybrid cells* were substituted for primary cultures. Binding was 7–60 times lower as compared with primary cultures; this was not unexpected, considering the lack of long-chain gangliosides. The ganglioside pattern differed with the lines used. GM_3 and GM_2 were found, and in some lines also GD_{1a}, but never GD_{1b} and GT_{1b}, which are known to bind the toxin best. Some loss (up to 29%) of binding was caused by neuraminidase, and serum removal raised toxin binding. However, the impression prevails that binding to the hybrid cells is significantly different from that to nerve cell cultures (YAVIN and HABIG 1984). The data give some weight to those of ZIMMERMANN and PIFFARETTI (1977), who first demonstrated binding of tetanus toxin to cultured neuroblastoma cells. They also assumed a partially neuraminidase-resistant binding. On the other hand, the low ganglioside content explains why DIMPFEL et al. (1977) failed to find an appreciable binding to nonneuronal cells.

Transplantation of gangliosides into the hybrid line 108 CC15 led to an enormous increase in their binding capacity (DIMPFEL et al. 1977). YAVIN (1984) extended this observation in the light of his three-step model. As the ganglioside-supported binding and its sequelae in these cells largely resemble the events described in nerve cell cultures, it has been suggested – without proof – that the interaction of the toxin with the cell membrane ganglioside is the most prominent and the earliest event in toxin translocation.

Uptake mechanisms in cell cultures may transport proteins in both endocytotic and nonendocytotic ways. To exclude the endocytotic process, LAZAROVICI and YAVIN (1985a, b) have coated human erythrocytes with gangliosides. However, there are various technical problems to be considered. First, the interaction between gangliosides and red cells is neither firm nor homogeneous. Part of

it is salt sensitive, a fact which has been assessed by the use of radiolabeled tetanus toxin. It has been argued that the binding of toxin to gangliosides is stronger than that of gangliosides to cells. If so, the data reflect the stability of the latter association. On the other hand, the same ganglioside might be embedded differently into different membranes, depending on their structure. This may then result in different affinities to toxin, and salt sensitivity would relate to toxin-to-ganglioside binding. There is no way out of this dilemma. The other measures used to distinguish bound from internalized toxin, for instance, treatment with trypsin, glutaraldehyde, or saturation experiments, are subject to other limitations, because the distinction between sequestered and internalized toxin is not unambiguous. A macromolecular complex between trypsin, ganglioside, and cellular protein(s) of 700000 daltons in ganglioside-supplemented red cells has been described. If it also exists in other toxin-sensitive cells, the complex might serve as a transducer between ganglioside binding and toxin action (LAZAROVICI and YAVIN 1985b).

Comparable studies with botulinum toxins are lacking.

4.3 Axonal Transport

4.3.1 Tetanus Toxin

Local tetanus has fascinated researchers for many years, and much of the older literature on the pathogenesis of tetanus (see KRYZHANOVSKY 1966, 1975a, b, 1981; WRIGHT 1955) deals with ingenious animal experiments for the assessment of the local events and with controversial interpretations of that puzzling phenomenon. After highly labeled ^{125}I-toxin had become available (HABERMANN 1970), the particular but not unique pharmacokinetics of tetanus toxin turned out to be the background of both local and general manifestations of tetanus. Briefly, locally produced or injected tetanus toxin enters the motoneurons in the periphery, ascends intraaxonally, and is then transported to some degree trans-synaptically to presynaptic neurons. This is true also for generalized tetanus (ERDMANN and HABERMANN 1977; PRICE and GRIFFIN 1977). The toxin barely passes the blood-brain barrier (HABERMANN and DIMPFEL 1973). The difference is that in local tetanus the toxin originates locally and ascends through the pertinent neurons, whereas in general tetanus it passes from the blood into the interstitial space, and from there ubiquitously by way of the motor nerve endings into the brain stem and the spinal cord.

We do not want to go into the pathogenesis of tetanus, which has been discussed previously by other authors (see Table 1); neither do we want to deal with results which have meanwhile become textbook knowledge. It would be tempting to go into the details of axonal transport, as many of its features have been elucidated by the aid of tetanus toxin. The reader is referred to reviews by SCHWAB and THOENEN (1977) and SCHWARTZ (1979). The former draw particular attention to extrinsic macromolecules, among them tetanus toxin.

In terms of this review, axonal transport may be regarded as an extension of internalization. Conversely, axonal transport gives final proof for internaliza-

tion but does not furnish proof for any intracellular action. It can be used by many agents, provided they (a) enter the nerve ending and (b) are not degraded. Tetanus toxin shares the ability to enter (a) with many other proteins, it is very stable against proteolysis during long-term intracellular exposure (see p. 151), and thus meets condition (b).

Entrance into cells may be viewed from two aspects, i.e., the role of the membrane and the role of the entering protein. Morphologically resting membranes, typically those of erythrocytes, are impermeable to any protein. Other membranes are in constant turnover, for instance those of kidney and intestinal microvilli. They pinocytose and must, for reasons of economy, reintegrate the envelope of the pinocytotic vesicles into the cell surfaces. The same is true for synaptic structures (for comparative considerations, see HABERMANN 1977). Among the proteins, we may distinguish those adsorbed to the cell surface from those remaining in free solution. The former will be internalized automatically during the renewal of the membrane. The higher their affinity and the faster the shuttle, the more they might be prone to axonal transport. The responsive process is adsorptive pinocytosis. As mentioned previously for tetanus toxin, pharmacokinetic receptors would fit nicely into this view. Other proteins must be pinocytosed by vesicular seclusion of their solvent. This endocytosis in bulk is less efficient and less specific.

Are uptake and the ensuing axonal transport linked with neuronal activity? Although obvious at first glance, the hypothesis is not supported by facts. KRISTENSSON and OLSSON (1978) unilaterally paralyzed the vibrissae region in mice with nearly lethal doses of *botulinum A toxin*. After complete paralysis, horseradish peroxidase was administered to the poisoned region, and its distribution was checked by electron microscopy. Surprisingly, incorporation into organelles of the axon terminal and retrograde transport to the cell bodies occurred about equally on both sides. The same was true when horseradish peroxidase had been applied systemically after unilateral paralysis. With a similar approach, KEMPLAY and CAVANAGH (1983) came to the same result.

Radiolabeled *tetanus toxin* was injected into the gastrocnemius muscle of mice that had been paralyzed by a previous local administration of botulinum A toxin. Again, ascent into the spinal cord was not impaired by the poisoning (HABERMANN and ERDMANN 1978) but even increased, probably owing to the longer persistence of the toxin in the paralyzed muscle.

The two separate approaches show that transmitter release is not required for uptake and transport. This view is supported by the ascent through sensory neurons (STÖCKEL et al. 1975) which do not pinocytose. The process which transports proteins inward is not strictly linked with the outward transport for transmitters. Alternative hypotheses are given by KRISTENSSON and OLSSON (1978). Thus, the riddle of internalization remains.

Neuronal ascent is a pharmacokinetic prerequisite for central toxicity, but it cannot be equated with toxicity. For instance, a toxoid prepared by mild formaldehyde treatment still ascends (HABERMANN 1973a; WELLER et al. 1986). Moreover, tetanus toxin does not influence adrenergic synapses in situ (AMBACHE et al. 1948a, b) but is nevertheless transported centrally through sympathetic postganglionic axons (see SCHWAB and THOENEN 1977). Binding to the

respective neurons greatly favors the retrograde transport. Tetanus toxin, having binding sites on all neuronal matter, ascends not only through motor fibers but also through sensory and adrenergic fibers (STÖCKEL et al. 1975). In contrast, nerve growth factor migrates through its targets, i.e., the sensory and adrenergic neurons, only. Central neurons also internalize ^{125}I-tetanus toxin. It is transported both antero- and retrogradely when injected into the rostral part of the n. caudatus of the rat (SCHWAB et al. 1977). The axonal transport can be saturated by an excess of protein, which fact indicates that pharmacokinetic receptors are involved. Cross-competition was of restricted specificity. Saturation has been described for nerve growth factor and wheat germ agglutinin, which may interact with carbohydrate moieties at the synapse (DUMAS et al. 1979a).

Which part, if any, of tetanus toxin is responsible for its axonal transport? According to a classical hypothesis (for review, see HABERMANN 1978), the toxin contains several domains, one of them responsible for binding to gangliosides or brain tissue. This binding site was located to the C-fragment (see p. 110). As binding was linked with transport, the assumption was obvious that this fragment is also the "transporter" for the whole toxin molecule.

Initial tests seemed to support the elegant hypothesis. As in the binding assay, C-fragment, but not the residual B-fragment, was found to substitute for tetanus toxin in ascent (BIZZINI et al. 1980b; MORRIS et al. 1980). When the C-fragment had been linked to B by aid of a disulfide bridge, it carried B with itself. It was apparently not crucial for transport studies whether the former fragment had been prepared by an intrinsic protease (originally called BIIb fragment) or by papain (called IIc fragment in Bizzini's nomenclature; BIZZINI et al. 1981). Radiolabeled material was found in the pertinent nerve cell bodies following ascent. This was the classical view on fragment C.

However, this so-called binding fragment appears to behave variably, depending on the system used for axonal transport. According to the publications by Thoenen's group, transport took place from the anterior eye chamber of the rat to the ganglion cervicale superius, where accumulation was registered. Akert's group used the oculomotorius pathways in rats and monkeys, without giving quantitative data. More practicable, quantitative, and closer to the pathogenesis of tetanus is the ascent from the m. gastrocnemius to the spinal cord (HABERMANN 1970, 1972). Even in the pioneering experiments with this system (BIZZINI et al. 1977), the unilateral ascent of fragment BIIb was of disappointingly low capacity. Less than 1/100000 of the radioactivity injected were transported.

Recent quantitative data, which have been gathered in the same system to resolve the inconsistencies, have furnished new insights into the link between binding and axonal transport (Table 10). The central observation is that fragment C and BIIb ascend weakly, whereas a carefully prepared toxoid does so quite well (WELLER et al. 1986).

Binding behavior is the key to understanding the discrepancies. Fragment C or BIIb do not mirror tetanus toxin in this respect. As already described by GOLDBERG et al. (1981) for binding studies in dilute (0.025 *M*) Tris acetate buffer, there is a preferential competition by the respective counterpart, i.e.,

Table 10. Influence of structure on binding and axonal transport of tetanus toxin (HABERMANN and ALBUS 1986; WELLER et al. 1986)

	Toxicity (% of original)	Ascent to spinal cord	Binding to brain in		Binding to kidney (physiol. salt)
			Physiol. salt	Low pH, low osmolarity	
Tetanus toxin	100	+++	+++	+++	+++
Tetanus toxoid (mildly toxoided)	1	++	++	++	n.t.
Fragment C or BIIb	<0.01	(+)	(+)	+++	+++

n.t., not tested

fragment C by radiolabeled fragment C, and tetanus toxin by radiolabeled tetanus toxin. This already indicates nonidentity of the binding sites. More importantly, if we, unlike the authors mentioned, run the binding assay with brain membranes in physiological solutions, fragment C (or BIIb) hardly competes any longer with ^{125}I-toxin (HABERMANN and ALBUS 1986; WELLER et al. 1986). In contrast, mildly toxoided toxin competes well with tetanus toxin in physiological solution, although it has lost about 95% of its toxicity. Only those toxin derivatives ascend well from the peripheral rat muscle to the spinal cord that compete with radiolabeled toxin for binding in physiological solution. Mildly toxoided toxin moves nearly like native toxin (HABERMANN 1973a), but the transport of fragment C is about 50 times less. Obviously, only those binding sites which resist physiological salt solution are relevant in pharmacokinetics (WELLER et al. 1986). The precondition lowers the chances for the proposed use of fragment C as a carrier for drugs or antibodies from the periphery into the spinal cord.

There is a second, no less important aspect. Fragment C mimics some features (MORRIS et al. 1980) of toxin binding, but not all. Other parts of the toxin molecule have to cooperate in order to establish the full properties of tetanus toxin in both binding and transport. Fragment C is a necessary, but not a sufficient requirement even for toxin binding.

The need for the toxin molecule as a whole depends not only on the binding conditions, as exemplified with brain matter, but also on the substrate: For instance, fragment C competes like toxin with ^{125}I-toxin on kidney membranes but not on brain membranes in physiological solutions (HABERMANN and ALBUS 1986). The differences are compiled in Table 10.

Due to its high affinity for neuronal membranes, tetanus toxin may be regarded as a temporary membrane protein which is picked up for axonal transport like any other complex formed with membrane constituents. It is well known that lectins ascend through neurons (STÖCKEL et al. 1977; GONATAS et al. 1979; HARPER et al. 1980; TROJANOWSKI et al. 1981; FABIAN and COULTER 1985). Moreover, antibodies against dopamine β-hydroxylase migrate centripetally in adrenergic neurons originating from the iris but, as expected, not in motor

or sensory axons (FILLENZ et al. 1976). Apparently, the enzyme is temporarily exposed during exocytosis and may bring the antibody into a position appropriate for internalization. Antibodies against synaptic components are transported retrogradely, too (WENTHOLD et al. 1984). It is not known whether the antibody-antigen mixtures dissociate during internalization or transport.

Tetanus toxin has been reported to promote the transport of a monoclonal antibody directed against the toxin. Low-affinity interaction with toxin appeared to be a precondition, because a high-affinity monoclonal antibody prevented the ascent (HABIG et al. 1983), as did polyclonal antibodies (HABERMANN 1972). However, the ligation technique used did not distinguish between intra- and periaxonal movements. The question of whether there is an axonal cotransport of tetanus toxin with monoclonal antibodies remains thus far unsettled.

It is also unknown whether tetanus toxin comigrates with its synaptic binding sites. In situ labeled gangliosides of the mono- to tetrasialo types migrate both ortho- and anterogradely through the sciatic motoneurons of the rat, and so do glycoproteins (AQUINO et al. 1985).

The *morphological correlates* of axonal transport and its driving forces are still under debate. Even electron microscopy would not help, because proteins enriched within the endoplasmic reticulum or in vesicles may rest at a depot, whereas the transporting compartment may stay invisible (see SCHWARTZ 1979).

In contrast to albumin, tetanus toxin can carry gold particles (200 nm) with itself; owing to their size, they would hardly pass the membrane without adsorptive pinocytosis. Some hours after injection into the anterior eye chamber of the rat, the preloaded particles were selectively associated with membranes of nerve terminals and preterminal axons in the iris. Intracellularly, the tracer was located mainly in SER-like compartments (SCHWAB and THOENEN 1978). Localization of "free" tetanus toxin within the various compartments of the neurites, and thus of the subcellular pathway of its axonal transport, has not yet been achieved.

In a variety of anatomical systems in rats, FABIAN and COULTER (1985) investigated wheat germ agglutinin, which binds to glucosamine and sialic acid residues, and three other lectins which are specific for mannose (concanavalin A, pisum sativum and lens culinaris agglutinin). Like tetanus toxin (see Sect. 4.3.1) they underwent both anterograde and retrograde axonal transport. Morphological studies indicated that whenever an agglutinin was transported anterogradely, it also moved transsynaptically to neuronal somata in the respective terminal field.

In this pharmacokinetic context tetanus toxin may be regarded as a lectin which is very specifically directed against certain gangliosidic structures.

4.3.2 Botulinum Toxins

As compared with tetanus toxin, data on the axonal transport of botulinum neurotoxin are comparatively meager, and are restricted to type-A toxin. Nothing is known about a possible trans-synaptic transport (see Sect. 4.4). Without doubt, the toxin is internalized on the neuromuscular junction (see DOLLY et al.

1984a, b). Upon intramuscular injection, small amounts of ^{125}I-neurotoxin appeared in the respective part of the spinal cord (HABERMANN 1974). WIEGAND et al. (1976) traced the toxin upon its intramuscular (m. gastrocnemius) injection in cats. Radioactivity was found ipsilaterally in the pertaining spinal segments. Long-term exposure revealed some fibers in the ventral roots enriched in radioactivity. The radioactivity in the peripheral nerve and in the spinal cord was too low for autoradiography. However, functional changes indicated that some toxin must have not only ascended but also acted (HAGENAH et al. 1977; WIEGAND and WELLHÖNER 1977). Recently we (WELLER and HABERMANN, to be published) have localized ^{125}I-botulinum A neurotoxin in the ipsilateral ventral horn of spinal cord in rats with local botulism. Ascent was quantitatively less, but distribution within the grey substance was indistinguishable from that of tetanus toxin.

4.4 Trans-synaptic Transport of Tetanus Toxin

Axonal transport of tetanus toxin is followed by trans-synaptic transport. If, as is generally assumed, tetanus toxin acts presynaptically within the spinal cord, it has to migrate from the motoneurons to their contacting interneurons. On its way from cell to cell it has to pass one membrane and at least to enter another. Like the features of axonal transport, this final event is not unique for tetanus toxin. Transneuronal transport has been described for horseradish peroxidase in the visual system of the house fly (NÄSSL 1981). In rats a variety of axonally transported molecules underwent intercellular transfer. TROJANOWSKI and SCHMIDT (1984) compared horseradish peroxidase and its conjugates with wheat germ agglutinin, cholera toxin, and B-subunit of cholera toxin in the tongue-hypoglossus pathway and in the visual system of the rat. They concluded that (a) horseradish peroxidase migrated intra- but never transcellularly, (b) all conjugates migrated transcellularly in the visual system, and (c) none of them did so in the tongue-hypoglossal system. Apparently, both the transported material and the assessed neuronal pathways are relevant for the occurrence of transneuronal transport.

A complex between tetanus toxin and horseradish peroxidase ascends from the eye bulb through adrenergic neurons into the cervical ganglion of the rat. In contrast to other proteins investigated, which end in the lysosomes of the pericaryon, a substantial fraction of the toxin-peroxidase complex is found in presynaptic terminals (SCHWAB et al. 1979). Using very large amounts of radiolabeled tetanus toxin in the same system, DUMAS et al. (1979b) localized the radioactive material within the presynaptic nerve and identified it by disc gel electrophoresis and subsequent autoradiography. Similarly, ^{125}I-tetanus toxin that had ascended through rat motoneurons was partially transferred into adjacent presynaptic terminals, as shown by electron-microscopic autoradiography. Glial cells remained free, like the cell bodies of interneurons and dorsal horn cells (SCHWAB and THOENEN 1976). Without trans-synaptic transport of its toxin tetanus would probably not exist as a disease, and antibodies administered intracisternally should not be more protective than those applied by the usual

routes (see REY et al. 1981 regarding this pathway in therapeutics). A confirmation of trans-synaptic transport came from the use of antibodies. ERDMANN et al. (1981) injected first nonlabeled tetanus toxin into the gastrocnemius muscle of rats, and ^{125}I-F(ab)$_2$ fragments into the cerebrospinal fluid at various times thereafter. The fragments did not enter the motoneurons but intercepted the toxin molecules, putatively on their trans-synaptic way. Thus, the motoneurons appeared decorated with radiolabeled antigen-antibody complexes. Trans-synaptic transport has also been described for BIIb-fragment, which is closely related to fragment C (see Fig. 3a). Following injection of ^{125}I-labeled BIIb-fragment into the extraocular eye muscles of monkeys, motoneurons were strongly labeled after 1.5–6 days, and a discrete cell soma labeling was found in all areas known to make contact with the primary stations. As the fragment is nontoxic, the trans-synaptic movement cannot be regarded as inherent to toxicity. Amounts of fragment BIIb can be used that allow its detection by light-microscopic autoradiography (BÜTTNER-ENNEVER et al. 1981a, b).

5 Pharmacodynamics of Isolated Systems

It is generally acknowledged now that tetanus is due to a preferential inhibition of central inhibitory synapses (for recent reviews, see KRYZHANOVSKY 1981; MELLANBY 1984; MELLANBY and GREEN 1981; WELLHÖNER 1982), and that botulism results from inhibition of peripheral cholinergic synapses (for review, see SIMPSON 1981). These statements on the diseases need no longer be discussed. However, the preceding chapters have stressed the many similarities between the clostridial neurotoxins. One wonders why so closely related toxins should lead to such distinct disease entities as botulism and tetanus. The present chapter does not deal with the whole-body toxicology. Instead, it looks at the elementary processes. It will be shown that their considerable analogies justify putting tetanus and botulinum neurotoxins into one group, despite the clinical differences of poisoning.

Three main complementary techniques are available for neurotoxicology in vitro. *Neurochemistry* (see Sect. 5.2) allows the study of not only action but also binding on the same system. As compared with neurophysiology, its results are easier to translate into quantitative terms; its techniques are more flexible and allow a broader application. Some discrete effects such as enzyme inhibition or phosphorylation would escape detection by other than biochemical techniques. *Electrophysiology* (see Sect. 5.3) is superior in time resolution of the data; its objects are generally better preserved, and its results are mostly closer to the in vivo situation. However, its access is limited to phenomena at the membrane level. *Membrane biophysics* (see Sect. 5.1) has so far not found much application to research on clostridial neurotoxins. Its models are quite remote from the natural events of poisoning, and it is feared that the crucial targets and/or the signal chains of the toxins are lost upon preparation of liposomes or artificial membranes. However, should it turn out that tetanus toxin acts directly on membranes, biophysical studies will become crucial.

One may ask about the value of *morphological* techniques in the analysis of the actions of clostridial neurotoxins. Without doubt they are relevant in the study of toxin distribution within organs and/or within organelles (see p. 127, 135). However, even electron-microscopic or immunofluorescence techniques did not reveal any changes directly linked with toxicity. What has been seen, for instance chromatolysis in tetanus (TARLOV et al. 1973), may be a consequence of functional alterations and not typical of the toxins. Axonal sprouting, for example, is not unexpected after long-lasting paralysis by botulinum (DUCHEN and STRICH 1968; DUCHEN 1970, 1971) or tetanus (DUCHEN and TONGE 1973) toxin. Even so, its role in the resumption of nerve activity is at least dubious (KRISTENSSON and OLSSON 1978). In addition, the reports on altered shape and number of vesicles (for review, see MELLANBY and GREEN 1981) are often controversial.

5.1 Membrane Events

Gangliosides are integral parts of neuronal membranes, and can bind tetanus and various, if not all, botulinum neurotoxins (see Chap. 4.1). With the ascent of biophysics, membrane perturbations by tetanus toxin, possibly mediated by glycolipids, were sought. The data cannot be assembled to a definitive picture yet. CLOWES et al. (1972) were the first to use lipid bilayer membranes consisting of lecithin, cerebroside, and gangliosides. Tetanus toxin in concentrations as high as 6.7 μg/ml did not change their electrical properties, although the membrane resistance broke down faster under the applied potential of 50 mV. The membranes also became thicker (from 79 Å to 119 Å) under the influence of the toxin, as shown by reflectivity measurements. The authors concluded that the effect of the toxin was restricted to the membrane surface.

BOROCHOV-NEORI et al. (1984) reinvestigated the possible effect of tetanus toxin on lipid bilayers. They applied tetanus toxin to an asymmetric planar bilayer under voltage clamp conditions. One side had been coated with a monolayer of asolectin, the other with asolectin supplemented with total brain gangliosides. Nothing happened when the toxin (25 μg/ml) made contact with the former layer. However, its (10 μg/ml) exposure to the ganglioside surface led within minutes to the formation of cation channels (conductance 46 ± 3 pS), which persisted in the open state for several minutes. The important result was that tetanus toxin apparently not only reacted with the gangliosides but also changed the membrane properties. However, any extrapolation concerning the mode of the toxin's action on its natural substrate would be premature. The membrane investigated cannot do anything but decrease its electrical resistance, whereas the nerve cell membrane can respond in many ways.

BOQUET and DUFLOT (1982) did not require gangliosides. They exposed single-walled, K^+-loaded asolectin vesicles to tetanus toxin and its fragments at various pH values. At pH 5 fragment B, but not fragment C, and, to a minimal degree, whole tetanus toxin promoted the efflux of K^+. As albumin also did so, albeit in a higher concentration range, the relevance initially appeared to be dubious. However SIMPSON and HOCH (1985) confirmed that fragment B

from tetanus toxin raised the membrane conductance. Residual toxicity, as measured on the mouse diaphragm, did not run parallel with this property.

Boquet and his group further emphasized the role of low pH in the toxin effects. According to BOQUET et al. (1984), tetanus toxin promoted the K^+ release at pH 3.5 and below. When the asolectin vesicles were coated with ganglioside GD_{1b} (but not with GM_3), the limiting pH value was about 1 unit higher. Binding of triton to tetanus toxin was also pH dependent in the same region. Therefore, it was concluded that, as with diphtheria toxin, low pH exposes a hydrophobic domain of tetanus toxin, thus mediating penetration into the cell membrane. Like diphtheria toxin, tetanus toxin should then enter into an acidic, perhaps lysosomal, cell compartment, which process might serve as a precondition of poisoning. Thus, Boquet's hypothesis implies both entrance and action.

ROA and BOQUET (1985) tried to locate the site(s) that link the toxin with asolectin vesicles. The sequences should thereby become protected against enzymatic attack. Peptic digestion of toxin bound at pH 3 (but not at pH 7) indicated the "survival" of two peptides (mol. wt. 27000 and 21000 respectively) which could be allocated into the β_2 fragment, i.e., that part of the heavy chain which is connected with the light chain to form fragment B (see Fig. 3). Neither the light chain nor the C-fragment were protected; this may, but need not be taken as evidence against their participation in binding or internalization on asolectin vesicles. As elegant as the experiments are, they fail to prove the biological relevance of the apparently hydrophobic toxin residues, and they do not answer the question of whether the two peptides represent the translocating apparentus (i.e., an extra binding site, besides the ganglioside-binding C-fragment) or translocated domains of the toxin.

According to CABIAUX et al. (1985), phosphatidylinositol appears to interact not only with diphtheria but also with tetanus toxin, provided pH is at 4. The observation is based on an increase in turbidity of small unilamellar vesicles made from phosphatidylcholine and phosphatidylinositol (9/1, w/w). The change in turbidity was probably due partly to fusion, partly to aggregation. Two facets of this work require comment. First, gangliosides are not required, although they are commonly regarded as toxin receptors. Second, phosphatidylinositols are of great interest as putative binding sites of tetanus toxin, since they are hydrolyzed to inositol-trisphosphate, which is a good candidate for being an intracellular messenger (BERRIDGE and IRVINE 1984).

A major difference between tetanus and diphtheria toxin on bilayer membranes still has to be settled. If we follow BOROCHOV-NEORI et al. (1984), tetanus toxin acts from the side coated with gangliosides. Diphtheria toxin, however, produced voltage-dependent channels at acidic pH when the bilayers had been exposed to phosphoinositides, but these lipids had to be applied to the contralateral side (DONOVAN et al. 1982). This toxin appears to intrude into an ordinary bilayer, and requires an "anchor" at the contralateral side in order to form a channel.

A third version of the interaction between clostridial neurotoxins and membranes has recently been introduced by HOCH et al. (1985). In contrast to BOROCHOV-NEORI et al. (1984), they found no promotion by gangliosides, and unlike

BOQUET et al. (1984), the efficacy of the toxins was negligible at symmetric pH values of 4 or 7 (although tetanus toxin fragments elicited more channel activity at pH 4). Their main observation was that a pH gradient (*cis* pH 4, *trans* pH 7) massively promoted the formation of large channels. Tetanus toxin, its heavy chain, its fragment B, and the heavy chain of botulinum B toxin were all active, as was diphtheria toxin, but the respective light chains, the complete botulinum B neurotoxin, and fragment C from tetanus toxin were not. Positive voltages applied to the *cis* compartment opened the channels and negative voltages closed them. The data may be reconciled with the assumption that, following internalization, the toxin must reach an acidic intracellular compartment, perhaps lysosomes, in order to form tunnels into the more neutral cytoplasm. The size of the tunnels is assumed to allow the transit of a still hypothetical active toxin fragment. Thus, the working hypothesis refers more to the pharmacokinetics than to the pharmacodynamics at the membrane level. Following this view, internalization should be a two-step process, leading first into the lysosomes and then into the cytoplasm.

The question of whether tetanus toxin also raises the permeability of biomembranes (and not only of artificial ones) has not yet been settled. The toxin might render the membrane core leaky, thereby raising the permeability to ions. So far, it has not been reported that tetanus toxin changes the resting potential of intact nerve cells, either in culture or in situ. The accumulation in synaptosomes of the lipophilic cation tetraphenylphosphonium, which is an indicator of the membrane potential, was lowered by very high concentrations (22 μg/ml) of tetanus toxin, but raised by lower (2 μg/ml) concentrations. The data are difficult to interpret, in particular because a contamination of the toxin with traces of the cytolytic tetanolysin has not been excluded (RAMOS et al. 1979). Nevertheless, they agree with the observations mentioned before that the electrical resistance of lipid bilayers can be reduced. The loss in membrane potential of synaptosomes was not seen with tetanus toxoid or with fragments B or C (MORRIS et al. 1980), which fact indicates some specificity.

We must refer here to the still unexplained observation that tetanus toxin, in admittedly high concentrations, promotes the release of noradrenaline (see p. 147); this could be due to or at least linked with an enhanced Na^+ entry. Upon poisoning in vivo the toxin also renders striatal slices more leaky for other radiolabeled transmitters (COLLINGRIDGE et al. 1980).

Data on the effects of *botulinum* toxins on artificial membranes are sparse. HOCH et al. (1985) proposed that the heavy chain of B toxin, but not the whole toxin molecule or its light chain, promoted the production of pores under the influence of a pH gradient. DONOVAN and MIDDLEBROOK (1985) reported a channel formation by C toxin when the pH was lowered from pH 7.5 (no effect), reaching a maximum at pH 6.0; they did not mention sidedness.

Does the interaction between tetanus toxin and membranes resemble the events registered with other toxins? In fact, every toxin studied appears to be handled individually by the cell. The pentameric B (from "binding") domain of cholera toxin remains in the membrane, whereas its ADP-ribosylating A (from "active") moiety enters the cell (for discussions, see VAN HEYNINGEN 1984). Ricin passes through the membrane bilayer via a more direct pathway.

Diphtheria toxin penetrates the membrane better at low pH, as does tetanus toxin (see above), probably by way of endocytosis. Pore formation by diphtheria B chain is thought to be insufficient to allow the entrance of diphtheria A chain. It is hypothesized that the diphtheria pore results from a dimeric structure $(AB)_2$ of the toxin (ZALMAN and WISNIESKI 1984). With both ricin and diphtheria toxin it is assumed that intracellular processing occurs in lysosomes, but there is no evidence that clostridial neurotoxins behave alike.

So far, we have dealt with nonphysiological, nonspecific channels. Only one group has reported on the blockade of a voltage-dependent channel by tetanus toxin (HIGASHIDA et al. 1983; SUGIMOTO et al. 1983). They used cells from NIE115, a neuroblastoma strain from mice minimally equipped with long-chain gangliosides. A relatively rapid (10–20 min latency), sensitive (65 ng/ml toxin) decrease of Ca^{2+} spikes was seen, however only under artificial conditions (Na^+-free salt medium). The data are at variance with those of BIGALKE et al. (1981a), who did not find any inhibition of Ca^{2+} uptake into particulate brain by tetanus or botulinum A toxin, and they are not compatible with the failure of both toxins to inhibit Ca^{2+} influx into motor nerve endings (DREYER et al. 1983).

Much effort has been devoted so far to the putative interaction of clostridial neurotoxins with enzymes. Previous approaches (see WELLHÖNER 1982) have not fulfilled the initial promises, and others have not been published. In our laboratory, we have looked for the interaction of tetanus toxin with choline acetyltransferase, protein phosphorylation, the aggregation of microtubules, Na^+, K^+-ATPase, changes in adenylate and guanylate cyclase activity in slices, and Ca^{2+} uptake – with no positive results. SMITH and MIDDLEBROOK (1985) tried another approach with a more promising result. Briefly, they reported that botulinum A, C, and D toxins and tetanus toxin partially inhibited (60%) both the soluble and the particulate guanylate cyclase of permeabilized guinea pig synaptosomes and NG 108-15 cells. So far, a link between this interesting observation and the inhibition of transmitter release is still being sought.

5.1.1 Clostridium botulinum C_2 Toxin as a Membrane-Active Botulinum Toxin

The membranal actions of this toxin are unique and deserve separate consideration. First, it is produced by some strains of *C. botulinum* types C and D (see Sect. 2.2) which at the same time may harbor phages involved in the production of C_1 and D toxins respectively. The second peculiarity rests in its structure. C_2 strains produce two proteins similar in size to the chains of the clostridial neurotoxins but not linked by disulfide bridges or noncovalent bonds (OHISHI et al. 1980a). Most important of all, in contrast to all other botulinum toxins, C_2 toxin is not a neurotoxin (SIMPSON 1982) but a cytolytic agent rendering many types of membranes leaky. To complicate the relations still further, both components have to be present in order to elicit the full effect (OHISHI et al. 1980b). Given intraperitoneally to mice, the two separate components I (size of light chains) and II (size of heavy chains) were hardly toxic (<10 LD_{50} per mg protein). When administered together in a ratio of 1:2 on a protein

basis (close to the equimolar ratio), toxicity was 5×10^3 LD_{50} per mg protein. The two components did not need to be injected at the same site (OHISHI 1983a). Component II had to be trypsinized to exert the full effect. Even under optimal conditions, toxicity of the mixture was lower than that of the classical neurotoxins.

Poisoning manifested as a general circulatory collapse with lung hemorrhage (SIMPSON 1982). Rise of vascular permeability turned out to set a good example for protein complementation as defined by HABERMANN and BREITHAUPT (1978). When the individual components were given alone, leakage of dye was present at the injection sites of II but not at those of I. The latter component, injected intravenously, potentiated dye leakage due to II (OHISHI 1983a). Toxin C_2 also led to fluid accumulation when instilled into the intestinal loop of mice. This activity was inhibited by antibodies against component I or II. The secretory response was positive when the tissue had been exposed to II, rinsed, and then exposed to component I. The reverse was not true (OHISHI 1983b). On the basis of the present data it can not be decided whether binding of II prepares for the secretory response, which is then induced by the action of I, or whether component I might promote the actions of II. Cytotoxicity was also evident from morphological changes in eight human cell lines exposed for 24 h to a mixture of components I and II. The effect was sensitive against anti-I and anti-II sera and occurred with concentrations between 10 ng/ml and 2.5 µg/ml (OHISHI et al. 1984). Despite their different targets, it is tempting to assume that C_2 cytotoxin may serve as a model for the actions of the clostridial neurotoxins. Component II of C_2 toxin may reflect the properties of their heavy chains, with respect not only to molecular weight but also to binding properties.

A novel hypothesis regarding the mode of involvement of component I in *C. botulinum* C_2 poisoning has been promoted by SIMPSON (1984d), who has found that it ADP-ribosylates both natural and synthetic (polyarginine) substrates. Unlike cholera toxin, it does not activate adenylate cyclase. Its natural substrate in various cells has been identified as actin, which is the biochemical basis of microfilaments. Functional changes of the cytoskeleton may well explain the cytotoxicity of C_2 toxin (AKTORIES et al. 1986). Enzymatic activity in one, even atypical, *C. botulinum* toxin may indicate a general mechanism underlying the mode of action of neurotoxic clostridial toxins, whose light chains might transfer ADP-ribose, too. However, their substrate and its function still have to be determined.

5.2 Neurochemical Studies on Transmitter Release

5.2.1 Studies In Vivo and in Isolated Preparations

5.2.1.1 Tetanus Toxin

OSBORNE and BRADFORD (1973) first applied biochemical techniques to the study of inhibition by tetanus toxin of transmitter release. Initially they exposed synaptosomes from the medulla and spinal cord of rats to toxin in vitro for up

to 40 min prior to electrical stimulation, without success. However, when the synaptosomes were from rats poisoned in vivo, and then, for the sake of completeness, poisoned in vitro as well, the release of the inhibitory amino acids glycine and GABA and of the excitatory compound aspartate was depressed. Despite its preliminary character, the work included crucial features of future experiments. In particular, it showed that the effect of tetanus toxin was nonspecific as to the transmitter released and that the transmitter content was apparently not much changed by the toxin. While their first statement was immediately accepted, the reports on transmitter content were controversial for some time. The moderate increase of GABA and glutamate in poisoned synaptosomes (OSBORNE and BRADFORD 1973) might have been secondary to reduced stimulus-induced efflux. Effects on glycine concentrations in the spinal cord of the cat were absent in interindividual comparison (JOHNSTON et al. 1969). Differences in glycine and GABA content were minor in left/right comparisons in cats with unilateral local tetanus (SEMBA and KANO 1969), and absent (with respect to glycine) under comparable conditions in rats (FEDINEC and SHANK 1971). Thus, it can now be stated that decreased transmitter release is not caused by a diminished storage due to tetanus toxin.

Basically, neurochemical studies in local tetanus are beset with pitfalls. Toxin has to ascend from the injected region and has to be transported trans-synaptically. This means that only a minor percentage of neurons will be afflicted, and their alterations might disappear among the bulk of nonpoisoned cells. Extreme dosages would lead to generalized tetanus, which precludes intraindividual comparison between poisoned and intact areas, for instance in unilateral administration. Finally, changes in one neuron might be only secondary to changes in a supplying neuron. The way out of the dilemma was sought in work with isolated systems. Two approaches were tried: (a) poisoning in vivo with subsequent study of the surviving tissue in vitro, as pioneered by OSBORNE and BRADFORD (1973), and (b) direct poisoning in vitro.

Approach (a) was motivated by the observation that injection of tetanus toxin into the substantia nigra of rats led to marked turning. The time to onset depended on the amounts of toxin administered and could be as short as a few minutes and as long as 4 days. Duration was more than 3 weeks. The direction, depending on the injection site, was ipsilateral (MCGEER et al. 1980; COLLINGRIDGE and DAVIES 1980, 1982a) or both contralateral and ipsilateral (JAMES and COLLINGRIDGE 1979; COLLINGRIDGE and HERRON 1985). The reason why both ipsilateral and contralateral turnings were seen is not entirely clear, but it is probably linked to multiple topographical and/or biochemical targets. If tetanus toxin primarily inhibits GABA release, it should interrupt the so-called inhibitory GABA loop. The dopaminergic cells should fire more strongly and thereby produce a contralateral turning, which should be abolished by intraventricular 6-hydroxydopamine. Ipsilateral turning is assumed to depend on the interruption of inhibitory thalamonigral and tectonigral systems (see FOCÁ et al. 1984). The direction of turning might also depend on the preferential poisoning of the pars compacta or pars reticulata. Another explanation would be that toxin injected into the s. nigra is axonally transported into neuronally connected regions, for instance the striatum, thereby reaching and inhibiting,

among others, the dopaminergic nerve endings. Independent of the interpretations, the system registers a specific manifestation of tetanus toxin in vivo. The target tissue can be isolated and investigated for biochemical changes later on. It is well known that the s. nigra is richly endowed with inhibitory, GABA-ergic synapses, which might be appropriate substrates for the toxin.

The turning response was not changed by prior treatment of rats with 6-hydroxydopamine (McGeer and McGeer 1979; McGeer et al. 1980). Striatal dopamine and homovanillic acid were raised when injection into the rostral s. nigra had led to contralateral turning, but not when injection into the caudal s. nigra had elicited ipsilateral turning (James and Collingridge 1979). Therefore, the dopaminergic system was thought not to be of major importance in the latter case. Glycine uptake into synaptosomal fractions was not significantly influenced either, and there was no asymmetry in other parameters of neostriatal transmitter systems such as content of choline acetyltransferase, tyrosine hydroxylase, norepinephrine, and 5-hydroxytryptamine (McGeer and McGeer 1979; McGeer et al. 1980). The McGeer group also reported the dopamine and homovanillic acid concentrations to be normal. However, transmitter release was depressed. Collingridge et al. (1979, 1980) injected tetanus toxin (500–2000 mouse LD) into the s. nigra or the corpus striatum of rats. The brains were dissected 6 h later. The uptake or the K^+-evoked release of labeled transmitters from the treated structures was then compared in vitro with that from the corresponding contralateral structures. There was no effect of injected tetanus toxin on the content of endogenous amino acids or on the uptake of GABA, dopamine, or 5-hydroxytryptamine into slices from tissue poisoned in vivo. In contrast, the Ca^{2+}-dependent, potassium-evoked release of radiolabeled GABA from such slices was reduced by 29%, and that of dopamine by 27%, the latter in contrast to the expectations based on the in vivo experiments mentioned, but stressing the nonspecific attack by tetanus toxin (see below). The release of labeled acetylcholine and 5-hydroxytryptamine was not influenced. Later, Collingridge and Davies (1982a, b) showed the effect of tetanus toxin to be reversible. Following injection of doses as low as 1–5 mouse LD, circling was maximal after 1 week and lasted 2–3 weeks. Moderate inhibition of GABA release in slices from in vivo-poisoned s. nigra run parallel with the course of circling behavior.

The gross changes in motility were also reflected by the electrophysiology of nigral neurons. Striatal-evoked inhibition of reticulata and compacta (Collingridge and Davies 1980) neurons was reduced during the circling period. As expected, neither the sensitivity to GABA nor that to other inhibitory transmitters was changed by locally administered tetanus toxin (Collingridge and Davies 1982a). The main problem with microinjections of tetanus toxin is its spread by axonal transport (Schwab et al. 1977), which makes its localization more difficult as compared with nontransported drugs. Nevertheless, there is good evidence that tetanus toxin inhibits GABA-ergic transmission in the s. nigra, and that this inhibition is largely responsible for the symptoms resulting from intranigral injection of the toxin. It would be interesting to know whether radiolabeled tetanus toxin persists in the injected area for the duration of circling.

COLLINGRIDGE et al. (1981) also observed an inhibition of GABA release by about 30% from rat hippocampal slices incubated in vitro with about 1 μg/ml toxin for 3 h. In later experiments with nigral slices concentrations of toxin between 100 ng and 1 μg/ml were sufficient, with the incubation times extended to 4 h (COLLINGRIDGE and DAVIES 1982b). One observation of these authors, presented in extenso by COLLINGRIDGE and HERRON (1985), raises particular interest because it may link the interaction between tetanus toxin and slices with that between the toxin and the neuromuscular junction (see Sect. 5.3.2.1). The three-step model of toxin action, resulting from work on the latter system, implies that the toxin first contacts the membrane and then is "internalized", as a precondition for action. Accordingly, treatment of nigral slices with the toxin could be shortened from nearly 4 h to less than 1 h without loss of efficacy, provided the slices were then incubated in the absence of toxin for the amount of time saved. Thus, the presence of free toxin is apparently no longer required in the later phase of poisoning, indicating that the onset of blockade of transmitter release is not governed by the duration of toxin exposure alone.

Electrophysiological studies on slices might be regarded as an intermediate between the neurochemical and behavioral work on the nigrostriatal system in situ and the cell culture investigations to be mentioned later. The advantage of slices lies in their ordered structure as compared with the statistical arrangement of neurons in tissue culture. Their disadvantage rests in their relative thickness, which impedes penetration of the toxin, and in their restricted viability as compared with the duration of poisoning by tetanus toxin. COLLINGRIDGE and HERRON (1985) poisoned hippocampal slices in vitro with huge toxin concentrations (25 μg/ml). The poisoned slices responded to Schaffer collateral-commissural stimulation with multiple population spikes, which were nearly absent in controls. Intracellular recordings from CA1-pyramidal cells showed that toxin suppressed the inhibitory postsynaptic potential moiety of the synaptic response, which then consisted of delayed excitatory postsynaptic potentials. Ca^{2+} spikes were apparently well preserved, a fact which argues against a blockade of Ca^{2+} channels by the toxin (see p. 140).

No comparable approach was tried with *botulinum* neurotoxins, perhaps because it was anticipated that they would inhibit only the central cholinergic synapses, like those in the periphery. Inhibition of cholinergic or noncholinergic synapses has never been shown by neurophysiological experiments on brain slices, although the data argue for a sensitivity of some central synapses toward botulinum A toxin in cats (WIEGAND and WELLHÖNER 1977; HAGENAH et al. 1977).

The approach (b) of the Giessen group was to replace in vivo poisoning by in vitro exposure of particulate brain preparations to tetanus and botulinum A neurotoxins. This extended the range of possible manipulations – for instance, variation of time, temperature, and other conditions of incubation, addition of antiserum at various times, and simultaneous measurement of binding and action. The advantages had to be weighed against three drawbacks. In contrast to the experiments with local injections, we had to dispense with a direct link between the in vivo and the in vitro situation. Moreover, the duration of the experiments was limited by the lability of the particulate prepara-

tions from brain. Finally, the restricted duration of the experiments had to be compensated for by raised toxin concentrations.

Two procedures were used. The basic aspects were established by a *superfusion* technique. Briefly, the particulate brain was preloaded with the radioactive transmitter to be studied, washed, then exposed to toxin for about 2 h, and finally loaded on a Sephadex column and superfused with or without a pulse of high K^+. Under the conditions chosen, the toxins did not measurably interfere with the re-uptake of transmitter during the incubation period, and the decrease in particle stability during 2 h of incubation was tolerable. In the so-called *batch* procedure, exposure to the toxins and depolarization were performed in the same vessel. Its relative simplicity is advantageous; however, transmitter uptake may interfere with the measurement of release.

The basic observation was that tetanus toxin partially inhibited the release of *many* transmitters – however, with differing potency and efficacy. Concordant with the earlier neurophysiological studies as reviewed by MELLANBY and GREEN (1981) and WELLHÖNER (1982), inhibitory transmitter systems, i.e., those with glycine and GABA, were most sensitive to tetanus toxin, inhibition of release being detectable with 1–10 ng/ml toxin. Higher concentrations were required to inhibit the release of noradrenaline or acetylcholine (BIGALKE et al. 1981 a, 1981 b; HABERMANN 1981 b). The experimental design mimicked in vivo poisoning in many respects. For instance, a dose-dependent latency preceded the onset of the inhibition of release, in accordance with the slow onset of poisoning in vivo. The toxin effects depended on temperature, like the effect on the end-plates of the diaphragm (see p. 155). Once manifest, they became irreversible, even when a large excess of antitoxin was tried, again like those on end-plates or like tetanus toxin poisoning in the living animal (see WRIGHT 1955; HABERMANN 1978; WELLHÖNER 1982) or in primary nerve cell cultures (HABIG et al. 1986). From the experiments with particulate preparations it also became evident that the binding step and the paralytic step of the toxin are different in their properties. The former was fast, largely independent of temperature, largely reversible by antibodies, and partially prevented by pretreatment with neuraminidase. The reverse was true with inhibition of transmitter release, which was delayed, not reversed by antibodies, not influenced by neuraminidase, and strongly dependent on temperature. Neuraminidase is known to degrade those gangliosides which fix the toxin (see p. 118). Nevertheless, neuraminidase was not able to prevent the inhibition by tetanus toxin of the transmitter release – again mirroring the inefficacy of the enzyme at the neuromuscular junction. We should not rush to conclusions and dismiss gangliosides as true receptors. It is possible that the discrepancy merely indicates an excess of binding sites, or that binding of the toxin to GM_1 molecules is sufficient for its action, either to the pre-existing ones or to those resulting from degradation by neuraminidase of long-chain gangliosides.

The number of toxin-sensitive transmitter systems has recently been extended. Not only inhibitory but also excitatory amino acids are subject to the toxin's effects. For instance, D-aspartic acid can be regarded as a stable substitute for glutamate, which is quickly metabolized. Its release is impeded by tetanus toxin (ALBUS and HABERMANN 1983). The toxin also partially inhibits the release

of neuromodulators, as exemplified by methionine-enkephalin-like activity (JANICKI and HABERMANN 1983). With every transmitter system the influence of time and temperature, the limited inhibition by delayed antibody treatment, and the inefficacy of neuraminidase were the same as described.

It is doubtful that tetanus toxin is specifically targeted against release processes. It also partially inhibits choline uptake, with the same dependence on time and temperature and the independence from neuraminidase treatment and antibodies as described above (HABERMANN et al. 1981). Inhibition of choline uptake might be regarded as secondary to inhibition of acetylcholine release. However, this link has often been discussed but never shown unambiguously. A direct membranal effect of the toxin cannot be dismissed. If it exists, tetanus toxin loses another mark of its presumed specificity.

5.2.1.2 Botulinum Toxins

Botulinum neurotoxins are known to act on peripheral cholinergic synapses of organs either isolated or in situ. They inhibit the transmitter release on the neuromuscular junction, on peripheral parasympathetic synapses, and in vegetative ganglia (for review, see SIMPSON 1981). Botulinum A toxin is about five times more potent than tetanus toxin on the electrically driven myenteric plexus-ileum strip of the guinea pig (BIGALKE and HABERMANN 1980). The course of paralysis and its sensitivity to antibodies as well as to agents which increase nerve-evoked transmitter release are quite similar to those with tetanus toxin poisoning. Previous claims of inhibition by botulinum A toxin of peripheral sympathetic nerve endings remained unconfirmed (see SIMPSON 1981). In fact, only cholinergic peripheral synapses have been generally accepted as targets for botulinum toxin. Neither cholinergic nor noncholinergic central synapses have been investigated thoroughly in situ.

In contrast, a broad range of release phenomena in minced brain and spinal cord have been established as sensitive to botulinum A toxin. Specificity was absent, as the toxin, like tetanus toxin, inhibited the release of glycine, GABA (BIGALKE et al. 1981 b), noradrenaline (BIGALKE et al. 1981 b; HABERMANN 1981 b), and met-enkephalin-like material (JANICKI and HABERMANN 1983), in addition to acetylcholine (WONNACOTT and MARCHBANKS 1976; BIGALKE et al. 1981 a, 1981 b; BIGALKE and HABERMANN 1981). However, its efficacy and potency on particulate brain was very much lower than that of tetanus toxin, at least with respect to glycine and GABA release and to inhibition of acetylcholine release. If one arranges the inhibition of acetylcholine release by tetanus and botulinum toxins according to the test systems, the following series ensues:

1. Neuromuscular junction (botulinum A toxin $\sim 500 \times$ tetanus toxin)
2. Guinea pig ileum (botulinum A toxin $\sim 5 \times$ tetanus toxin)
3. Particulate preparation (tetanus toxin $\sim 10 \times$ botulinum A toxin)

Thus, the discrimination index (1 vs 3) is about 5000. It is not yet established whether the in vitro effects of botulinum A toxin mirror its potency and efficacy

in vivo. In contrast to tetanus toxin, injections into brain tissue in situ did not lead to reactions typical of botulinum toxin. It is unknown whether the central cholinergic synapses in situ share the tremendous sensitivity of the peripheral synapses handling the same transmitter.

In two aspects the analogy between the toxins extends beyond release. First, GUNDERSEN and HOWARD (1978) poisoned brain slices with botulinum toxin, prepared synaptosomes, and found choline uptake diminished. They proposed (in contrast to WONNACOTT and MARCHBANKS 1976) that botulinum toxin altered the acetylcholine levels in a pool important for modulating both choline uptake and acetylcholine release. Tetanus toxin behaved similarly, and was about ten times more potent (HABERMANN et al. 1981). Second, relatively high concentrations of tetanus toxin promote the outflow of endogenous or radiolabeled noradrenaline from rat synaptosomes (KRYZHANOVSKY et al. 1980). The same was true with botulinum A neurotoxin (HABERMANN 1981 b).

In summary, high concentrations of tetanus toxin and botulinum A toxin appear to produce a relatively nonspecific membrane alteration (see Sect. 5.1), which manifests itself not only in the inhibition of release of many transmitters, but also in the promotion of the release of noradrenaline and in the inhibition of choline uptake. In addition, there must be biochemical guiderails which favor the interaction of low concentrations of tetanus and botulinum A toxin with specific transmitter systems in vivo or in isolated organs. In vivo, the preferential axonal transport of tetanus toxin might be one, but not the only factor that determines specificity. The conclusions are restricted to botulinum A toxin, as no other botulinum toxin has been assessed.

5.2.2 Cell Cultures

Slices, particulate preparations, and synaptosomes are only "better than nothing" for work with clostridial neurotoxins. They suffer damage during preparation. Their active life is only a few hours, whereas the toxins act for a long time. They die during poisoning. Tremendous toxin concentrations are required to shorten the incubation times to a tolerable length. It is doubtful whether the results can be extrapolated to the in vivo situation, where much smaller toxin concentrations are expected.

Work with cell cultures makes it possible to lower the toxin concentrations and to prolong the times of exposure. However, choice among the cells is restricted. Most tumor lines lack the ability to store and to release transmitters. For those that do not, the ganglioside pattern required for toxin binding is deficient. Binding and internalization studies on such cultures (see p. 129) should be supplemented with release data. On the other hand, primary cultures have been used extensively for binding, sequestration, and internalization of tetanus toxin (see Sect. 4.2.3). Our knowledge of the electrophysiology of the cultures and of their changes due to the toxins has advanced far during the past few years (see Sect. 5.3.1). Some groups have studied uptake and release of neurotransmitters in context with binding to and electrophysiology of neuronal cultures.

5.2.2.1 Tetanus Toxin

BIGALKE et al. (1978) exposed primary nerve cell cultures from embryonic rat to tetanus toxin. Pretreatment with the toxin diminished the de novo formation of acetyl [^{3}H] choline and partially suppressed the loss of acetyl [^{3}H] choline upon K^+ depolarization.

PEARCE et al. (1983) pretreated cerebellar cultures with tetanus toxin for 18–20 h before loading them with [^{3}H] GABA and putting them into a superfusion chamber. The toxin-treated cultures ceased to release [^{3}H] GABA upon receiving a pulse with high K^+. GABA uptake was not measurably influenced. Binding of toxin was seen during the first few days in vitro, whereas release by K^+ did not occur before the seventh day in culture. Not the basal, but only the K^+-evoked release was toxin sensitive. The data support the hypothesis of a multi-step process from binding to action and add a developmental aspect.

WENDON and GILL (1982) observed that the hybrid line NG 108-15, once preloaded with [^{3}H] choline, released [^{3}H]acetylcholine upon K^+ stimulation. Tetanus toxin abolished the K^+ effect. The cells had been grown in U-tubes and superfused. The assay might be useful for comparing tetanus and botulinum toxins. Beyond this confirmatory statement on transmitter release, they questioned whether exposure to toxin might alter the ADP-ribosylation or phosphorylation of cellular proteins. Use of a cell line was advantageous, as the glial cells in primary cultures might have blurred any effects on neurons. At the level of resolution, neither phosphorylation nor the ADP-ribosylation pattern was changed upon exposure to 5 μg toxin. Thus, tetanus toxin does not fit into the group to which cholera toxin or diphtheria toxin belong. However, it is worth to note that the toxin reacts with the hybrid line which is equipped with only minimal concentrations of toxin binding sites (DIMPFEL et al. 1977).

PC 12 cells, with or without pretreatment with nerve growth factor, have been tried repeatedly as targets for tetanus toxin. The cells used in our laboratory did not respond. However, FILIOGMENI and GRASSO (1985) reported that their NGF-stimulated, but not their naive PC 12 cells bound tetanus toxin saturably. They then promoted noradrenaline and dopamine release by veratridine and high K^+ and found it about 30% inhibited by micromolar (!) concentrations of tetanus toxin. Transmitter release by latrotoxin was not inhibited. Provided the concentration of tetanus toxin selected was not too high (no data given) and the work is reproducible elsewhere (we failed), this system could prove to be a valuable tool for the study of toxin binding, transmitter release, and the link between them.

5.2.2.2 Botulinum Toxins

On primary nerve cell cultures, BIGALKE et al. (1978) found botulinum A toxin more potent than tetanus toxin (see Sect. 5.2.2.1) in suppressing de novo formation of acetyl [^{3}H] choline and its release by K^+ depolarization. Isolated bovine adrenal medullary cells maintained in culture secrete catecholamine upon exposure to various secretagogues, like acetylcholine, veratridine, high K^+, Ba^{2+}

or the calcium ionophore A 23187. Previous exposure to botulinum D toxin, but not to botulinum A, B, E or tetanus toxin, nearly abolished the response independent of the stimulus. Inhibition proceeded for days and could be elicited by concentrations as low as about 100 MLD/ml (KNIGHT et al. 1985). The data are important in three points. (a) They show that at least botulinum D toxin inhibits not only cholinergic, but also adrenergic transmission, as expected from the studies with particulate brain (see p. 145). (b) The various botulinal toxins are functionally not equivalent. (c) After poisoning the plasma membrane of the cells were made leaky to Ca buffer using intense electrical fields. Unlike in non-poisoned controls, the Ca dependent release of catecholamine was still inhibited in toxin-treated cells. Therefore, not the Ca^{2+} entry but the response to intracellular Ca^{2+} is blocked.

5.3 Neurophysiological Studies

Two systems have been particularly valuable in elucidating the mode of action of clostridial neurotoxins: primary nerve cell cultures and the neuromuscular junction. Both models have served physiologists for many years and are well evaluated in many respects. Some technical aspects of their use in the context of clostridial neurotoxins must be considered. The experiments on slices so far performed with clostridial neurotoxins have been discussed on p. 144.

5.3.1 Primary Cultures

Primary neuronal cultures have some advantages for research on clostridial toxins. They mimic many basic features of neural tissue in vivo. Neurons in culture can be kept in vitro for as long as several months, allowing the long-term poisoning required. In contrast to intact animals or to slices, the culture system provides much easier access of know toxin concentrations to neuronal membranes and their synaptic structures without any axonal transport or diffusion through dense neuropil. Compared with cell lines, neurons in culture are much richer in long-chain gangliosides (DIMPFEL et al. 1977), which are putative receptors for the toxins. Their main disadvantage in neurochemical terms is their heterogeneity, which can be overcome only partially by preparative skills, for instance, isolated growth of cell populations from ventral or dorsal horns of the spinal cord. To ensure survival of neurons, fibroblasts and glial cells must be present. Deprivation of these cells has been attempted repeatedly, but always to the disadvantage of the neurons. In order to filter out true neuronal effects of the toxins, one may use histochemical techniques or study specific neuronal functions, for instance, uptake or release of transmitters. In terms of neurophysiology, cultured neurons are excitable and synaptically linked and yield primitive functional networks. Since individual cells are impaled, functional differences, e.g., between dorsal root ganglia cells and motoneurons, can be determined. Functioning neuronal networks are indispensable for work with clostridial neurotoxins, because they act, apparently exclusively, on synaptic functions. For

this reason, all attempts to study these agents on cell lines must be regarded with caution unless the results have been confirmed with primary neuronal cultures. For instance, an effect on the Ca^{2+} spike (see p. 140) could not be demonstrated on neurons in primary cell cultures (Bigalke, personal communication). Selective action of the toxins on synapses is in good agreement with the data collected in vivo. In summary, primary nerve cell cultures furnish information on how membranes, synapses, and neuronal networks respond to clostridial toxins. Experience with more differentiated cell cultures, for instance, the thin explants developed recently by GÄHWILER (1984), is still lacking.

5.3.1.1 Tetanus Toxin

The overall response of primary neuronal cultures mimics tetanus as a disease in many respects (BERGEY et al. 1983). Even after exposure to very high concentrations of toxin (10 μg/ml), there is an obligatory latent period of about 30 min before the onset of the convulsant action of tetanus toxin. Latency depends on the toxin concentration and can last about 500 min with 10 pg/ml, which is about 0.1 mouse LD/ml. At low concentrations the toxin action starts with a burst-like increase in excitatory activity, followed by organized paroxysmal depolarizing events (PDEs). They are characterized by sudden depolarizing shifts of the membrane potential, accompanied by a train of triggered action potentials often lasting for seconds before returning to resting membrane potential. With time, the events often occur at quite regular frequencies (10–40/min), while the spontaneous activity in the interburst periods disappears (BERGEY et al. 1983). In the final stage the toxin action results in progressive disappearance of the PDEs together with all other synaptic activity (BERGEY et al. 1981; DREYER et al. 1984). Thorough washing and addition of antitoxin fail to restore the spontaneous synaptic activity. The neurons remain in the silent state as long as the cells contain toxin. Only after several weeks, when internalized toxin molecules are eliminated, is the blockade of synaptic transmission reversed (HABIG et al. 1986).

Paroxysmal depolarizing events are not a peculiarity of tetanus toxin poisoning. Strychnine (10^{-4} mol/l and less), which inhibits glycine receptors, produces PDEs within a few minutes, and washing normalizes the pattern of spontaneous synaptic activity again (BERGEY et al. 1983). Other convulsants, such as bicuculline and picrotoxin, which antagonize the inhibitory postsynaptic action of GABA also produce PDEs rapidly (MACDONALD and BARKER 1978; HEYER et al. 1981). These findings show not only the differences between them and tetanus toxin in time course, but also suggest that blockade of inhibitory synapses is the common cause.

Tetanus toxin preferentially reduces the inhibitory postsynaptic potentials (IPSPs) in mouse spinal cord neurons in culture (BERGEY et al. 1986). Recordings from synaptically connected cell pairs revealed marked diminution in amplitude of evoked IPSPs coincident with the onset of PDEs at a time when monosynaptic excitatory postsynaptic potentials (EPSPs) were relatively unaffected. The preferential reduction of the IPSPs by the toxin apparently increases the effectiveness

of the EPSPs. This disinhibition would lead to an imbalance in the overall activity of the neuronal network, shifting the neurons toward a more excitable state, expressed by paroxysmal depolarizations.

The main difference between convulsants like strychnine and tetanus toxin is merely that the convulsants act postsynaptically and the toxin presynaptically. The presynaptic locus of action for tetanus toxin has been established in various experiments. First, neither the postsynaptic sensitivity of neurons to exogenously administered putative neurotransmitters like glutamate, GABA, and glycine (CURTIS and DEGROAT 1968; CURTIS et al. 1973; BERGEY et al. 1981) nor the electrical properties (membrane potential, input resistance, spike height, rate of rise of action potential, etc.) of the neurons were affected by the toxin. Second, quantal analysis of the late effect of tetanus toxin on the monosynaptic EPSP revealed a reduction in quantal content without reduction in quantal size (BERGEY et al. 1986). Third, tetanus toxin inhibited the release of various transmitters from synaptosomal preparations (BIGALKE et al. 1981 b).

It is not yet known which specific transmitter system in primary nerve cell cultures is preferentially blocked by tetanus toxin, but evidently, inhibitory connections are generally more sensitive than excitatory synapses, and the disequilibrium ultimately leads to the PDEs. Decrease in amplitude of evoked IPSPs parallels the onset of the convulsant action, and decrease of excitatory transmission is linked with the final silencing of the cells (BERGEY et al. 1983, 1986).

As with particulate preparations (see p. 145), neuraminidase treatment did not prevent the tetanus toxin effects, although it had converted the long-chain gangliosides into additional GM_1 (BIGALKE et al. 1986).

Why does tetanus toxin poisoning last so long both in vivo and in cell cultures? Two hypotheses may be offered, and one of them can be rejected. According to the rejected hypothesis, tetanus toxin is a "hit-and-run" drug immediately exerting a persisting effect before it disappears. The other possibility is that the toxin itself persists for the duration of poisoning. The latter hypothesis is substantiated by direct measurements of radiolabeled toxin, and by the characterization by disc gel electrophoresis of the toxin itself and its chains. This approach has already been tried successfully with rat and cat spinal cord in local tetanus (HABERMANN et al. 1977), where radiolabeled toxin persisted for the duration of the disease (HABERMANN 1972). On cell cultures, the half-life of intact tetanus toxin was about 5 days, and the half-lives of the heavy and light chains were within the same time range (HABIG et al. 1986). Obviously, the duration of tetanus is linked to the persistence of the intact toxin. There is no evidence that it requires processing in vivo in order to acquire biological activity, in contrast to numerous other high-molecular-weight toxins (see p. 126). As in every other system susceptible to tetanus toxin, antitoxin is inactive against poisoning in cultures once convulsant action becomes manifest. Apparently, the toxin has been "internalized", dependent on time, into a compartment wherein antitoxin no longer "sees" it. The underlying multi-step concept of poisoning will be dealt with in extenso in the context of the neuromuscular junction. Here it should be stated that the cell culture mirrors not only the pharmacodynamics but also the pharmacokinetics of tetanus toxin in the live animal, with the great advantage that both can be studied in the same subject.

5.3.1.2 Botulinum Toxins

Botulinum A neurotoxin also changed the pattern of spontaneous synaptic activity in neurons of primary cultures (BIGALKE et al. 1985). Whereas the so-called passive membrane parameters were not altered by botulinum or tetanus toxin, three major differences between their actions were found at the synapses. First, neuraminidase diminished the botulinum A toxin effects more than tenfold but those of tetanus toxin not at all (BIGALKE et al. 1986). Second, the PDEs typical for tetanus toxin action were induced in 50% of the cells only, and even here they were less regular in shape and frequency as compared with the responses to tetanus toxin. Synaptic activity could still be recorded during the interburst periods. Forty percent of the neurons did not show any prominent burst-like activity. In these cells the frequency of action potentials was progressively reduced, while IPSPs and EPSPs disappeared. The conclusion was that botulinum A toxin lacks selective action on inhibitory synapses (BIGALKE et al. 1985). Third, the sensitivity of inhibitory and excitatory transmitter release processes to botulinum A toxin was in the same dose range as that required for inhibition of EPSPs by tetanus toxin. The IPSPs were about 10000 times less sensitive to botulinum A than to tetanus toxin (BIGALKE et al. 1985).

Contrary to the botulinum A toxin effect on peripheral synapses, that on synapses in nerve cell cultures was not limited to cholinergic connections, because neither atropine nor *d*-tubocurarine mimicked the toxin. Toxin-treated neurons devoid of spontaneous and nerve-evoked synaptic potentials still responded to iontophoretic application of the putative neurotransmitters glutamate, glycine, and GABA, thus suggesting a presynaptic locus of action for botulinum toxin (BIGALKE et al. 1985).

The experiments mentioned show some similarities, but also differences between tetanus and botulinum toxin in quantitative and qualitative terms. Profound differences between botulinum toxins and between tetanus toxin and botulinum A toxin became apparent by analyzing the single quanta release at the neuromuscular junction (see p. 157). Strict comparisons are difficult to make with spinal cord neurons in culture for technical reasons, namely to record events produced by single quanta. Nothing is known about the effects of botulinum toxin types B–E in primary nerve cell cultures.

Why, then, are the very poisonous botulinum toxins relatively inactive at central synapses when injected into the living animal? There are three possible reasons. First, the ascent of radiolabeled toxin from the periphery to the spinal cord is less (see HABERMANN 1974) as compared with tetanus toxin. Second, the affinity of botulinum A toxin for purified gangliosides is less than that of tetanus toxin. It may be repeated that binding of botulinum A toxin can be measured in low-pH, low-ionic-strength buffer, but hardly in a physiological medium (HABERMANN et al. 1985; BIGALKE et al. 1986). Binding to solid-phase ganglioside columns (HABERMANN and TAYOT 1985) or synaptosomal columns (HABERMANN 1976) is quite feasible with tetanus toxin, but botulinum A toxin is much less affine. Tetanus toxin derivatives with low affinity hardly ascend to the brain (WELLER et al. 1986). Botulinum toxin appears to behave in this respect exactly like these pharmacologically inert derivatives. In summary, the inertia of botulinum A toxin in vivo (except on cholinergic synapses) may be

linked with deficit in ganglioside binding and the ensuing transport. This limit can be overcome in vitro simply by raising the toxin concentration. However, a third possibility may rest in the lesser sensitivity of central synapses.

5.3.2 Neuromuscular Junction

It has long been suggested that the symptoms of botulism are due to an impairment of the cholinergic transmission of motor and parasympathetic nerves. With the isolated rat phrenic nerve-hemidiaphragm preparation introduced by BÜLBRING (1946), BURGEN et al. (1949) demonstrated for the first time the presynaptic action of botulinum toxin on neuromuscular junctions.

Although the main targets of the action of tetanus toxin are the central inhibitory synapses whose inhibition results in spastic paralysis of skeletal muscles, clinical observations (for review see HABERMANN et al. 1980) and experiments with in vivo administration of tetanus toxin demonstrated that the toxin also affects the peripheral cholinergic transmission (AMBACHE et al. 1948a; KAESER and SANER 1969; DIAMOND and MELLANBY 1971; MELLANBY and THOMPSON 1972; DUCHEN and TONGE 1973; KRYZHANOVSKY 1973).

The experimental approaches towards botulinum and tetanus toxin bear different accents with respect to methodology. Biochemical work has concentrated on tetanus toxin rather than on the botulinum toxins (see this review). The situation is quite the opposite with respect to electrophysiological experiments. The action of botulinum toxin has been studied extensively at neuromuscular junctions, while the action of tetanus toxin has been examined at spinal cord neurons of intact animals, and only occasionally at neuromuscular junctions. Recently, the analysis of both neurotoxins at the synaptic level was advanced using in vitro poisoning of spinal cord neurons in culture by botulinum toxin (see Sect. 5.2.2.2) and of mammalian motor end-plates by tetanus toxin (HABERMANN et al. 1980).

Neuromuscular junctions serve as synaptic models to explore actions of drugs or toxins on neurotransmission because of their easy access for electrophysiological experiments and morphological studies. Various transmitter systems can be studied, such as the cholinergic system of skeletal neuromuscular junctions, cholinergic parasympathetic and noradrenergic sympathetic transmission to smooth muscle cells (e.g., mouse vas deferens, piloerector muscles of the cat's tail), and finally the inhibitory and excitatory neuromuscular junctions of crayfish muscle, with GABA and glutamate respectively as putative transmitters. Most results are obtained from motor end-plates of frog and mammals, in particular the isolated rat phrenic nerve-hemidiaphragm preparation (SIMPSON and colleagues). Other preparations often used are the m. extensor digitorum of rats (THESLEFF and colleagues), the m. sartorius of the frog (KATZ and MILEDI and colleagues), and the m. abductor superficialis of goldfish (MELLANBY and colleagues). To analyze the effects of botulinum toxin and tetanus toxin under identical experimental conditions, the phrenic nerve-hemidiaphragm preparation of the mouse has proved very suitable because of the easy access of the end-plate region for the high-molecular-weight toxins (HABERMANN et al. 1980; DREYER and SCHMITT 1981, 1983).

The data presented in the literature do not allow a plain comparison. Many experiments were performed after local toxin injection in vivo, with several drawbacks. For one thing, one may not know for certain the degree of neuromuscular block achieved. For another, the experiments are often performed days after the toxin injection. During this period changes in the properties of MEPPs and EPPs may occur independent of the primary action of the toxin, resulting instead from denervation-like processes at the pre- and/or postsynaptic side of the neuromuscular junction (see, for example, THESLEFF 1960; TONGE 1974; ANTONY and TONGE 1980; SELLIN and THESLEFF 1981; COLMEUS et al. 1982). It is difficult, if not impossible to separate the acute action of the toxins from long-term effects on neuromuscular transmission possibly induced by the drastic reduction of the rate of quantal release. Finally, the results from different authors are often difficult to compare because they were obtained (a) with different species and muscles which may have unequal sensitivity to the toxins; (b) at different temperatures which have a strong influence on the release probability; and (c) with different types of botulinum toxin which, as electrophysiological methods recently showed (see p. 157), affect the quantal transmitter release quite differently.

5.3.2.1 Three-Step Mechanism of Action

The steps from the administration of botulinum A toxin to the paralytic effect have been studied by SIMPSON (1974, 1980; see review 1981) on the isolated rat phrenic nerve-hemidiaphragm. He postulated a sequential three-step mechanism of action (SIMPSON 1980):

1. The binding of botulinum toxin molecules to specific receptors on the presynaptic membrane.
2. The translocation of the toxin molecules into or across this membrane.
3. The paralysis, leading to the blockade of transmitter release.

These steps have characteristic properties and can be separated by appropriate experimental conditions. The *binding* of toxin molecules to the presynaptic membrane is irreversible and only slightly dependent on temperature. Administration of specific antitoxin after the completion of the binding step does not prevent the paralytic step of botulinum toxin, but only delays the onset of paralysis. It is concluded that a sufficient number of molecules move within a short time from their binding sites into a deeper compartment. This was the reason for inserting a *translocation* step between the binding and the paralysis. At this stage it appears that the botulinum toxin molecules become embedded in the presynaptic membrane, such that they are no longer available for antitoxin binding, or undergo conformational changes, such that they lose their antigenic determinants. The translocation step does not depend on transmitter release but can be accelerated by nerve stimulation. In later publications Simpson prefers the term "internalization step". However, we still do not know whether the toxin molecules responsible for the blockade of acetylcholine release have to pass into the nerve terminal or only to be incorporated into the presynaptic membrane. The translocation step is followed by a *paralytic* step during

which the toxin molecules block transmitter release. The blockade has been characterized by a strong dependence on temperature, with a Q_{10} of about 4 and the requirement of transmitter release (SIMPSON 1980). However, in our own experiments the paralytic step of botulinum A toxin did not depend on nerve stimulation (DREYER and FUNK, in preparation).

A similar model of three sequential steps is considered for the action of tetanus toxin (SCHMITT et al. 1981). Differences between the two toxins rest mainly in the translocation step. With tetanus toxin this step depends absolutely on transmitter release. Apparently, the irreversibly bound tetanus toxin molecules can still be inactivated by antitoxin in the absence of quantal release, but not after the translocation step is completed by nerve-evoked quantal release. The third (paralytic) step for tetanus toxin is not influenced by nerve stimulation. It strongly depends on temperature with a Q_{10} value of more than 4. The temperature must be increased for a short time only (about 10 min at 37° C). Once this last step had started the development of blockade of transmitter release could no longer be stopped by reducing the temperature to below 20° C.

5.3.2.2 Electrophysiological Experiments

Electrophysiological recordings provide a direct method for studying the paralytic step of the neurotoxins, i.e., the blockade of quantal release of acetylcholine (ACh). Motor end-plates allow the examination of pre- and postsynaptic changes in the neuromuscular transmission process.

The release of ACh from motor nerve terminals can be divided into three types: (a) the nerve-evoked synchronous release of multiple quanta of ACh revealed by end-plate potentials (EPPs); (b) the spontaneous unsynchronized quantal release revealed by miniature end-plate potentials (MEPPs); (c) the spontaneous nonquantal release from intramuscular nerve fibers and terminals. The actions of the neurotoxins will be discussed in these terms.

It is well documented that the cholinergic transmission at neuromuscular junctions is impaired by botulinum toxin (for example: BROOKS 1956, in the guinea pig; THESLEFF 1960, in the cat; HARRIS and MILEDI 1971, BOROFF et al. 1974, and MOLGO and THESLEFF 1984, in the frog; SPITZER 1972, CULL-CANDY et al. 1976, LUNDH 1983, and THESLEFF et al. 1983, in the rat; TONGE 1974, KAO et al. 1976, KRIEBEL et al. 1976, and DREYER and SCHMITT 1981, in the mouse). Tetanus toxin also blocks the neuromuscular transmission (MELLANBY and THOMPSON 1972, in the goldfish; KRYZHANOVSKY 1973 and BEVAN and WENDON 1984, in the rat; DUCHEN and TONGE 1973, HABERMANN et al. 1980, and DREYER and SCHMITT 1981, 1983, in the mouse).

There is general agreement that upon treatment with tetanus toxin or botulinum A toxin at 37° C, mammalian motor end-plates are characterized by (a) a low rate of spontaneous MEPPs accompanied by a shift of the mean amplitude to lower values and (b) 100% failure to elicit any quantal release by single nerve stimuli (0.1 Hz). Neither neurotoxin alters the invasion of the terminal branches by the nerve action potential or impairs presynaptic Na^+, K^+, and Ca^{2+} currents (GUNDERSEN et al. 1982; DREYER et al. 1983). Neither the postsyn-

aptic ACh receptors, nor the enzyme acetylcholinesterase in the synaptic cleft, nor the synthesis and storage of ACh are affected (see GUNDERSEN 1980; SIMPSON 1981).

Nerve-Evoked Quantal Transmitter Release. The very low release probability of evoked quanta at end-plates treated with clostridial neurotoxin can be enhanced (a) by increasing the extracellular Ca concentration, (b) by increasing the frequency of nerve stimulation, (c) by reducing the temperature, and (d) by potassium channel blockers such as tetraethylammonium or 4-aminopyridine.

ad a) *The Influence of* Ca^{2+}. At normal end-plates the mean quantum content (m) of nerve-evoked EPPs does not vary linearly with the extracellular Ca^{2+} concentration, but instead increases with a power of about 3–4. This is interpreted as a cooperative action of calcium ions on the releasing process. The transmitter release at neurotoxin-poisoned end-plates also depends on Ca^{2+}, but the relationship between quantum content and external Ca concentration shows only a reduced power of about 1.3–1.5 for botulinum A toxin (CULL-CANDY et al. 1976; LUNDH 1983) and of about 1 for tetanus toxin (BEVAN and WENDON 1984). Much higher Ca levels are required to allow a certain release of quanta. Thus, the transmitter-releasing process is much less affected by the extracellular Ca concentration in toxin-treated terminals than in normal ones. Since the Ca influx into the nerve terminals is not impaired by the neurotoxins, it may be suggested that the efficacy of intracellular Ca concentration to evoke quantal release is decreased.

ad b) *Nerve Stimulation.* Differences in the quality of action of botulinum A toxin and tetanus toxin become obvious when the nerve is stimulated with higher frequencies. In muscles poisoned with *botulinum A toxin* in vitro the failure rate of quantal release in response to 50-Hz nerve stimulation is higher than 99% (DREYER and SCHMITT 1981, 1983); this contrasts with other studies (SPITZER 1972; CULL-CANDY et al. 1976), probably owing to differences in the completeness of toxin action. Further, it has been reported that repetitive nerve stimulation (20–100 Hz) increases the frequency of MEPPs, at least at some end-plates (BROOKS 1956; SPITZER 1972; CULL-CANDY et al. 1976). A similar finding by HARRIS and MILEDI (1971) may be due to the use of botulinum toxin type D. For toxin type A no increase in MEPP frequency was observed during repetitive nerve stimulation by TONGE (1974) and by DREYER and SCHMITT (1981, 1983).

In *tetanus toxin*-treated end-plates, however, 50-Hz nerve stimulation produces a remarkable increase in quantal release with a delay of about 2 s after the onset of nerve stimulation. The release of MEPPs does not stop immediately after the cessation of stimulation (DREYER and SCHMITT 1981, 1983; BEVAN and WENDON 1984). That tetanic nerve stimulation facilitates the frequency of quantal release has already been described (MELLANBY and THOMPSON 1972; DUCHEN and TONGE 1973; KRYZHANOVSKY 1973), but it was interpreted as an increase in spontaneous MEPPs. DREYER and SCHMITT (1981, 1983) favor the hypothesis that tetanus toxin disturbs at least one mechanism which synchro-

nizes the release of several quanta in response to the depolarization of the nerve terminal. The MEPPs may be confined to the phasic, but distorted secretion process for the following reasons: (a) The increase of MEPP frequency during tetanic stimulation strongly depends on Ca^{2+} concentration. (b) A burst-like release of two or more quanta occurs more often than one would expect in a purely random process. (c) The responses are not randomly distributed between the succeeding nerve stimuli as would be expected in spontaneous release. The latencies between the nerve impulses and the beginning of the responses are widely distributed over about 20 ms, however, with a strong peak around a value (about 3 ms) which is about twice the latency found in normal or botulinum A toxin-treated end-plates (DREYER and SCHMITT 1983; BEVAN and WENDON 1984).

Generally, the latency represents a synaptic delay due to Ca entry and the subsequent fusion process of vesicles with the presynaptic membrane at the "active zones". It has been suggested that phasic release is related to a chemical reaction associated with the binding of Ca^{2+} inside the nerve terminal (BARRETT and STEVENS 1972; DATYNER and GAGE 1980). Reducing the extracellular Ca concentration does not affect the time course of the latency histogram, but it lowers its magnitude. In contrast, tetanus toxin changes the latency distribution, suggesting that the toxin does not reduce Ca^{2+} entry into terminals.

It has been reported that tetanic nerve stimulation of tetanus toxin-poisoned end-plates sometimes restores the quantal release evoked with a synaptic delay, as in normal end-plates (DUCHEN and TONGE 1973; KRYZHANOVSKY 1973; BEVAN and WENDON 1984). However, a synchronous transmitter release was not observed by DREYER and SCHMITT (1981, 1983). Our negative findings may result from a more complete block of transmitter release due to the in vitro poisoning procedure.

One may take the asynchronous release of evoked quanta for the most obvious difference between the action of tetanus toxin and botulinum A toxin on transmitter release. Thus, it is surprising to learn that other types of botulinum toxins, such as type B (SELLIN et al. 1983b), type D (HARRIS and MILEDI 1971; our own unpublished observations on mouse motor end-plates), type E (SELLIN et al. 1983a), and type F (SELLIN et al. 1983c), act more like tetanus toxin than like botulinum A toxin in this respect.

ad c) *Temperature Dependence.* A striking result in botulinum A toxin-poisoned end-plates is the extremely high temperature sensitivity of quantal transmitter release. A Q_{10} value of about 40, independent of the frequency of nerve stimulation, can be estimated from the results with botulinum A toxin at the mouse hemidiaphragm (DREYER and SCHMITT 1983). LUNDH (1983) reported a similar increase in mean quantum content with a Q_{10} of about 45 (30° to 20° C) for the rat m. extensor digitorum longus, whereas the Q_{10} value of unpoisoned muscles blocked by high Mg^{2+} concentration was only 2. At the frog neuromuscular junction the quantum content increased with a Q_{10} of about 6 (14° to 4° C) (MOLGO and THESLEFF 1984).

In contrast to botulinum toxin-poisoned muscles, where the temperature change was already effective at a low rate of nerve stimulation (0.5 Hz), stimula-

tion of tetanus toxin-treated nerve endings at 1 Hz still failed to elicit any quanta over the temperature range of 37°–20° C. Only when the frequency of stimulation was raised above 5 Hz did the quantum content increase in response to temperature reduction. The Q_{10} value was much lower (about 3) (DREYER and SCHMITT 1983). For both tetanus toxin and botulinum A toxin the pronounced release of quanta at lower temperatures could readily be reversed by heating and induced again by cooling.

ad d) *Potassium Channel Blockers.* The mean quantum content can be increased not only by downward shifting of the temperature, but also by the application of potassium channel blockers such as tetraethylammonium (TEA) or 4-aminopyridine (4-AP). In botulinum toxin-poisoned end-plates, 0.2 m*M* TEA increased the mean quantum content of EPPs sixfold and 0.6 m*M* 23-fold (CULL-CANDY et al. 1976). 4-AP, which is known to be more potent than TEA, augmented the release of quanta 100- to 150-fold in the concentration range between 100 and 150 μ*M* (LUNDH et al. 1977; DREYER and SCHMITT 1981). Like the temperature shift, 4-AP was less effective at terminals treated with tetanus toxin. It increased the quantum content only by a factor of 6 (DREYER and SCHMITT 1981). The facilitatory effects of the potassium channel blockers TEA and 4-AP may be due to a prolongation of the nerve action potential, thus increasing the amount of calcium entering the nerve terminal. The same may hold for the temperature effect, taking into account the longer duration of presynaptic currents at 23° C as compared with 37° C (DREYER et al. 1983). As has been pointed out by DUDEL (1983), membrane depolarization of the terminal directly controls quantal release in addition to the intracellular Ca concentration. So the effectiveness of temperature reduction or of 4-AP may be due not only to an increase of Ca^{2+} influx but also to the prolongation of the presynaptic membrane depolarization itself. The effect of K^+ channel blockers is not restricted to poisoned end-plates; it can also be demonstrated in normal unpoisoned end-plates. For example, in the presence of 30 m*M* Mg^{2+} (m = 0.03), concentrations as low as 10 μ*M* of 4-AP increased the mean quantum content by a factor of about 180, while the same concentration augmented the EPP amplitude at *d*-tubocurarine end-plates only by a factor of 3 (LUNDH 1978). Apparently, the increase in quantum content also depends on the initial value of the quantum content itself. This may be the reason why in tetanus toxin-treated end-plates the augmentation of evoked quanta by temperature change or 4-AP is less than in botulinum toxin-treated end-plates, since the quantal content of evoked release is higher during tetanic nerve stimulation.

It has been reported that quantal transmitter release in botulinum toxin-poisoned end-plates can be restored by decreasing the temperature or, more strongly, by the application of potent K^+ channel blockers and that this is accompanied by a restoration of twitch responses of previously paralyzed muscles (LUNDH et al. 1977; LUNDH 1978). Using mouse hemidiaphragm poisoned in vitro with botulinum A toxin, we were not able to restore twitch tension by reducing the temperature to 20° C, nor at 37° C by the application of even 2.5 m*M* 4-AP. Only the combined use of 4-AP and temperature reduction was effective (unpublished observations). Again, the divergence of these observations

shows that in vitro (as compared with in vivo) poisoning blocks transmitter release more effectively. Certainly, the release probability can be significantly increased by the procedures and drugs mentioned; nevertheless, the mean quantum content remains considerably low (about $m = 5$) compared with the 150–200 quanta released per nerve impulse at normal end-plates. This indicates that the manifestation of poisoning by the neurotoxins is still present.

Spontaneous Quantal Transmitter Release. Much attention has been paid to the influence of *botulinum toxins* on the properties of spontaneous MEPPs. For this discussion it is necessary to know the features of MEPPs at normal end-plates. In a variety of studies on neuromuscular junctions it has been observed – and it is now generally accepted – that at least three different populations of MEPPs exist. The MEPP amplitudes of the most frequent population are distributed with a bell-shaped variation in the size of the amplitude (normal MEPPs). The second population (about 10% of the total) is composed of the so-called small-mode MEPPs (small MEPPs) with amplitudes one-third to one-quarter of the normal ones. The amplitude distribution is skewed and fuses with the normal MEPP distribution. The origin and significance of the small MEPPs have been discussed in detail (HARRIS and MILEDI 1971; KRIEBEL et al. 1976; for reviews GUNDERSEN 1980; TREMBLAY et al. 1983).

The third population consists of the "giant" MEPPs, characterized by a slower time course and an amplitude at least twice the value for normal MEPPs (COLMEUS et al. 1982). In normal end-plates the "giants" are very rare and, if they exist at all, comprise no more than 1% of the total. This population of MEPPs appears more frequently (a) in end-plates after several days of botulinum A toxin poisoning (SELLIN and THESLEFF 1981; KIM et al. 1984), (b) in regenerating neuromuscular junctions (COLMEUS et al. 1982), and (c) within minutes in response to the drug 4-aminoquinoline (MOLGO and THESLEFF 1982). These "giants" will not be discussed further because they are apparently not a direct effect of botulinum toxin but rather secondary to blockade of neuromuscular transmission.

There is general agreement that the MEPP frequency of end-plates completely blocked by botulinum A toxin decreases to about 1% or less of its original value. Simultaneously, the mean amplitude of MEPPs becomes smaller (SPITZER 1972; TONGE 1974; CULL-CANDY et al. 1976; KRIEBEL et al. 1976; DREYER and SCHMITT 1981). According to KRIEBEL et al. (1976), botulinum A toxin decreased the frequency of the normal MEPPs to zero, leaving the mean amplitude of this fraction of MEPPs unchanged. The small MEPPs were more resistant, albeit only partially, to botulinum toxin. Thus, the percentage of small MEPPs increases, and this leads to a reduction of the mean overall MEPP amplitude and to a skewed amplitude distribution.

The reports on spontaneous quantal transmitter release from *tetanus toxin*-poisoned end-plates are divergent. While the MEPP frequency was found unchanged in rat soleus neuromuscular junctions (WENDON 1980; BEVAN and WENDON 1984), MELLANBY and THOMPSON (1972) reported the complete absence of transmitter release in muscles of the goldfish. DUCHEN and TONGE (1973) found only a relatively slight effect of tetanus toxin on the MEPP frequency

in slow (soleus) and fast (extensor digitorum longus) skeletal muscle of the mouse. In contrast, KRYZHANOVSKY (1973), HABERMANN et al. (1980), and DREYER and SCHMITT (1981) registered a significant reduction in the frequency to about 10% of the normal value in mouse diaphragm. A similar disagreement was found for the mean amplitude of MEPPs. While BEVAN and WENDON (1984) observed an unchanged amplitude distribution, a change from a bell-shaped distribution to a skewed one was shown by DUCHEN and TONGE (1973) and DREYER and SCHMITT (1983). The most probable reason for these different findings is again the method of intoxication. For example, we observed a strong reduction of the MEPP frequency in soleus end-plates after in vitro poisoning (unpublished observations), contrary to results obtained by BEVAN and WENDON (1984). Apparently, in vitro poisoning of the toxin produces a more effective block of transmitter release.

Using an in vitro poisoning procedure, DREYER and SCHMITT (1983) registered the amplitude distribution of MEPPs from the same end-plate before and after paralysis by tetanus toxin. It was shown that tetanus toxin preferentially blocks the release of normal amplitude MEPPs without affecting the frequency of the small ones. In contrast, botulinum toxin strongly inhibits both the normal and the small MEPPs, thus leading to a frequency of MEPPs of less than 1% of the original value. This suggests that tetanus toxin preferentially inhibits one of at least two different vesicle populations or release mechanisms which generate small and normal MEPPs, while botulinum A toxin affects a step of the transmitter release process which is common for the generation of both modes of MEPPs. The leftward shift in the distribution of MEPP amplitude which can be observed in botulinum A toxin- *and* tetanus toxin-poisoned nerve terminals is not due to an increased appearance of small amplitude MEPPs, but rather to blocking of a population of MEPPs of relatively high amplitude.

The action of botulinum toxin type D on the MEPPs has been studied at the frog neuromuscular junction (HARRIS and MILEDI 1971). As with the more thoroughly investigated neurotoxins, MEPP frequency was lowered and their amplitudes were diminished. Repetitive nerve stimulation raised the frequency of MEPPs which had an amplitude distribution comparable to those of normal MEPPs. Similar observations have been made for botulinum A toxin (SPITZER 1972; BOROFF et al. 1974; CULL-CANDY et al. 1976). The same effect was observed in tetanus toxin-poisoned end-plates (DREYER and SCHMITT 1983); 50 Hz nerve stimulation increased the release of small MEPPs, and even more of normal MEPPs which previously had been blocked by tetanus toxin action. The amplitude distribution of these evoked quanta was similar to the histogram obtained from spontaneous MEPPs before toxin action at the same end-plate. This finding also explains why MELLANBY and THOMPSON (1972) and KRYZHANOVSKY (1973) reported an unchanged mean amplitude of MEPPs at tetanus toxin-treated end-plates; they measured the mean amplitude of MEPPs elicited during tetanic nerve stimulation.

The other types of botulinum toxin such as B, E, and F have not been examined in detail. A tetanus toxin-like action on the spontaneous MEPPs has been reported for the action of β-bungarotoxin (LLADOS et al. 1980).

Much attention has been paid to agents and procedures which normally cause a strong increase in the MEPP frequency. Their effects on neurotoxin-treated end-plates might help to improve our understanding of the mechanisms of toxin action.

First of all, changes in the extracellular Ca concentration which affect the MEPP frequency at normal end-plates failed to do so in botulinum A toxin-treated (CULL-CANDY et al. 1976) and tetanus toxin-treated ones (DREYER and SCHMITT 1983; BEVAN and WENDON 1984). Calcium ionophores A 23187 or X 537, which induce an influx of calcium into the nerve terminals and cause a massive quantal release with depletion of synaptic vesicles, failed to elicit any increase in MEPP frequency at terminals fully blocked by botulinum A toxin (CULL-CANDY et al. 1976; KAO et al. 1976). This finding, together with the results obtained for the Ca dependence of evoked release, supports the idea that, compared with normal terminals, a step requiring Ca^{2+} in the transmitter release process has a lowered sensitivity to intracellular Ca concentration due to the action of the neurotoxin. The same conclusion has been drawn for the mode of action of botulinum D toxin on bovine chromaffine cells (KNIGHT et al. 1985).

However, this hypothesis fails to explain the effect of black widow spider venom on botulinum toxin-poisoned nerve terminals. It is believed that α-latrotoxin, the major component of black widow spider venom, induces uptake of Ca^{2+} and other divalent cations across the presynaptic membrane by inducing a nonconventional cation channel of low ionic specificity. This toxin is the substance most potent in causing a massive release of transmitter from nerve terminals. Correspondingly, it depletes the terminals from synaptic vesicles and other intraterminal organelles. When black widow spider venom is added to end-plates completely blocked by botulinum A toxin, it still produces a tremendous release of quanta as in normal end-plates (CULL-CANDY et al. 1976; KAO et al. 1976; SIMPSON 1978; DREYER et al. 1984). A similar effect has been reported with brown widow spider venom (PUMPLIN and DEL CASTILLO 1975). Surprisingly, black widow spider venom was much less potent on tetanus toxin-poisoned end-plates and caused no alterations in the ultrastructure of the nerve terminals (DREYER et al. 1984).

High potassium solution is often used to simulate high-frequency nerve stimulation. HARRIS and MILEDI (1971) reported that K^+ depolarization of poisoned nerve terminals causes a small increase in MEPP frequency which seems to take many minutes to develop fully. These quanta belong to the MEPP population of normal amplitude. However, they used botulinum toxin type D, which is more tetanus toxin-like in its action. THESLEFF et al. (1983) observed that terminals poisoned with botulinum A toxin are completely insensitive to depolarization with 20–30 m*M* potassium. In the same rat skeletal muscle KIM et al. (1984) found a slight increase in MEPP frequency; however, they worked with a muscle preparation only partially paralyzed by botulinum A toxin.

In *tetanus toxin*-treated slow (soleus) and fast (extensor digitorum longus) skeletal muscles of the mouse, high potassium concentration failed to increase the frequency of MEPPs, although repetitive nerve stimulation was effective in this respect (DUCHEN and TONGE 1973). The authors did not speculate on why the end-plates responded so differently.

Raising the osmolarity of the extracellular solution enhances MEPP frequency in normal end-plates. The mechanism of the osmotic effect is not fully understood, but it is probably due to the mobilization of Ca^{2+} from intracellular stores such as the mitochondria. In contrast, in botulinum toxin-poisoned end-plates the already low rate of MEPPs further decreased by a factor of 3 (THESLEFF et al. 1983), while for tetanus toxin no change in MEPP frequency has been reported (BEVAN and WENDON 1984) in high-osmolarity solution. Again the latter result is contrary to own unpublished observations showing a further decrease in MEPP frequency by a factor of 3.

Lanthanum, which is known to increase the MEPP frequency in normal neuromuscular junctions tremendously, led to a reappearance of spontaneous MEPPs in goldfish muscles where tetanus toxin had entirely blocked neuromuscular transmission (MELLANBY and THOMPSON 1981). This finding has been confirmed by BEVAN and WENDON (1984) at rat soleus neuromuscular junctions. Exposure of tetanus toxin-treated terminals to 100 μM lanthanum caused about a sixfold increase in frequency, which is, however, much less than the 100-fold increase seen in control experiments.

Summarizing the results concerning the influence of drugs on MEPP frequency, one may state that end-plates poisoned with tetanus toxin and botulinum A toxin react in similar ways, with the exception of the resistance of tetanus toxin effects to black widow spider venom. However, other releasers have not been compared on tetanus toxin- and botulinum toxin-treated end-plates under identical experimental conditions with respect to toxin, species, muscle, and poisoning procedure. It is necessary to know in more detail the mechanism of action of the various releasers and/or of the neurotoxins. We are far from this ideal. At least the results with black widow spider venom support the hypothesis that tetanus toxin and botulinum A toxin act at different steps of the transmitter-releasing process. This assumption was further supported by double-poisoning experiments studying the effect of tetanus toxin on the nerve-evoked transmitter release of end-plates which were completely blocked by a preceding treatment with botulinum A toxin (DREYER et al. 1984). Tetanus toxin was able to change the well-synchronized responses during 50 Hz nerve stimulation of botulinum A toxin-poisoned end-plates into an asynchronous release of quanta typical for its action.

Spontaneous Nonquantal Release. Evidence has accumulated for a continuous nonquantal leakage of ACh from a cytoplasmic pool of the nerve terminals into the extracellular fluid. It is believed that under resting conditions quantal release of ACh constitutes no more than 5% of the total ACh release (for references, see DOLEZAL et al. 1983). Thus, most of the spontaneous output of ACh is due to a nonquantal component of release. Although this special release process is usually ignored, it can be demonstrated by electrophysiological techniques (KATZ and MILEDI 1977; VYSKOCIL and ILLES 1977). It is probably responsible for a small depolarization in the end-plate region, which can be recorded as a hyperpolarization after local application of *d*-tubocurarine to anticholinesterase-treated end-plates. Since different release mechanisms are presumably involved in quantal and nonquantal release, it is of interest to know

if the neurotoxins also affect nonquantal release. While such experiments are still lacking for tetanus toxin, some have been performed with botulinum A toxin.

Using biochemical methods, a reduced spontaneous release of ACh by about 50% has been observed. The decrease could not be explained quantitatively by the block of spontaneous quantal release (POLAK et al. 1981; GUNDERSEN and JENDEN 1983). However, using an electrophysiological technique, STANLEY and DRACHMAN (1983) reported that the nonquantal ACh release persisted in botulinum A toxin-poisoned end-plates whereas the quantal release was almost completely blocked. The situation is complicated by the report of DOLEZAL et al. (1983) on a decrease of the spontaneous release of ACh by botulinum A toxin. The authors used a combined biochemical and electrophysiological approach. However, they did not work with typical botulinum toxin-poisoned end-plates, since the frequency and the mean amplitude of MEPPs were not changed. Furthermore, the onset of toxin action occurred within 10 min and, more surprisingly, at 20° C, a temperature at which it should take days to complete the paralytic step of the toxin action. The authors suggested that botulinum A toxin acts by binding to some receptors related to the nonquantal release and localized on the outer surface of the presynaptic membrane. Provided the effect was specific (they used a huge concentration of 1 μg/ml), it would force the conclusion that botulinum A toxin has two different sites of action at the nerve terminal. One of them only might be related to the binding and/or translocation step. However, the interpretation of this putative inhibitory effect of botulinum A toxin on nonquantal release is difficult, because we know neither the molecular mechanism of nonquantal ACh release nor that of botulinum toxin action.

6 The Problem of Specificity

The fundamentally different pictures of poisoning suggest a high degree of specificity of tetanus toxin and botulinum toxins. On the other hand, this review has pointed out numerous similarities between them. So the different aspects must be dealt with individually for each toxin.

As for tetanus toxin, we have to follow the statement of MELLANBY and GREEN (1981) that, despite its high potency, it is not specifically directed against any particular transmitter. Given the appropriate conditions, the release of every transmitter investigated can be depressed, although this does not always occur in vivo, where, for instance, adrenergic transmission is resistant against the toxin (see AMBACHE et al. 1948a, b, for rabbit iris; own unpublished data on the cat nictitating membrane). Neither is any region of the mammalian central nervous system resistant to tetanus toxin, provided the toxin gains access to it. Since the blood-brain barrier is largely impermeable, systemically administered toxin will reach only those areas that are neuronally linked with the periphery. This is its natural pathway. However, when it is injected directly into the parenchyma of brain or spinal cord, foci of excitation arise that putative-

ly stem from a pronounced depression of inhibitory influences. (The compilation of the pertinent data is beyond the scope of this review. They have been listed by WELLHÖNER 1982.) Poisoned areas may then work as dispatch stations for general or local excitation (see the comprehensive work of KRYZHANOVSKY 1981). The synopsis is that (a) the substrate for tetanus toxin must be a universal constituent of neurons, (b) the reaction between toxin and substrate occurs whenever toxin and neurons gain sufficient contact, and (c) the manifestation of symptoms is less a matter of specificity of the toxin than of the specific function exerted by the cell investigated. Even the term "neuronotropy" should be used with caution, as the toxin is not exclusively bound to neurons. There are many extraneuronal binding sites (see p. 119) which share some properties with those on neurons. The difference is that these binding cells do not respond.

The situation is different with botulinum A toxin. Admittedly, many properties of tetanus toxin can be recognized, however modified. Binding to gangliosides is present, but affinity, at least to botulinum A toxin, is lower than to tetanus toxin. Ascent is less (see Sect. 4.3.2), and local botulism following injection into cerebral tissue has never been proven. On the other hand, botulinum A toxin affects many transmitter systems in vitro, including the release of noncholinergic transmitters in primary nerve cell cultures.

In contrast, botulinum toxin is at least 1000 times more potent than tetanus toxin on neuromuscular synapses in situ. At least at the neuromuscular junction, strong similarities exist between tetanus toxin and various types of botulinum neurotoxin such as B, D, E, and F with respect to the action on quantal transmitter release. Interestingly, although these types of toxin act in a tetanus toxin-like manner, they are again much more potent than tetanus toxin on motor nerve terminals. A future classification of botulinum toxins should cover (a) their comparison with tetanus toxin, together with (b) the assessment of their specific (i.e., presynaptically anticholinergic) effects, and (c) comparison between the various types of botulinum toxin.

7 Current Status and Outlook

Hardly any group of neurotoxins involves so many aspects of the neurosciences as those of clostridial origin. Nearly a century has passed since detection of the neurotoxic power of the culture fluids. Despite continuing efforts, none of the problems that fascinated the earlier researchers has been solved. A milestone in history was toxoiding, which opened the way to immunization. Progress in biochemistry led to the establishment of the molecular weight and the general structure of tetanus and botulinum toxins and their remarkable similarities. Without radiolabeled toxins, the axonal and trans-synaptic transport could not have been proven, although the former had been postulated as early as 1903 by MEYER and RANSOM. Similarly, radiolabeled toxins were indispensable for binding studies. They had been initiated as early as 1898 (MARIE; WASSERMANN and TAKAKI), long before ligand studies became a must in nearly every pharmacologist's oeuvre. In pharmacology on the living animal, SHERRINGTON's animal

Table 11. Shift to advanced techniques in research on clostridial neurotoxins

Topic	Historical approach	Recent approach
Toxin synthesis	Culture broth	Gene technology
Toxin structure	–	Biochemical techniques, gene technology
Antigenicity	Toxoiding, polyclonal antibodies	Monoclonal antibodies
Binding studies	Dissipation of toxicity	^{125}I-toxin
Transport studies	Distribution of toxicity	^{125}I-toxin
Neurophysiology	Nervous system in situ	Isolated system, such as the neuromuscular junction and nerve cell cultures

experiments (1905) on the loss of inhibition marks the start of the electrophysiological approach. Work in vivo extended into the 1970s but is slowly on the wane now in favor of isolated systems. The neuromuscular junction became more and more important as a tool for the in vitro study of not only botulinum but also tetanus toxins on a fully differentiated single synapse. The introduction of primary nerve cell cultures furnished insights into the interaction between the toxins and primitive neuronal networks, as well as into cellular pharmacokinetics. Finally, novel developments in the fields of monoclonal antibodies (not covered in this review) and gene technology have refined our view on the structure and biosynthesis of the toxins.

A look at Table 11 gives an impression of the methodological progress, but it tells nothing about increases in fundamental knowledge. Without denying that there have been advances in this respect, we are painfully aware of their limits. For instance, without knowledge on the three-dimensional structure we are still unable to describe the toxigenic and binding groups of the macromolecules, to localize the immunogenic determinants, and to understand the interactions and functions of the chains. Due to the size of the clostridial neurotoxins, sequences should be (and are being) established by the arrangement of nucleotides in DNA, probably those of plasmids or phages, and more effort should be devoted to the crystallization of the toxins. So far, none of the neurotoxins has been grown as crystals useful for X-ray analysis. The binding studies hitherto performed suffer not only from a lack of knowledge of the protein structure mentioned; we also do not know what is the structure of their receptor. Is it represented by a single ganglioside molecule or by a cluster of them? Are glycoproteins involved, in addition to the identified gangliosides? Which moiety of the ganglioside molecules (or of glycoproteins) contacts the toxin molecules? How is the binding translated into action? Internalization can no longer be doubted, but what is the morphological basis of entry, of intracellular and of trans-synaptic transport? And the most important questions: What happens at the membrane during translocation, and what happens within the cell after internalization? Mere entry is not sufficient to elicit toxicity, as has been shown with toxoided toxin; some other membranal and/or enzymatic processes must be involved. Intracellular injections of toxins are urgently required in order to circumvent the step of internalization. A lot of information is available about

peripheral and central effects of the neurotoxins. There has been much speculation about their mechanism of action, mainly concerning calcium. We act as if we know how calcium is handled in the nerve terminals, using terms like "Ca^{2+} influx," "intracellular Ca^{2+} level," "uptake," "storage," and "sequestration." However, at the moment we have to accept the fact that there is no real hint of any molecular mechanism of action of the neurotoxins at this level. Neurophysiology displays only the waves on the cell surface as quantal release, but hardly what happens in the depth of the cell.

The spin-off of the work with clostridial neurotoxins in other fields of science is considerable but might be still improved. So far, it has forwarded the basic knowledge of toxoiding. Tetanus toxin has been used as a tool to distinguish neurons from accompanying cells. Botulinum toxin is being used in ophthalmology. The concept of axonal transport rests largely, that of trans-synaptic transport nearly completely on studies with tetanus toxin. Perhaps exploitation of gene technology will provide novel insights into the coding and processing of information stored in large plasmids and phages and their exchange. Site-directed mutagenesis in systems admissible for experimentation might help in producing defined derivatives of the toxins.

Note Added in Proof

p. 121–122: Data for type B neurotoxin are similar to those for type A neurotoxin. K_D was 0.3–0.5 nM, $B_{max} = 30–60$ fmol/mg protein for the high affinity binding site, together with a much larger, heterogeneous population of low affinity binding sites. Like with type E toxin, trypsinization did not alter the affinity of the native type B neurotoxin, although it raised toxicity about 15 fold. – Specific binding of type B neurotoxin was nearly completely abolished by preincubation of synaptosomes with neuraminidase. Their further analysis, also by heat treatment and trypsinization, indicated two classes of binding sites. However, heat treatment may have disturbed the arrangement not only of proteins but also of lipids, and trypsinization may have destroyed the carrier function of synaptosomes (EVANS DM, WILLIAMS RS, SHONE CC, HAMBLETON P, MELLING J, DOLLY JO (1986). Botulinum neurotoxin type B. Its purification, radioiodination and interaction with rat-brain synaptosomal membranes. Europ J Biochem 154:409–416). Therefore the nature of the binding site(s) is still in dispute, like those for type A neurotoxin which appear to be closely correlated (WILLIAMS et al. 1983).

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Proteus mirabilis: Taxonomic Position, Peculiarities of Growth, Components of the Cell Envelope

K. KOTELKO

1 Introduction

Microorganisms of the *Proteus* group were first described and defined by HAUSER in 1885 (*P. vulgaris* and *P. mirabilis*) and redefined by WENNER and RETTGER in 1919 (according to WILSON and MILES 1964). In the following years other forms were isolated and named *P. morganii* and *P. rettgeri*.

This group of facultatively anaerobic gram-negative rods is widely distributed in nature and plays an important role in the decomposition of organic matter of animal origin. Representatives of the *Proteus* group frequently occur in the intestine of man and animals; besides their saprophytic life, *Proteus* bacilli give rise, under favorable conditions, to pathological events and are

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then considered facultative pathogens. *P. mirabilis* is the species most frequently encountered in clinical cases.

Proteus bacteria are typical members of the Enterobacteriaceae, resembling in many ways the coliform bacilli and other gram-negative rods, though differences do exist in some essential metabolic abilities.

Characteristic features of the *Proteus* group are the proteolytic activities under aerobic or facultatively anaerobic conditions, the capacity for oxidative deamination of amino acids, the production of volatile amines in a medium containing acid-hydrolyzed casein or in nutrient broth, and the ability to decompose urea to ammonia and carbon dioxide.

Like other enterobacterial rods, *Proteus* bacilli possess a cell envelope composed of an outer membrane and a peptidoglycan layer. Essential components of the outer membrane are lipopolysaccharides, proteins, and phospholipids. They also contain the enterobacterial common antigen (ECA).

The major groups of outer-membrane proteins identified in *Salmonella* and *Escherichia coli* were also found in *Proteus* bacteria. In contrast, capsular antigens were not detected in *P. vulgaris* and *P. mirabilis* strains. The facility with which L-forms can be induced in *Proteus* bacilli (mainly in *P. mirabilis*) is one of the characteristics unique to these microorganisms. The cyclic morphological changes resulting in the phenomenon of swarming are also unique properties of *P. vulgaris* and *P. mirabilis*.

The main purpose of this review is to summarize our knowledge of *Proteus* bacteria, particularly with respect to some special features of this group of microorganisms. *P. mirabilis* and *P. vulgaris* will form the basis of the discussion.

2 Classification of *Proteus*

In the last decade, the description of the genus *Proteus* was that of Bergey's Manual of Determinative Bacteriology (BUCHANAN and GIBBONS 1974) which included five species: *P. vulgaris* as the type species, *P. mirabilis, P. rettgeri, P. morganii,* and *P. inconstans*. It must be stressed, however, that this classification remained subject to discussion; many opponents advocated the reintroduction of *Morganella* and *Rettgerella* as separate genera in the family Enterobacteriaceae.

The present edition of the Manual (KRIEG and HOLT 1984) contains many changes concerning nomenclature and classification of Enterobacteriaceae. Several genera have been introduced with regard to the arbitrary DNA-relatedness groupings. This basis is still far from perfect, but may suggest the possible phylogenic divergence within the 14 main genera included in the family Enterobacteriaceae.

The values of guanine + cytosine (G + C) for all genera in this family range between 38 and 60 mol%. The base composition of *Proteus* DNA was established by Falkow as early as 1962 – 39 mol% for *P. vulgaris, P. mirabilis,* and *P. rettgeri* and 50 mol% for *P. morganii*.

Independently of the investigation of DNA-base composition and of the studies of DNA-relatedness within the *Proteus* group (BRENNER et al. 1978) some other cell constituents of microorganisms belonging to the Enterobacteriaceae were compared in the last decade.

The comparison of 16S rRNA partial sequences allowed Woese and his group (reviewed by STACKEBRANDT and WOESE 1979; FOX et al. 1980) to propose a common *Escherichia/Proteus* branch in the genealogical tree of Eubacteria. The comparison of other ribosomal components such as 5S rRNA or ribosomal proteins in Enterobacteriaceae (HORI 1976; HORI and OSAWA 1978) and the investigation of homology of the gene coding for outer membrane lipoprotein performed by NAKAMURA et al. (1979) confirmed the particular position of the genus *Proteus* in the family. There has been a proposal to classify the lipoproteins of Enterobacteriaceae into the following three groups according to the degree of homology with *E. coli* lipoprotein: *E. coli, Shigella, Salmonella* and *Citrobacter; Enterobacter, Klebsiella, Serratia* and *Erwinia; Proteus.*

Within the family Enterobacteriaceae immunological investigations on the homology of some enzymes in comparison with the corresponding *E. coli* enzymes have been performed. Generally, no or only incomplete cross-reactions of the enzymes were demonstrable with bacteria outside this family, but even within the Enterobacteriaceae cross-reactions were markedly weaker in *Erwinia* and *Proteus* compared with those in more closely related enterobacteria (COCKS and WILSON 1972; REINERS and WILSON 1975; REYES and ROCHA 1977).

Similarly, interrelationships within the genus *Proteus* were studied. Ureases, as the most characteristic *Proteus* enzymes, were investigated. Results of serological studies performed by GUO and LIU (1965), comparative analyses using polyacrylamide gel electrophoresis by SENIOR et al. (1980), and physicochemical investigations by ROSENSTEIN et al. (1981) led to some interesting conclusions. The pattern of urease isoenzymes were in good agreement with the relatedness within the genus *Proteus* based on DNA affinity studies as revealed by BRENNER et al. (1978). The ureases of *P. morganii* strains, indistinguishable from one another in their electrophoretic pattern, proved to be quite distinct from those of the remaining *Proteus* species, and this was also reflected in such properties as enzymatic activity (K_m for urea), activation energy (E_A), and molecular weight. Although each *Proteus* strain was characterized by a unique pattern of urease properties, those of *P. mirabilis* and *P. vulgaris* were very similar.

The most convincing data which justify the profound revision of the classification of the genus *Proteus* were derived from DNA-relatedness studies (BRENNER et al. 1978). Together with some additional information mentioned above, they constitute the basis for the new taxonomic proposals.

The current description of the genus *Proteus* is that of Bergey's Manual of Systematic Bacteriology (KRIEG and HOLT 1984). The genus *Proteus* comprises three species, namely *P. vulgaris* (type species), *P. mirabilis,* and *P. myxofaciens*. The last species is included because of its phenotypic similarity (thin film of growth on solid media) and to its relatedness by DNA/DNA hybridization to *P. vulgaris* and *P. mirabilis. Proteus rettgeri* was revealed to be more closely related to *Providencia alcalifaciens* and to *Providencia stuartii* than to

P. vulgaris and *P. mirabilis,* and is included in the genus *Providencia. Proteus morganii* is placed in the previously existing genus *Morganella.*

3 Swarming Growth

3.1 General

HAUSER (1885), who identified the genus *Proteus,* also discovered its unusual ability to swarm away from a central inoculum point on solid media. He related this phenomenon to striking morphological changes of the rods, and these properteis inspired him to name the discovered bacilli "*Proteus*". Proteus, the custodian of Poseidon's seals, sly and spiteful, was clever enough never to be discovered or caught, owing to his frequent changing of appearance. Today, 100 years after Hauser's findings, the phenomenon of swarming is still not fully understood.

3.2 Characteristics of Swarming

Two species, *P. mirabilis* and *P. vulgaris,* exhibit spontaneous swarming (SMITH 1972). In contrast to the short rods (0.6 by 1–2 μm) found in broth cultures of *Proteus,* the growing culture on a suitable solid medium, when centrally inoculated, presents quite a different picture under the microscope. At the central point of growth the slightly enlarged cells divide normally, forming a typical colony. After a period of time depending on the strain and culture conditions, many cells near the perimeter of the colony undergo elongation, attaining a size of 0.7 by 20–80 μm. In her pioneer work, Hoeniger studied cellular changes manifesting themselves during swarming of *P. mirabilis* strains (HOENIGER 1964, 1966). She demonstrated that the striking elongation of short rods was concomitant with a 50- to 500-fold increase in the number of flagella per swarm cell, depending on the size of individual swarmers. Calculated per cubic micrometer of bacterial volume, the number of flagella amounted to a 10-fold increase (HOENIGER 1965).

This was later confirmed by ARMITAGE et al. (1978), who found a 50-fold increase in the number of flagella per unit area. The long, highly flagellated swarmers represent multinucleated, nonseptated filaments. The scarce long forms are also seen in broth cultures, but their formation is attributed to a type of variant to be mentioned below.

On suitable solid media, swarming manifests itself in cyclic changes of developing forms or, quoting WILLIAMS and SCHWARZHOFF (1978), "in periodic cycles of movement and consolidation". Consolidation is the term used for the phase in which the swarmers divide, giving rise to short cells, which in turn divide normally for some time.

In the macroscopic picture, the movement of larger numbers of cells manifests itself as zones or haloes covering the surface of the medium. The extent

and duration of the phenomenon depends to a high degree on culture conditions. On a relatively rich, complex medium at 25°–37° C (WILLIAMS and SCHWARZHOFF 1978), swarming starts after 4 to 6 h, continues for 1 to 3 h, and terminates with the first swarm band formed at a distnce of 1 cm from the central colony. The consolidation period follows, in which the large filaments divide into short rods. These in turn, after a number of normal divisions, give rise to new swarmer cells, and a second swarm band emerges from the edge of the first band. Finally, the whole surface of the agar plate may be covered by series of alternating dense and transparent concentric rings.

Three types of morphological variants of *Proteus* have been described by BELYAVIN (1951); according to the type of swarming response, they were designated as A, B, and C. The A phase represents the *Proteus* form growing as concentric bands on a plate inoculated in the center, the B phase consists of nonswarming (nonmotile in fluid media) rods, whereas the C phase is the growth form that covers the whole surface of a plate with a uniform halo and consists of motile rods when cultured in broth. COETZE and SACKS (1960) described variants designated as Y, Z, X, and W. The Y type is characterized by swarming in the form of concentric bands at room temperature but not at 37° C, even when the plate is inoculated with motile rods from a broth culture incubated at 37° C. The Z form corresponds to the C phase of Belyavin. The X and W phases do not possess the ability to swarm but demonstrate some degree of motility in fluid media.

On the basis of population-pressure experiments, COETZE (1972) postulated that all the morphological phases described by him are mutually convertible, and he succeeded in transducing swarming cells into motile, nonswarming mutants. He established the swarming locus and identified three sites within it, but allelism could not be proved. The products of the swarm locus, however, are not yet known.

3.3 The Role of Extracellular Slime in Swarming

The first data obtained by scanning electron microscopy of swarming were derived from *P. mirabilis* by VANDER MOLLEN and WILLIAMS (1977). The authors demonstrated slime covering swarm cells, large masses of helical flagella embedded in slime, and rafts of swarmers with slime (visible on the agar surface). Recently, STAHL et al. (1983) adapted several techniques to show the extracellular slime of *P. mirabilis,* which surrounds the swarming cells in considerable quantities. The observations seemed to indicate a relationship between swarming and the production of slime that could serve as a matrix for the migration of cells. Based on the results of staining with ruthenium red and Alcian blue, the slime is thought to be an acidic polysaccharide with a high content of water.

Three groups of nonswarming mutants have been described according to their cell morphology, number of flagella, and slime production (STAHL et al. 1983). The first group consisted of strains resembling the wild form in swarm cell formation, the large number of flagella per cell, and slime production.

In the second group of mutants, the swarm cells lacked the large number of flagella. The third group represented mutants lacking both flagella and slime layer. Nonswarming mutants lacking only the extracellular slime layer could not be obtained. These results led to the conclusion that in the phenomenon of swarming several independent events play a role; swarm cell formation, synthesis of large numbers of flagella, and slime production are each essential for swarming, but in addition, other, unknown changes seem to be required.

3.4 Structural and Metabolic Characteristics of Swarmer Cells

ARMITAGE et al. (1979) investigated cell wall differences between swarming, filamentous and nonswarming, short cells. According to their results, swarm cells may contain a lipopolysacharide with longer O-antigenic side chains than that of nonswarming cells, which contained predominantly short side chains.

This suggestion, based on the results of chemical analysis of the lipopolysaccharide extracted from the limited quantities of swarmers harvested directly from the respective zones of growth on the surface of agar plates, has recently been confirmed by SIDORCZYK and ZYCH (1985). From two *P. vulgaris* strains investigated, one was a profusely flagellated swarmer and the other was a nonflagellated strain, not able to swarm. Chemical and electrophoretic analyses showed that the nonflagellated short cells synthesize mainly low-molecular-weight lipopolysaccharide, whereas the motile short rods, which can transform into the long swarmers, produce both kinds of lipopolysaccharide (low and high molecular weight).

Freeze-fracture studies (ARMITAGE 1982) revealed differences between short and elongated cells of *P. mirabilis*. The rigid outer membrane of short rods did not cleave during freeze-fracture, and the electron paramagnetic resonance spin label profile of the isolated and purified outer membrane resembled that of other gram-negative bacteria. In contrast, swarmers fractured along both the outer and the inner membranes, the former exhibiting higher fluidity than the outer membrane of short cells. Interpretation of the results is difficult but the observation is noteworthy.

Alterations in metabolic activities of swarmers and short rods may also be taken into account. According to ARMITAGE (1981), the rates of incorporation of precursors into DNA, RNA, and protein were lowered in broth cultures immediately after translocation of swarming cells from agar swarm plates. At the same time, the oxygen uptake was markedly reduced, whereas the intracellular concentration of ATP remained unchanged; ARMITAGE (1981) is inclined to regard swarmers as bacteria not growing in the fluid medium, with a metabolic activity only sufficient to assure the motility of flagella.

The successful separation of short and swarm cells due to the use of suitable new media and to strains swarming across the whole surface without reverting to the short cells, opened of new possibilities of identifying enzymes connected with each cell types (FALKINHAM and HOFFMAN 1984). It was demonstrated, in the time period in which swarmers did not undergo consolidation, that the two cell types could be distinguished by their abilities to synthesize certain

enzymes. Tryptophanase was inducible only in short cells. Urease, the most characteristic *Proteus* enzyme, was shown to be constitutive in swarm cells and uninducible in short rods. It must be stressed, however, that this enzyme may not be under strong repression in normal, short cells; the urease activity was, however, demonstrated in *P. mirabilis* cultures in nutrient broth supplemented with urea (SENIOR 1980, 1983).

The fact that phenylalanine deaminase could be induced in both cell types prompted FALKINHAM and HOFFMAN (1984) to conclude that transcriptional and translational mechanisms were functional in short and swarm cells. The changes were also shown in the level of some outer membrane proteins, as well as in a decreasing level of cytochrome b and repression of synthesis of cytochromes a and c, manifesting themselves during swarm cell formation. These last results permitted speculation on the changes in transcription resulting in swarm cell development (FALKINHAM and HOFFMAN 1984).

3.5 Concluding Remarks

The swarming phenomenon manifests itself only on the surface of a suitable solid medium, and this limits the possibility of harvesting sufficient quantities of homogeneous swarmers free of short rods. This is of great importance for all analytical work, especially in the evaluation of differences in the chemical composition of lipopolysaccharides obtained from short rods and from swarmers. Regarding metabolic invesigations performed with "pure" swarmers translocated into a fluid medium, it must be stressed that they can exist as swarmers only up to the moment of consolidation. The fluid medium represents for them an unfavourable environment; in the common broth culture of *Proteus* bacteria, few elongated forms are present. It is therefore reasonable to consider such experiments preliminary, and the results should be evaluated with caution.

The recently described separation of *Proteus* swarm cells and short rods in the form of nearly homologous suspensions opens enlarged perspectives in searching for the differences between these two cyclically occurring, distinct forms of *Proteus* bacilli.

It appears that the key to understanding the swarming phenomenon is inherent in the process of swarm cell formation, induced possibly by agents which on solid media promote the cyclic sequence of changes shown by the majority of *P. vulgaris* and *P. mirabilis* strains.

Another question concerns the mechanism of swarmer migration. In a series of investigations, Williams' group convincingly demonstrated that the movement of swarm cells away from the central colony is caused by neither negative nor positive chemotaxis (reviewed by WILLIAMS and SCHWARZHOFF 1978), but nothing further is known. The mechanism of consolidation of swarmers, i.e., the septation of swarm cells and their division to produce normal short rods, is also poorly understood. This will probably continue to be the case as long as the primary process – swarmer formation – is not elucidated.

Questions can be posed about the physiological role of swarming. Is swarming of *Proteus* only a visible response to a physiologicaly artificial environment

(laboratory solid media), or an example of prokaryotic cell differentiation? Does it play a role in the natural environments of this microorganism, such as soil or the intestines of humans and animals? The secondary niches such as the urinary tract or other tissues invaded by facultatively pathogenic *Proteus* strains are, of course, of great importance in this context. The question then arises whether swarming can be regarded as an alternative to chemotaxis in the evolutionary development of *Proteus*.

For discussions of *Proteus* envelope antigens the phenomenon of swarming growth must be considered.

4 Cell Envelope

4.1 General

The development of modern techniques (electron microscopy, freeze fracturing, scanning electron microscopy), together with a variety of other physical and biochemical methods, has resulted in a fast evolution of views on anatomy, topography, and functions of the gram-negative cell envelope. According to the commonly accepted nomenclature, this complex architecture consists of outer membrane, intermediate rigid layer (peptidoglycan), and cytoplasmic membrane (for reviews, see FREER and SALTON 1971; COSTERTON et al. 1974).

Methods to separate the outer membrane (OM) and the inner, cytoplasmic membrane (CM) have been developed. The outer membrane was found to be composed of protein, phospholipid, and lipopolysaccharide; it represents a complex and unique structure which plays a vital role in the life of the gram-negative cell (for details, see the comprehensive book edited by INOUYE 1979b). Through specific and nonspecific channels of the outer membrane, nutrients and ions are passively transported into the periplasmic space, from which they are actively transported across the plasma membrane. The outer membrane also protects the cell against the entering of chemicals, antibiotics, and poisons.

Specific receptors for phages and colicins are located in the outer membrane; the primary role of the proteins is often to serve as specific receptors and channels for the nutrients indispensable for growth. The role of the outer membrane in conjugation events has also been established.

For the description of membrane proteins we have adopted the designations used by INOUYE (1979a), who divides the major proteins into three classes: matrix proteins-porins, ompA protein, and lipoprotein.

Matrix proteins are strongly but noncovalently associated with the peptidoglycan. They are characterized by a high content of β-helix in contrast to other membrane proteins with mainly α-helix content. They cover about 60% of the peptidoglycan outer surface in the form of a hexagonal lattice. Matrix proteins are involved in the formation of pores, which serve passive diffusion.

The second group, ompA, likewise occurs in large quantity in the outer membrane. This protein is required in F-pilus-mediated conjugation, and it is suggested that it also has a part in pore formation.

The lipoprotein, so far the best-investigated and also the most abundant protein of the outer membrane, is usually covalently attached to the peptidoglycan, but a free form has also been found. One of the known functions of lipoprotein is to maintain the integrity of the outer membrane structure, and it may serve as a marker of relatedness between microorganisms (as revealed by NAKAMURA et al. 1979; see Section 2).

Some minor proteins cannot be precisely defined. Under certain growth conditions, however, some of them are produced in quantities comparable to the amounts of major proteins. Many minor proteins have been recognized as receptors for phages and colicins.

The lipopolysaccharide, a component characteristic of the outer membrane of the gram-negative cell, had been investigated long before the outer membrane was identified. Nearly 50 years ago, BOIVIN and MESROBEANU (1935) obtained from *Salmonella typhi* a complex extract composed of lipopolysaccharide, protein, and lipid. Two important activities had been recognized as attributed to the Boivin-type antigen: toxicity and serological specificity (for review, see LÜDERITZ et al. 1971). Among the gram-negative organisms, the lipopolysaccharides of the enteric rods have been investigated most intensively, and among the family Enterobacteriaceae, the genus *Salmonella* has become a model subject for the study for two reasons: its importance in human and animal infections, and the existence of the Kauffman-White scheme, which classifies the genus serologically, based on O and H specificities of each species. Lipopolysaccharides constitute the endotoxins and the O antigens of gram-negative bacteria. It was shown that lipopolysaccharides, including those of groups remote from Enterobacteriaceae and differing in many details of their fine structure, possess a common general architecture. They represent amphipathic molecules composed of a polysaccharide (O-specific chains and core oligosaccharide) and a covalently linked lipid, termed lipid A. Through lipid A the lipopolysaccharide is anchored noncovalently in the outer membrane.

The list of the biological effects of endotoxins is long, and there exists a huge bibliography concerning lipopolysaccharide structures, biosynthesis, genetics, and functions (for recent reviews on lipopolysaccharides and lipid A; see WESTPHAL 1975; WILKINSON 1977; GALANOS et al. 1977a; KABIR et al. 1978; OSBORN 1979; RIETSCHEL and LÜDERITZ 1980; LÜDERITZ et al. 1982; RIETSCHEL et al. 1982a, b; ANDERSON and UNGER 1983; RIETSCHEL 1984; HOMMA et al. 1984).

4.2 Lipopolysaccharide

First observations of the presence of specific polysaccharides in *Proteus* bacteria were published by MEISEL and MIKULASZEK (1933). BENDICH and CHARGAFF (1946) described two polysaccharides extracted from *Proteus* OX19, an organism known to be agglutinated by typhus exanthematicus convalescent sera (reviewed by STACEY and BARKER 1960). KOTELKO and IZDEBSKA (1964) and KOTELKO et al. (1964) isolated a degraded polysaccharide from *P. mirabilis* strain 1959 according to Freeman's procedure (extraction of dried bacteria with acetic acid)

and identified the following sugar components: glucose, galactose, glucosamine, galactosamine and heptose. The same constituents and, in addition, 2-keto-3-deoxyoctonic acid (KDO; dOclA, 3-deoxyoctulosonate) were also found by NESBITT and LENNARZ (1965) in the lipopolysaccharide of *P. mirabilis* strain P18. The definite composition of the lipopolysaccharide of *P. mirabilis,* established in 1965 (KOTELKO et al. 1965; BAGDIAN et al. 1966), includes, aside from lipid A, galacturonic and glucuronic acids, galactosamine, glucosamine, glucose, two different heptoses recognized as D-glycero-D-mannoheptose and L-glycero-D-mannoheptose, and KDO. Also L-lysine and ethanolamine were identified as constituents. Glucuronic acid had been found also in *Proteus* OX19 by DZULYNSKA and MIKULASZEK (1954) and in *P. mirabilis* P18 by BERST and CHEMINAT (1965).

A classification of lipopolysaccharides into chemotypes according to their sugar compositions was introduced for *Salmonella* by KAUFFMANN et al. (1960). Subsequently, more than 200 lipopolysaccharides derived from various genera of gram-negative bacilli have been classified in this way (reviewed by LÜDERITZ et al. 1971).

The serological classification of *P. mirabilis* and *P. vulgaris* has been elaborated by PERCH (1948) and KAUFFMANN (1966) and is known as the Kauffmann-Perch scheme (KAUFFMANN 1966). In these two *Proteus* species, 49 O antigens (32 in *P. mirabilis*) and 19 H (flagella) antigens have been identified. A number of new serotypes have been identified recently by LARSSON and OLLING (1977), and PENNER and HENNESSY (1980). A comprehensive review of *Proteus* serology was given by LARSSON (1984).

The existence of the Kauffmann-Perch scheme permitted a systematic study of the lipopolysaccharides of representative test strains of all O serotypes of *P. mirabilis* and *P. vulgaris*.

Lipopolysaccharides were obtained according to Westphal's procedure (WESTPHAL et al. 1952; WESTPHAL and JANN 1965) and analyzed for their sugar constituents. The results led to the classification of chemotypes as shown in Table 1.

The composition of the lipopolysaccharides of *P. mirabilis* and *P. vulgaris* is characterized by the occurrence of one or two uronic acids, namely, glucuronic and galacturonic acids. Ribitol phosphate was identified in the lipopolysaccharide of *P. mirabilis* for the first time by GMEINER (1977), who detected 1:4 anhydroribitol (the product of acid degradation). Lysine, which occurs in more than half of the lipopolysaccharides, represents another unusual component (KOTELKO et al. 1965; JONES 1973; RADZIEJEWSKA-LEBRECHT 1974; SIDORCZYK et al. 1975).

In about two-thirds of the *Proteus* chemotypes, L-glycero-D-mannoheptose coexists with a second heptose, D-glycero-D-mannoheptose (KOTELKO et al. 1965; BAGDIAN et al. 1966; KOTELKO et al. 1975).

According to their composition, *P. mirabilis* and *P. vulgaris* lipopolysaccharides can presently be divided into 16 chemotypes; 37 serotypes are clustered in two chemotypes. *Proteus mirabilis* strain 1959, whose lipopolysaccharide has been studied intensively, belongs to chemotype XIV. This strain and strain RMS-203 were not serotyped by KAUFFMANN and PERCH.

Table 1. Chemotypes of *Proteus mirabilis* and *P. vulgaris* lipopolysaccharides (KOTELKO et al. 1975; SIDORCZYK et al. 1975)

Chemo-type	Lipopolysaccharide constituents												O-Serotypes	
	Glucuronic acid	Galacturonic acid	L-D-heptose	D-D-heptose	KDO	Glucose	Glucosamine	Galactosamine	Galactose	Rhamnose	Ribitol	Lysine	*P. mirabilis*	*P. vulgaris*
I		+	+		+	+	+					+	32	–
II		+	+		+	+	+		+			+	28	–
III		+	+		+	+	+	+				+	23	–
IV		+	+		+	+	+	+	+			–	38	–
V		+	+		+	+	+	+	+		+	+	16	–
VI		+	+	+	+	+	+					+	–	39
VII		+	+	+	+	+	+		+			+	36	–
VIII		+	+	+	+	+	+	+				+	–	12, 23
IXa		+	+	+	+	+	+	+	+			+	11, 13, 14, 24 26, 31, 48	19, 31
IXb		+	+	+	+	+	+	+	+			–	5, 9, 10, 18, 19 35, 40, 41, 43	2, 21, 34, 42
X		+	+	+	+	+	+	+		+		+	49	–
XI		+	+	+	+	+	+	+	+		+	+	33	–
XII		+	+	+	+	+	+	+	+	+		–	–	25
XIII	+	+	+	+	+	+	+		+			–	–	46
XIV	+	+	+	+	+	+	+	+				+	27, 1959[a] RMS-203[a]	–
XVa	+	+	+	+	+	+	+	+	+			+	3, 6, 7, 29, 30	1, 4
XVb	+	+	+	+	+	+	+	+	+			–	17, 20	8, 15, 37, 44, 45, 47
XVI	+	+	+	+	+	+	+	+		+		+	–	22

L-D-heptose, L-glycero-D-mannoheptose; D-D-heptose, D-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctonic acid

[a] These strains have not been serotyped

4.2.1 O-Specific Polysaccharide

The structure of the immunodeterminant group of the lipopolysaccharide of *P. mirabilis* 1959 has been elucidated by GROMSKA and MAYER (1976). Among the split products obtained by mild acid hydrolysis and separated by paper chromatography and electrophoresis, an oligosaccharide was found containing equimolar amounts of D-galacturonic acid, D-galactosamine, and L-lysine. Its

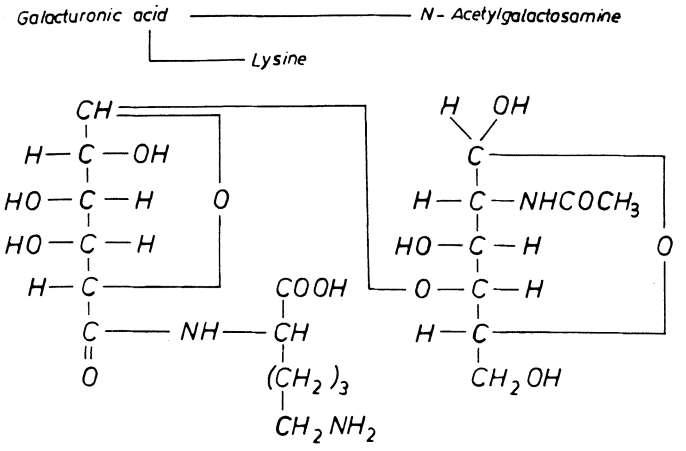

Fig. 1. Structure of the immunodeterminant oligosaccharide of *Proteus mirabilis* 1959 lipopolysaccharide (GROMSKA and MAYER 1976)

structure was established as D-galacturonyl-1,4-D-galactosamine with lysine attached to the carboxyl group of galacturonic acid by its α-amino group (Fig. 1). The high inhibitory activity of this oligosaccharide in the homologous system (lipopolysaccharide/O-antiserum) convincingly indicated its immunodeterminant role in the serological specificity of *P. mirabilis* 1959. It should be stressed that noncarbohydrate constituents such as amino acids are unusual components of O antigens and have not been detected as constituents of immunodeterminants in O-specific polysaccharides (for the immunodetermining role of lysine, see below).

The investigation of other oligosaccharides obtained from the lipopolysaccharide of *P. mirabilis* 1959 (dashed lines of Fig. 2; GROMSKA 1974) led to the structure of the repeating unit of this polysaccharide (Fig. 2).

The results were confirmed with the aid of a phage specific for the lipopolysaccharide of *P. mirabilis* 1959 (KACA and KOTELKO 1981, 1983). The phage "otto" had been studied by S. Stirm's group, who found it capable of degrading lipopolysaccharide (RIEGER-HUG et al. 1977). This phage was propagated on *P. mirabilis* 1959, purified and concentrated. In phage inactivation tests, the lipopolysaccharide was effective in small doses ($PhI_{50} = 0.03$ μg/ml), but high doses of the isolated O-specific polysaccharide, which was free from lipopolysaccharide contamination (D. Krajewska, unpublished data), also inhibited phage infectivity.

As a result of phage otto-mediated hydrolysis of the lipopolysaccharide of *P. mirabilis* 1959, three oligosaccharides could be isolated from the reaction mixture in a yield of about 90% based on the O-specific chains. The oligosaccharides (O1, O2, O3) were shown to contain the same sugars in the same molar ratios as the O-specific polysaccharide. Reduction of the O1, O2, and O3 oligosaccharides resulted in a decrease in the content of galactosamine by about one-sixth, one-fourth, and one-half respectively, indicating that galactosamine represents the reducing terminus.

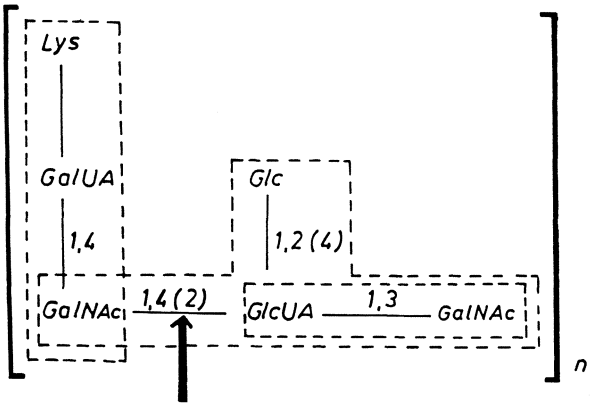

Fig. 2. Structure of the chemical repeating unit of the O-specific polysaccharide of *Proteus mirabilis* 1959 (GROMSKA 1974; GROMSKA and MAYER 1976). The *arrow* indicates the site of phage-mediated hydrolysis, the *dashed lines* mark the oligosaccharides that have been isolated. *Lys,* lysine; *Glc,* glucose; *GlcUA,* glucuronic acid; *GalUA,* galacturonic acid; *GalNAc*, N-acetylgalactosamine

The smallest oligosaccharide liberated by the phase enzyme represents a repeating unit; its calculated molecular weight (1156) was confirmed by filtration on a Bio-Gel P10 column with the aid of suitable reference compounds. On the same column the molecular weight of the O-specific polysaccharide was estimated as 17400; thus, the calculated (average) number of repeating units is about 15 (KACA and KOTELKO 1983). The site of phage-provoked hydrolysis was found to be the 1,4 (or 1,2) linkage between galactosamine and glucuronic acid (arrow in Fig. 2). It could be shown that the complete structure of the O-specific polysaccharide was indispensable for this cleavage.

Lysine was found to be present in 20 of 32 *P. mirabilis* serotypes (KOTELKO et al. 1975). For a study of the question whether lysine would act as an immunodominant structure, four lysine-containing lipopolysaccharides (*P. mirabilis* 1959, O3, O7, O27) in comparison with two lysine-lacking lipopolysaccharides (*P. mirabilis* O20, O43) were tested for serological cross-reactivity by the passive hemagglutination test. The results are summarized in Table 2.

These studies revealed two pairs of strongly cross-reacting lipopolysaccharides: 1959 and O7 on the one hand, and O27 and O3 on the other. Only weak (or no) cross-reactions occurred between these two groups. The two lysine-lacking lipopolysaccharides (O20 and O43) cross-reacted neither with each other nor with the other groups. It was found that approx. the same amount of lysine was needed to inhibit the precipitation of 1959 antiserum by polysaccharide 1959 as was needed to inhibit the O7 system. Furthermore, modification of the free ε-amino group of lysine residues by N-acetylation drastically decreased the serological activity of both lipopolysaccharides, 1959 and O7. In the case of the two other lysine-containing lipopolysaccharides, O27 and O3, a contribution of lysine to their serological specificity could not be unequivocally established (GROMSKA et al. 1978).

The structure of the lipopolysaccharide of *P. mirabilis* serotype O27, whose polysaccharide contains galacturonic acid, glucosamine, glucose, L-lysine, L-

Table 2. Serological cross-reactivity of *Proteus mirabilis* lipopolysaccharides containing or not containing lysine, as determined by passive hemagglutination tests (GROMSKA et al. 1978)

Lipopolysaccharide from *P. mirabilis*		Antisera against *P. mirabilis* Serotype					
Serotype	Chemotype	1959	O7	O27	O3	O20	O43
Reciprocal titer							
1959	XIV (Lysine$^+$)	10240	5120	–	–	–	–
O7	XVa (Lysine$^+$)	5120	5120	–	–	–	–
O27	XIV (Lysine$^+$)	80	320	10240	40	–	N.D.
O3	XVa (Lysine$^+$)	–	–	1280	5120	–	–
O20	XVb (Lysine$^-$)	–	–	–	–	10240	–
O43	IXb (Lysine$^-$)	–	–	–	–	–	640

Dash indicates that no agglutination was obtained with highest serum concentration

alanine, ethanolamine (and heptose), has been partially investigated (GROMSKA and KRAJEWSKA 1981). Four oligosaccharides obtained by mild-acid hydrolysis and purified by paper electrophoresis have been analyzed. An acid-labile constituent of these oligosaccharides has been recognized as N-acetylglucosamine; the strict correlation existing between its release and the decrease of serological activity indicated the dominant role of this sugar in the O specificity of strain O27. Both amino acids, lysine and alanine, are amide-linked. Lysine is probably linked to the carboxyl group of the hexuronic acid through its α-amino group. The lipopolysaccharides 1959 and O27, however, do not cross-react with each other.

GMEINER (1977) has investigated the lipopolysaccharide derived from *P. mirabilis* D52. The results of partial hydrolysis and periodate oxidation of the polymer before and after dephosphorylation with hydrofluoric acid allowed Gmeiner to propose the structure of the chemical repeating unit as Gal-1,3(or1,4)-GlcNAc-1,3-Glc-1,3-GlcNAc, with galactose substituted at C-3 by ribitol-5-phosphate, and glucose substituted at C-6 by ethanolamine phosphate (Fig. 3). The serological cross-reactions existing between the lipopolysaccharides of *P. mirabilis* D52, O16, and O33 are believed to be caused (at least partly) by ribitol phosphate. The degree of cross-reactivity was found to decrease in the order D52/O33 > D52/O16 > O33/O16. It should be mentioned that the lipopolysaccharides O16 and O33, which contain ribitol phosphate and lysine, do not exhibit cross-reactivity with the lipopolysaccharides O7 and O14, which contain lysine but no ribitol phosphate (GMEINER et al. 1977).

4.2.2 Core Oligosaccharide

Lipopolysaccharide-defective R mutants are most valuable for structural investigations of the core. In the case of *Proteus*, however, difficulties are encountered in the identification and isolation of such mutants: To obtain separate colonies,

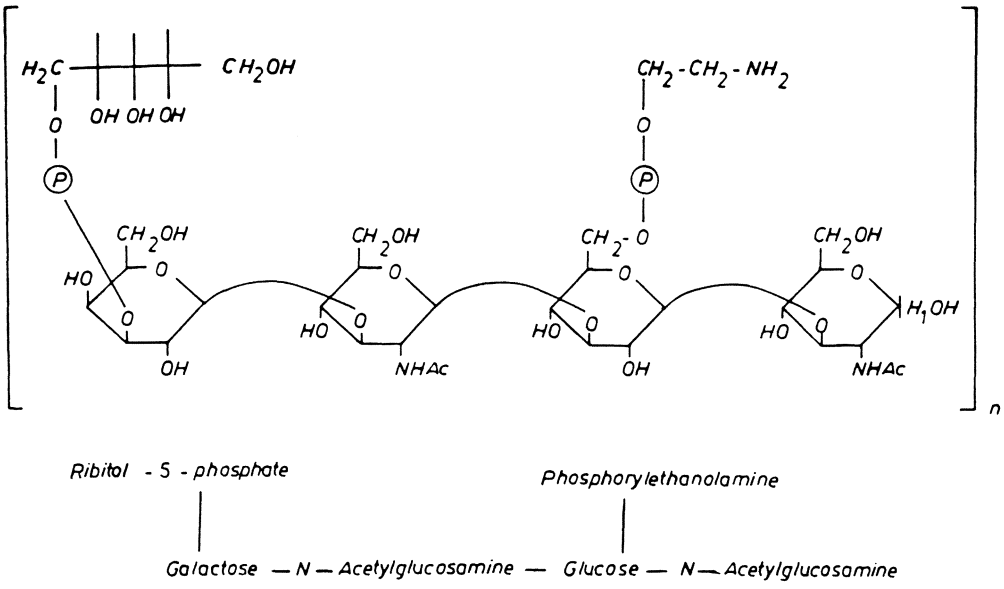

Fig. 3. Structure of the chemical repeating unit of the O-specific polysaccharide of *Proteus mirabilis* D 52 (GMEINER 1977)

one must suppress swarming by means of chemicals which disturb the integrity or the activity of flagella; ethyl alcohol, boric acid, phenol, or surface-active detergents in suitable concentrations have been used for this purpose. Another disadvantage is that the colonies of R mutants do not have a "rough" appearance but resemble the smooth colonies of the S-form wild strains. The colonial morphology cannot, therefore, be used in the selection of R mutants. Out of the tests proposed by SCHMIDT et al. (1969a, b) for the isolation of *Salmonella* R mutants, the most useful ones for a differentiation between S and R forms of *Proteus* were (a) the Brown test with crystal violet, (b) the gradient plate test with crystal violet, and (c) the thermostability test of suspensions (KOTELKO et al. 1974). The induction of mutants was performed either by means of UV irradiation or by action of methyl-p-toluenesulphonate.

The first R mutants were isolated from *P. mirabilis* 1959 by the aid of the phage Pm16 obtained from sewage, which provoked confluent lysis of the wild strain 1959. A phage-resistant, heptoseless mutant, R45, was isolated. Two other phage-resistant mutants contained galacturonic acid in their lipopolysaccharides: R13 represented a Ra mutant, and R14, a mutant lacking D-glycero-D-mannoheptose (KOTELKO et al. 1974).

From *P. mirabilis* RMS-203, whose lipopolysaccharide resembles that of *P. mirabilis* 1959 in chemical composition, ARAI et al. (1975) and NAKAHARA et al. (1975), using phages as a tool of selection, isolated heptoseless mutants. They tested suspensions of clones directly for the presence of heptose by the Dische-Osborn method. Table 3 lists the presently available *P. mirabilis* R mutants and the sugar composition of their lipopolysaccharides.

Table 3. Sugar composition of lipopolysaccharides derived from R mutants of *Proteus mirabilis* 1959 S, O28 S, and RMS-203

Lipopolysaccharides and oligosaccharides from	Sugar constituents								
	GlcUA	GalN	GalUA	GlcN	Glc	Gal	Heptoses		KDO
							DD	LD	
P. mirabilis 1959 S[a]	+	+	+	+	+		+	+	+
Core oligosaccharide[b]			1	1	1		1	2	+
R110 (Ra)			1	1	1		1	2	+
R13 (Ra)			1	1	1		1	2	+
R14			1	1	1			2	+
R51					1			2	+
R45 (Re)									+
P. mirabilis O28 S[c]		Gal	+	+	+	+		+	+
Core oligosaccharide[b]			1	1	1			2	+
R4					1			2	+
P. mirabilis RMS-203[d]	+	+	+	+	+	+		+[e]	+
N-434 (Re)									+

GlcUA, glucuronic acid; GalN, galactosamine; GalUA, galacturonic acid; GlcN, glucosamine; Glc, glucose; Gal, galactose; DD-Heptose, D-glycero-D-mannoheptose; LD-Heptose, L-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyotonic acid; 1 or 2-molar ratios

[a] KOTEŁKO et al. 1974, 1977
[b] Fraction II derived from S-form lipopolysaccharide; see text
[c] RADZIEJEWSKA-LEBRECHT et al. 1980; RADZIEJEWSKA-LEBRECHT 1983
[d] NAKAHARA et al. 1975; ARAI et al. 1975
[e] Configuration of heptose was not determined

It is noteworthy that the *P. mirabilis* 1959 R mutants are resistant to the action of the following phages, which have been used in the classification of *Salmonella* R mutants: Felix O1, 6SR, and the R phages: Br2, Br10, Ffm1, Ffm3 and P22 (SCHMIDT and LÜDERITZ 1969).

Chemical analysis of the oligosaccharide derived from the lipopolysaccharide of *P. mirabilis* 1959 mutant R110 showed identity in composition and molar ratios of the constituents with an oligosaccharide, designated fraction II (KOTELKO et al. 1977). This fraction was obtained on Sephadex G-50 filtration of the degraded polysaccharide isolated from the lipopolysaccharide of *P. mirabilis* 1959 (S form) and is considered to represent the complete Ra core oligosaccharide. Therefore, mutant R110 was classified as a Ra type.

The structure of the complete *Proteus* R110 core has not yet been determined. It differs, however, from other enterobacterial cores by the absence of galactose and the presence of galacturonic acid, which probably occupies a terminal position in the core (KOTELKO et al. 1983); this is concluded from results of methylation analysis performed on the lipopolysaccharide after carboxyl reduction, i.e., conversion of galacturonic acid into galactose.

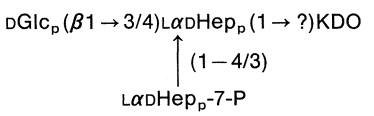

Fig. 4. Structure of the glucose-heptose region of the core of *Proteus mirabilis* O28 (analytical results with mutant R4; RADZIEJEWSKA-LEBRECHT et al. 1980)

In contrast to the lipopolysaccharide of *P. mirabilis* 1959, that of *P. mirabilis* O28 (S form) contains only one kind of heptose (Tables 1, 3). The mutant lipopolysaccharide R4 (from *P. mirabilis* O28) yielded an oligosaccharide with glucose, L-glycero-D-mannoheptose, KDO, and phosphate (RADZIEJEWSKA-LEBRECHT 1974). The combined data obtained by methylation analysis and nuclear magnetic resonance (nmr) studies allowed the proposal of the structure of the glucose-heptose region as shown in Fig. 4 (RADZIEJEWSKA-LEBRECHT et al. 1980). That this is not the complete core of *P. mirabilis* O28 and that the mutant R4 represents a core-defective mutant became obvious when the degraded polysaccharide of the lipopolysaccharide of the wild-type parent strain was separated on Sephadex G-50. The oligosaccharide (fraction II) isolated in this way represents the complete core and contains galacturonic acid and glucosamine, in addition to glucose, L-glycero-D-mannoheptose, KDO, ethanolamine, and phosphate (RADZIEJEWSKA-LEBRECHT 1983). This oligosaccharide was analyzed by a procedure analogous to that used with mutant R110 and was also found to be terminated by galacturonic acid, which, according to preliminary results, is linked to the side chain L-glycero-D-manno-heptose (Radziejewska-Lebrecht, unpublished data). A terminal position for galacturonic acid has also been revealed in a third *Proteus* core oligosaccharide, viz. that of *Proteus* O27 (Krajewska and Radziejewska-Lebrecht, unpublished data). The structure of this core includes N-acetylgalactosamine instead of N-acetylglucosamine.

In summary, the core oligosaccharides derived from members of two distinct chemotypes (representing three different serogroups), II and XIV are characterized by the presence of the same terminal acidic sugar constituent, galacturonic acid. In the lipopolysaccharide of serotype O28 (II), a single type of heptose, L-glycero-D-mannoheptose, is present, whereas in those of O27 and 1959 (XIV) an additional type, D-glycero-D-mannoheptose, exists. It is noteworthy that galacturonic acid represents a constituent of both the O-specific chains and the cores of all the *P. mirabilis* lipopolysaccharides so far investigated (GROMSKA 1974; GROMSKA and MAYER 1976; GROMSKA and KRAJEWSKA 1981; KOTELKO et al. 1983; RADZIEJEWSKA-LEBRECHT 1983).

4.2.3 Lipid A

The structure of lipid A has been studied with the Re mutant R45, derived from *P. mirabilis* 1959 by applying methods that have led to the lipid A structure of other Enterobacteriaceae. The proposed structure of *Proteus* lipid A is shown in Fig. 5 (SIDORCZYK et al. 1983b).

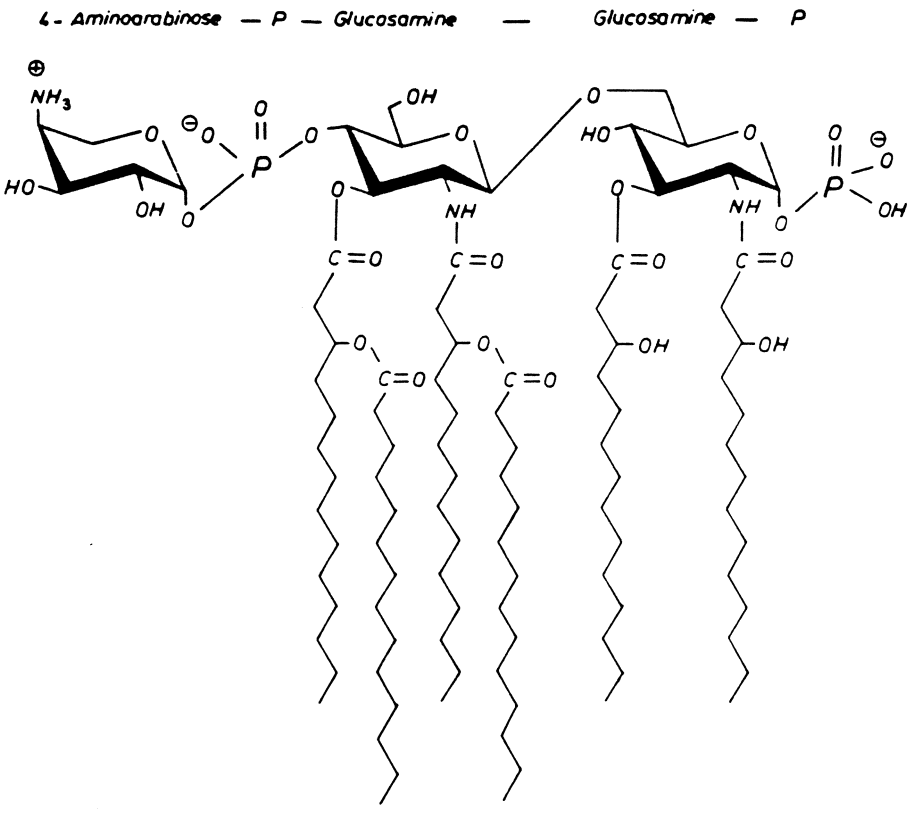

Fig. 5. Structure of *Proteus mirabilis* lipid A (analytical results with mutant R45; SIDORCZYK et al. 1983). The fatty acids are tetradecanoic and 3-hydroxytetradecanoic acid. Hexadecanoic acid partially substitutes the 3-hydroxytetradecanoic acid linked to position 2 of the reducing glucosamine (not shown)

The backbone of the lipid A consists of β-1,6-linked D-glucosamine disaccharide carrying two phosphate groups, one ester-bound to position 4′ of the non-reducing, and one linked to C-1 of the reducing, glucosaminyl residue. The same type of backbone structure has previously been revealed for other enteric bacteria as well as for various other groups of gram-negative microorganisms (for summaries, see RIETSCHEL et al. 1977; LÜDERITZ et al. 1978; RIETSCHEL et al. 1983).

In *P. mirabilis* the ester-bound phosphate is quantitatively substituted by a nonacylated 4-amino-L-arabinosyl residue in glycosidic linkage. In contrast to the lipid A structures of other enterobacteria (GALANOS et al. 1977a), the glycosidically bound phosphate group at C-1 appears not to be substituted.

In the *P. mirabilis* Re lipopolysaccharide, KDO (dOclA) is linked to the hydroxyl group at position 6′ of the nonreducing glucosaminyl residue. This hydroxyl group, therefore, carries the saccharide component in the complete lipopolysaccharide and is unsubstituted in free lipid A (SIDORCZYK et al. 1984). This situation at position 6′ seems to be a general principle in enterobacteria.

The backbone is substituted by long-chain fatty acids. As in *Salmonella* and *Escherichia coli,* only D-3-hydroxytetradecanoyl residues (4 mol/2 mol glucosamine) are attached directly to the backbone: 2 moles to the amino groups of the glucosamine units and 2 moles to hydroxyl groups, probably at positions 3 and 3′. Two moles tetradecanoic acid are ester-linked to the hydroxyl groups of the two 3-hydroxytetradecanoyl residues linked to the nonreducing glucosaminyl residue. Lipid A preparations always contain small amounts of hexadecanoic acid, which partially substitute the hydroxytetradecanoic acid at position 2 of the reducing glucosamine.

The calculated molecular weight of the structure presented in Fig. 5 is 1960.9. The molecule lacking 4-aminoarabinose and both phosphate groups would have a molecular weight of 1666.8. When free dephosphorylated lipid A was investigated in laser-desorption mass analysis, an abundant quasi-molecular ion, $(M+K)^+$, was detected at $m/z = 1703 \pm 3$ (LINDNER and SEYDEL, in preparation, cited according to SIDORCZYK et al. 1983). Correction of this value for K^+ ($=39$) yields 1664 ± 3, which correlates well with the above-calculated molecular weight of dephosphorylated lipid A. In the high mass range, a small peak $(M+K)^+$ occurred at $m/z = 1942 \pm 3$. This ion is interpreted as corresponding to *Proteus* lipid A molecules containing an additional hexadecanoyl residue (calculated molecular weight, 1905.2, plus $K^+ = 1944.2$). These findings support the structure of *P. mirabilis* lipid A as proposed in Fig. 5.

A question which remains to be elucidated concerns the nature and localization of an unknown component present in *Proteus* R45 lipopolysaccharide. This component is released by mild-acid hydrolysis, and in the amino acid analyzer it migrates similarly to aspartic acid. Preliminary investigations seem to indicate that this constituent is attached to a KDO unit rather than to lipid A (Sidorczyk, unpublished data).

Structural similarities of lipid A's of different origin are reflected in their serological cross-reactions (GALANOS et al. 1971a, b; 1977b). As expected, lipid A from *P. mirabilis* acts as a strong inhibitor of the homologous *Salmonella* lipid A/anti-lipid A hemolysis system. When coated to erythrocytes, it reacts strongly with anti-*Salmonella* lipid A antiserum in the direct passive hemolysis test. The reciprocal cross-reaction of *Salmonella* lipid A with anti-*Proteus* lipid A antiserum has also been demonstrated, as well as cross-reactions of *Shigella flexneri* and *S. sonnei* lipid A in the *Proteus* lipid A system. These results point to the great similarity of the immunodeterminant structures in these lipid A's (SIDORCZYK et al. 1978).

4.2.4 Heterogeneity of Lipopolysaccharides

It is generally accepted that lipopolysaccharides exhibit great microheterogeneity. This is true of all three regions, the O chains, the core, and lipid A. Main reasons for heterogeneity are incomplete substitution by small, often polar substituents, by side-chain sugars, by fatty acids (in lipid A), and others. Further reasons for microheterogeneity lie in the length of O-specific chains, which may vary in a lipopolysaccharide preparation from 0 to 40 repeating units

linked to the core. Therefore, unsubstituted core stubs (they represent the origin of core oligosaccharide fraction II; see Sect. 4.2.2) may be present in the mixture, sometimes amounting to as much as 30% (RYAN and CONRAD 1974; JANN et al. 1975; MORRISON and LEIVE 1975; PALVA and MÄKELÄ 1980; GOLDMAN and LEIVE 1980; KULAKOVSKA and ROMANOWSKA 1981; for review, see LÜDERITZ et al. 1982).

Since all the different molecular species of a lipopolysaccharide preparation form mixed micelles, they cannot be separated by the usual physical methods. It was, therefore, most surprising when GMEINER (1975) found that on phenol-water extraction of *P. mirabilis* D 52, two lipopolysaccharide fractions were obtained which differed in composition. Fraction I (LPS I) formed a pellet on ultracentrifugation, thus behaving like ordinary enterobacterial phenol-water-extracted lipopolysaccharide. However, this *Proteus* lipopolysaccharide fraction was highly enriched in lipid A and core sugars. Lipopolysaccharide fraction II (LPS II), obtained from the supernatant of ultracentrifugation, was rich in O-chain sugars. Similar results were obtained later with *P. mirabilis* strain O 27 (KRAJEWSKA and GROMSKA 1981). The yield of degraded polysaccharide and lipid A obtained from the two lipopolysaccharide preparations was typical: LPS I (pellet after ultracentrifugation) contained 35.5% polysaccharide and 56.0% lipid A (sum, 91.6%), whereas in LPS II (supernatant) 57.6% polysaccharide and 27.4% lipid A (sum, 85.0%) were detected. Both LPS I and LPS II could be separated by sodium dodecyl sulphate (SDS)-polyacrylamide-gel electrophoresis into a slowly, and a fast-migrating fraction. In crossed immunoelectrophoresis the slowly migrating fraction of LPS II gave a strong band of precipitation with homologous *Proteus* anti-O serum, whereas in the respective fraction of LPS I a much lower content of O-specific material was revealed. The fast-migrating fractions of LPS I and LPS II did not react with the O antiserum, indicating that they represent unsubstituted core-lipid A.

These observations seem to fit in with results which had been obtained much earlier (KOTELKO et al. 1965; 1968a, b; 1969). In general, when bacteria are extracted according to Freeman with acetic acid, polysaccharides are obtained which (owing to the cleavage of the KDO-lipid A linkage) represent mixtures of O-chain-substituted and unsubstituted core products. In the case of *P. mirabilis* 1959, however, it had been found that the first acetic acid extract (I) yielded a polysaccharide differing from the product of a second acetic acid extract (II). It could be shown by chemical and serological analyses that fraction I contained O-chain core polysaccharide, while fraction II resembled unsubstituted core (KOTELKO et al. 1974).

Although such fractions (LPS I and LPS II, Freeman fractions I and II) also occur in other enterobacteria, they have never been found separated as in the case of *Proteus*. The reason for this special behavior is not known, but *P. mirabilis* contains an unusual accumulation of charged constituents in the polysaccharide part of the lipopolysaccharide. This may lead to increased solubility in water and a decreased aggregation of LPS II, thus allowing the simple separation of the fractions by ultracentrifugation.

In this context, the recent observation of GOLDMAN et al. (1982) must be mentioned. These authors have identified two fractions extracted from *E. coli*

O111 as representing a true lipopolysaccharide containing an average of 12 repeating units and a (capsular-like) polysaccharide containing an average of 400 repeating units. It is possible that a similar situation exists in *P. mirabilis*.

4.3 Enterobacterial Common Antigen (ECA) in *Proteus*

The enterobacterial common antigen (ECA) represents a characteristic marker of Enterobacteriaceae, and its composition, structure, and occurrence have been intensively studied (for reviews, see MÄKELÄ and MAYER 1976; MAYER and SCHMIDT 1979; LUGOWSKI et al. 1983). GROMSKA and DEKA (1981) have tested extracts from 220 *P. mirabilis* and *P. vulgaris* strains for the presence of ECA by means of the passive hemagglutination technique, using rabbit antiserum obtained by immunization with a live culture of *E. coli* O14:K7(L). It was found that 147 isolates from patients with acute *Proteus* infections and 73 reference strains of *P. mirabilis* and *P. vulgaris* (including R mutants) were ECA-positive. Additionally, 130 *P. mirabilis* isolates from urinary tract infections were found to contain ECA (Gromska, unpublished data).

It has been demonstrated that ECA is virtually lacking in stable protoplast L-forms, while in unstable spheroplast L-forms reduced amounts of this antigen were detected in serological tests or by means of ferritin-labeled antibodies. Such L-forms have been used to confirm the exclusive localization of ECA in the outer membrane of enteric bacteria (RINNO et al. 1980).

4.4 Outer Membrane Proteins

4.4.1 Major Proteins

In the last two decades, techniques have been developed allowing the separation and isolation of outer and cytoplasmic membranes. Considerable information on the structure and functioning of the gram-negative outer membrane (OM) is now available. As far as proteins are concerned, it has been demonstrated that they occur in the outer membrane, as a small number of characteristic major proteins, in higher amounts than the remaining components such as lipopolysaccharide and phospholipid. Some of these proteins have been shown to be involved in nonspecific diffusion processes and to be responsible for the relative resistance of the outer membrane to surface-active substances like detergents. Questions concerning the OM structure and functions have been reviewed by INOUYE (1979a).

RAZIN's group was the first to investigate the proteins of *Proteus* bacilli. The comparative study of the outer and cytoplasmic membranes of *Proteus* (*P. mirabilis* 19) required the elaboration of new procedures that omit the ethylenediaminetetraacetate (EDTA) step, which would lead to unwanted spheroplasting of *Proteus* rods. Sonication was therefore employed, which resulted in the rupture of the cell envelope, exposing the rigid peptidoglycan layer to the action of lysozyme. Separation of membranes was achieved by centrifugation

in a linear 45%–60% sucrose gradient. The outer membrane was obtained in a relatively pure form, almost free from inner-membrane contaminants. The density was evaluated (1.22 ± 0.02 g/cm^3), and proteins, phospholipids, and lipopolysaccharide were found to constitute approximately 40%, 18%, and 30%, respectively, of the total (HASIN et al. 1975). The electrophoretic pattern of OM proteins resembled that of other enteric rods. Features of the outer and cytoplasmic membranes of *P. mirabilis* 19 have been compared (ROTTEM et al. 1975; HASIN et al. 1975; RAZIN et al. 1976). It was suggested that the protein and phospholipid components of the outer membrane of *P. mirabilis* are partly masked on the cell surface, probably by the O-specific chains of the lipopolysaccharide.

About 15 polypeptide bands have been revealed by gel electrophoresis of OM preparations (HASIN et al. 1975). The two major bands, termed C1 and C2, had molecular weights of 39000 and 38000, respectively. The C2 band, which is resistant to trypsin digestion, is thought to be the peptidoglycan-associated polypeptide, present in the outer membrane of all enterobacterial rods investigated so far. The other band (C1) is trypsin-sensitive and heat-modifiable, i.e., its migration rate depends on the thermal conditions of solubilization.

Three major proteins (molecular weights 39000, 36000, and 15000 respectively) were obtained from isolated cell walls of *P. mirabilis* 19. The 39000 protein was extracted with sodium deoxycholate, and subsequent extraction with acetic acid yielded the 36000 and the 15000 proteins (NIXDORFF et al. 1977). The first two proteins formed model membrane systems with cell membrane phospholipids. The 36000 protein is associated with peptidoglycan and is insensitive to digestion with trypsin in the native cell wall, while the 39000 protein is not associated with peptidoglycan and is trypsin-sensitive. Both proteins are involved in the formation of hydrophilic pores, mediating the penetration of hydrophilic molecules through model membrane phospholipid bilayers and protecting the membranes against the action of detergents. The 15000 protein was later shown to represent a lipoprotein (GMEINER 1981). The major proteins (39000 and 36000) could be stored in lyophilized form and have been characterized immunologically (BUB et al. 1980); both preparations reacted strongly with antisera against the purified cell walls of the homologous strain. The methods used for isolation, thus, yield preparations which retain their native antigenicity.

In comparative studies, the outer membranes of *P. mirabilis* 1959 and derived R mutants were isolated and their compositions determined (ROTTEM et al. 1979). The results of Table 4 show that the amounts of proteins and phospholipids (relative to lipopolysaccharide) in the outer membranes of the deeper R mutants (R51, R45) were about 40% lower than those of the other mutants, as estimated according to the content of KDO. However, there was no significant difference in the ratios of lipid phosphate to protein in the outer membranes of S and R forms.

The electrophoretic patterns of the OM polypeptides from S and R forms of *P. mirabilis* 1959, solubilized at 50° C, did not show any difference. However, the different susceptibilities of the isolated outer membranes to proteolysis (digestion with pronase and trypsin) increased from R110 (Ra) through R51 to

Table 4. Amounts of protein and phospholipid (relative to lipopolysaccharide) in the outer membrane (OM) of S and R strains of *Proteus mirabilis*, as estimated according to KDO content (ROTTEM et al. 1979)

Strain of *P. mirabilis*	KDO/OM (μg/mg)	Protein/KDO (mg/μg)	Lipid phosphate KDO (μmol/μg)	Lipid phosphate Protein (μmol/μg)
S1959	3.72	0.111	0.062	0.56
R13	5.10	0.110	0.060	0.55
R110	5.45	0.116	0.057	0.49
R51	7.97	0.071	0.033	0.46
R45	9.63	0.070	0.028	0.40

KDO, 2-keto-3-deoxyoctonic acid

R45 (Re), which was reflected by changes in electrophoretic patterns of the membrane polypeptides; in this property, *Proteus* R mutants resemble the known deep rough mutants of *E. coli* and *Salmonella typhimurium* (ROTTEM et al. 1979).

Proteolytic activity of enzymes on the isolated outer membrane of *Proteus* strains seems to be dependent on the length of the O polysaccharide chain. In smooth strains the polysaccharide chains probably mask the polypeptides, protecting them from proteolytic degradation (ROTTEM et al. 1979).

4.4.2 Antigenic Relationships Between OM Proteins

Recently, serological cross-reactions between OM proteins from *E. coli* and *Proteus* species (*P. vulgaris, P. mirabilis*) have been studied (HOFSTRA and DANKERT 1980). The SDS-polyacrylamide gel immunoperoxidase assay (SGIP; VAN RAAMSDONK et al. 1977) was adapted for the analysis of proteins in the range of 33000–42000 daltons. Antisera against isolated proteins I and II of *E. coli* O26 K60 were employed in cross-reactions with *Proteus* proteins. The OM protein I antiserum was shown to cross-react with OM protein I (higher molecular band) of other *E. coli* serotypes. In contrast, this antiserum reacted with lower-molecular-weight OM proteins of *P. vulgaris* and *P. mirabilis*. When the serum against *E. coli* OM protein II was applied, it cross-reacted with the lower-molecular-weight protein of *E. coli* serotypes, but in the case of the two *Proteus* species, it reacted with OM proteins of higher molecular weight.

These results seem to agree with earlier reports (LUGTENBERG et al. 1977; NIXDORFF et al. 1977; ROTTEM et al. 1979), which had demonstrated that in *P. vulgaris* and *P. mirabilis* the lower-molecular-weight OM protein is associated with peptidoglycan while the higher-molecular-weight fraction is not; this is the reverse of the situation in *E. coli* and other enteric bacteria.

4.4.3 Lipoproteins

The lipoprotein is covalently linked to the peptidoglycan in the cell walls of Enterobacteriaceae. Known as Braun's lipoprotein (for summary, see BRAUN

1975), it has been shown to be an essential factor maintaining the stability of the outer membrane-peptidoglycan complex. In the past, *P. mirabilis* was considered a species lacking this kind of protein (BRAUN et al. 1970). Lipoprotein could, however, be isolated from *P. mirabilis* strain 19 after growth to the early stationary phase and digestion of the peptidoglycan sacculi by use of endo-N,O-diacetylmuramidase from the fungus *Chalaropsis* (GMEINER et al. 1978). The lipoprotein preparation was purified by gel filtration and compared to the corresponding preparation from *E. coli* regarding the amino acid composition, the number of peptide cross-linkages, the remaining, covalently linked peptidoglycan subunits, and the fatty acid composition. The results established convincingly that a lipoprotein, linked covalently to peptidoglycan, is present in the *P. mirabilis* cell wall (GMEINER et al. 1978). The existence of a free form of lipoprotein in *P. mirabilis* cells was simultaneously reported (KATZ et al. 1978). As was shown subsequently (GMEINER 1979), the bound form of lipoprotein is entirely lacking in the exponential growth phase of *P. mirabilis,* but the amount of lipoprotein occurring in the stationary phase in *Proteus* cells is comparable to that present in *E. coli* and *S. typhimurium* cells (in all growth phases). It must be stressed that in *Proteus* cells the presence or absence of lipoprotein in the different phases of growth does not influence the structure of the peptidoglycan, which remains practically unchanged.

The conclusion from these observations was that, contrary to the situation in *E. coli,* the presence of rigid-layer-attached lipoprotein in *Proteus* is not decisive to the stability of the cell wall; this illustrates an important difference in the organization of the outer membrane of these two enteric bacteria.

Peptidoglycan-associated Lipoproteins. Two proteins, protein H from *Pseudomonas aeruginosa* and protein 21 K from *E. coli* K 12 (molecular weights, both 21 000) have been characterized by MIZUNO (1979) as peptidoglycan-associated lipoproteins (PAL). Similar proteins were found in other enteric and nonenteric bacteria (MIZUNO 1979). *Proteus* species seemed to be especially rich in PAL (molecular weight 18 000), which was isolated and intensively studied (MIZUNO 1981 a, b, c; GMEINER 1981; MIZUNO et al. 1982). PAL was shown to occur in the cell envelope as a lipoprotein firmly but not covalently linked to the peptidoglycan layer. The lipoproteins H, 21 K, and PAL resemble each other and Braun's lipoprotein in substantial properties. They contain covalently linked faty acids (about 3 moles per 1 mole of protein). The unique compound, glyceryl-cysteine, to which the fatty acids are covalently linked, occurs at the N-terminus of both proteins: Braun's lipoprotein and PAL of *P. mirabilis.*

Besides these similarities, there exist marked differences. The molecular weight of PAL is nearly three times that of Braun's protein; the amino acid composition of the latter includes 58 amino acid residues, while PAL of *P. mirabilis* contains about 160. It needs to be mentioned, however, that homologies in the structure of the amino-terminal regions do exist (Fig. 6). It has been proven that biosynthesis of PAL is controlled by a gene different from that of Braun's lipoprotein. Recently a region of PAL, termed 11 K fragment, has been isolated and shown to be associated with peptidoglycan; it is supposed to be protected by peptidoglycan against trypsin digestion. Since the N-terminal

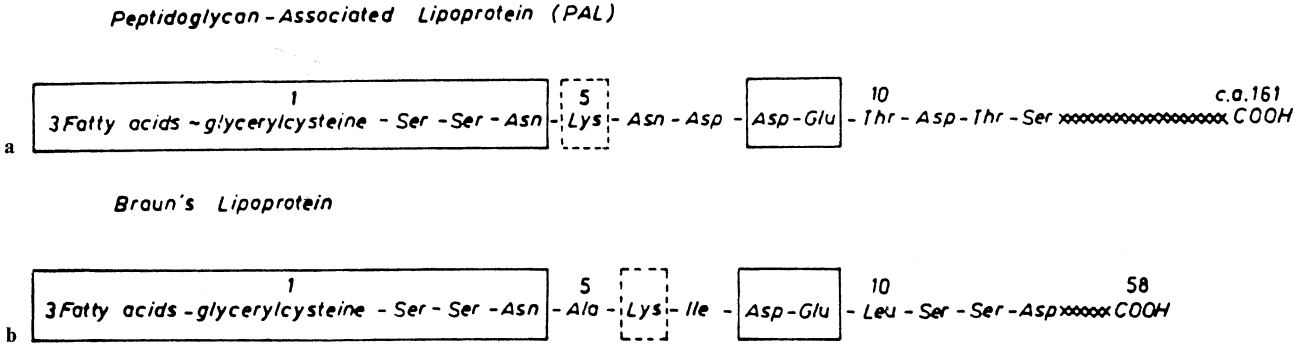

Fig. 6a, b. Amino terminal parts of the lipoproteins from **a** *Proteus mirabilis* (PAL) and **b** *Escherichia coli* (Braun's lipoprotein) (MIZUNO 1981c)

region is not involved in the interaction with peptidoglycan, it is presumed to play a role in the attachment of PAL to the outer membrane (MIZUNO et al. 1982).

It is too early to speculate on the biological role of this novel OM protein, but as it is present in all gram-negative bacteria investigated, PAL may represent an important factor in the functioning of the gram-negative cell envelope.

4.5 Peptidoglycan (Murein) and *Proteus* L-Forms

Bacterial L-form may be differentiated into two types: A, the stable, nonreverting protoplasts and B, the unstable spheroplasts, which easily revert to the bacterial form in the absence of the converting agent (DIENES and WEINBERGER 1951; TULASNE 1951). After this finding, it took another decade to prove that the cell wall is lacking in the stable, protoplast forms, while the unstable, spheroplast forms still contain it. Moreover, it could be shown that in different L-forms, isolated from the same strain, the defective cell wall may be blocked at different steps of the biosynthetic pathway (MARTIN 1964).

There have been reports on the occurrence of nonrevertible spheroplast L-forms which had been obtained from *P. mirabilis* strain 1959 by the aid of high doses of penicillin, and which could be cultured for many years. These L-forms grew as B-type colonies on soft nutrient agar, and as a layer on the surface of a nutrient broth without serum and penicillin. The material harvested from these cultures was a mixture of type-A and type-B forms, with significant amounts of cell wall constituents always detectable (KOTELKO et al. 1964, 1965).

Today it is commonly accepted that the L-phase (all morphologically distinct L-forms included) expresses the ability of many bacterial species to survive and to multiply under unfavorable conditions, either entirely without, or with a degraded, cell wall (for review, see HAYFLICK 1969; MARTIN 1983). The relative facility with which L-forms can be induced in *Proteus* bacilli, especially in *P. mirabilis,* is one of the characteristic properties of these microorganisms. It should be noted that the protoplast L-forms, which are devoid of peptidoglycan and the major OM proteins, contain small amounts of lipopolysaccharide in their cytoplasmic membrane (KROLL et al. 1980).

The basic structure of the peptidoglycan of *P. mirabilis* was identified as a polymer, built up from the disaccharide-tetrapeptide unit: N-acetylglucosaminyl-β-1,4-N-acetyl-(or N,O-diacetyl)-muramoyl-L-alanyl-D-γ-glutamyl-meso-diaminopimelyl-D-alanine. In the polymer, a portion of the disaccharide-peptide building blocks was found to be peptide-cross-linked. After specific enzymic digestion of the polymer with endo-N,O-diacetylmuramidase from *Chalaropsis,* the fragments disaccharide-peptide (monomers), peptide-cross-linked bis-[disaccharide-peptide] (dimers), and, in minor quantities, tris-[disaccharide-peptide] (trimers) were obtained. These fragments could be separated chromatographically into subspecies depending on the presence or absence of O-acetyl groups on N-acetylmuramic acid residues (FLECK et al. 1971; MARTIN and GMEINER 1979).

Unstable spheroplast L-forms which synthetize a defective peptidoglycan occur in cultures of *P. mirabilis* exposed to high concentrations of penicillin. Although this layer does not maintain the normal shape, and the cells are round and fragile, they do multiply and survive. Moreover, it was shown that the spheroplast "cell wall" contains cell wall material comparable in quantity to that in *Proteus* rods and closely resembling it, at least in the content of specific amino sugars, amino acids, and free amino groups, thus providing evidence for a normal degree of cross-linkage in their structure (MARTIN 1964; HOFSCHNEIDER and MARTIN 1968). It was then clear that *P. mirabilis* is able to evade the inhibiting action of penicillin. On the basis of previous investigations, however, it was demonstrated (MARTIN and GMEINER 1979) that penicillin modifies the structure of peptidoglycan. L-forms contain far fewer O-acetyl groups attached to muramic acid residues than do normal *Proteus* rods. Because the majority of peptide side chains in spheroplast peptidoglycan contained L-alanyl-D-γ-glutamyl-meso-diaminopimelyl-D-alanine tetrapeptides, it seemed that penicillin-sensitive enzymes such as peptidoglycan transpeptidase and DD-carboxypeptidase were not affected by the presence of this antibiotic in spheroplast L-form cultures. Two different membrane-bound enzymes, termed DD-carboxypeptidase/transpeptidase H and L, were recently isolated and purified from *P. mirabilis* (SCHILF and MARTIN 1980).

The two enzymes differ in several properties, but of prime importance is the difference in susceptibility of both enzymes to inactivation by penicillin. Enzyme H (molecular weight 49000) is highly sensitive to benzylpenicillin and exhibits permanent inactivation; it functions as an endopeptidase on peptide-cross-linked side chains of peptidoglycan. Enzyme L (molecular weight 43000) seems to be identical with the single active DD-carboxypeptidase previously revealed in the spheroplast L-form of *P. mirabilis* (MARTIN et al. 1976). This enzyme can be compared to the two membrane proteins of *Salmonella typhimurium,* which act as enzymes and as penicillin-binding proteins (SHEPHERD et al. 1977). It resembles also the multiple DD-carboxypeptidases and/or transpeptidases of *E. coli* (POLLOCK et al. 1974). It must be stressed that it is not easy to reconstruct in vitro the conditions of in-vivo peptide cross-linking. This probably explains the low efficiency of purified *Proteus* enzymes in artificial conditions. It is postulated that the continuous activity of enzyme L in vivo as DD-carboxypeptidase/transpeptidase may be one of the decisive factors involved in the manifestation of partial resistance of *P. mirabilis* to high penicillin concentrations. At present, penicillin-binding protein 5 (PBP 5) of *P. mirabilis,* acting as DD-carboxypeptidase/transpeptidase L, is considered to be the only enzyme identified so far which is capable of supporting peptide cross-linking and, in consequence, the peptidoglycan synthesis in L-forms of *Proteus* (SCHILF and MARTIN 1980). The in vitro synthesis of peptide-cross linked peptidoglycan from the suitable precursors by permeabilized spheroplasts of *P. mirabilis* (grown in the presence of penicillin) confirmed the role of PBP 5, which functions as transpeptidase in the synthesis of functionally defective peptidoglycan.

As mentioned above, the substitution in peptidoglycan of nearly two-thirds of the N-acetyl-muramic acid residues by O-acetyl groups accounts for the essential difference between peptidoglycan of *P. mirabilis* and that of other en-

teric bacteria studied to date. The presence of such a substituent, which can be considered a "natural marker," opens a new perspective for possible utilization of *P. mirabilis* as a model microorganism. The investigations of O-acetylated and non-O-acetylated subunits in *P. mirabilis* peptidoglycan, initiated recently by GMEINER and KROLL (1978), may advance the general knowledge of murein turnover, and elucidate some unsolved questions concerning sacculus elongation and septum formation in gram-negative bacilli.

5 Summary and Concluding Remarks

The *Proteus* group, while conforming to the general definition of the family Enterobacteriacecae, occupies a particular position when evaluated on the basis of molecular markers, adopted in modern molecular taxonomy. *P. mirabilis* and *P. vulgaris,* which are the main object of this review, have been shown to be closely related.

One of the most characteristic features of these two species is their ability to undergo drastic morphological changes on solid media, resulting in swarming. The detailed mechanism of this striking phenomenon remains obscure. The crucial question is whether this phenomenon is a particular response of *Proteus* bacilli to a physically strange environment like laboratory solid media, or perhaps a property which plays some role in natural environments of these microorganisms, in their saprophytic or facultatively pathogenic life.

Some substantial differences in the fine structure of the cell envelope between *Proteus* and other enteric bacteria have been revealed. Negatively charged constituents occur in *Proteus* (*P. vulgaris* and *P. mirabilis*) lipopolysaccharides in higher quantities than usual. Glucuronic and/or galacturonic acids are present in both O-specific polysaccharides and core oligosaccharides of *Proteus* lipopolysaccharides.

Taking into account the peculiarities of metabolism of these bacteria, e.g., the proteolytic activity under aerobic and facultatively anaerobic conditions as well as their ability to decompose urea, one could speculate that an electronegative compound such as lipopolysaccharide would protect the cell surface from effects of marked alkalization.

An essential difference between *Proteus* and other enterobacterial species (*E. coli, Salmonella*) is the lack of peptidoglycan-bound lipoprotein (Braun type) in the exponential phase of growth of *Proteus,* which suggests that the organization of the outer membrane in this organism is different and that the attachment of lipoprotein to the rigid layer is not decisive to the stability of the cell wall. It can be supposed that the novel kind of lipoprotein, PAL, which has been detected in *Proteus* in rather large amounts compared to other gram-negative bacteria and which is closely associated with peptidoglycan but not covalently attached to it, is involved in the stabilization of the *Proteus* outer membrane.

The substitution of a great part of the N-acetylmuramic acid residues by O-acetyl groups is a unique feature of *Proteus* peptidoglycan; in spheroplast L-forms the number of O-acetyl groups is reduced. It seems that the O-acetylated

and non-O-acetylated subunits in peptidoglycan of *P. mirabilis* may provide a useful model for investigations aiming at elucidation of some general principles of murein structure and turnover.

The partial resistance of *P. mirabilis* to high concentrations of penicillin has been recently postulated to be due to the activity of the enzyme, DD-carboxypeptidase/transpeptidase, so far the only identified enzyme capable of supporting peptide cross-linking in the presence of penicillin.

The role of *Proteus* species in natural environments as decomposers of organic matter of animal origin has been established, and their potential as facultative pathogens has been postulated for some years.

During the last decade, the changing picture of infectious diseases has provoked an increasing interest in the mechanisms of microbial pathogenicity. The rise in the number of antibiotic-resistant strains, complications resulting from nosocomial infections, the increasing importance of some facultative pathogens in the etiology of human and animal diseases, and the persistence of chronic infections have induced the investigation of factors of pathogenicity (for reviews, see SMITH et al. 1980; CHENG et al. 1981). In this context, a comprehensive study also of *Proteus* bacteria seems to be important.

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