Broth) was filtered on Millipore (0.45 μm), concentrated 50-fold by ultrafiltration (Amicon PM 10) and filtered on Sephadex G-100 (elution buffer: phosphate 0.1 *M*, pH 7.2); for the 11 strains, 1 hemolytic peak was found (apparent molecular weight 46,000 ± 3000). The hemolytic fractions were pooled, dialyzed against glycine 1%, pH 7 and electrofocused in a pH 5–8 gradient of Ampholine. 2 major and 1 minor hemolytic peaks were found, corresponding respectively to pI 6.54; 6.13 and 5.85. A second isoelectricfocusing of each major peak confirmed these values. Moreover, by Sephadex gel filtration, the same apparent molecular weight (46,000) was found for each pooled fraction. Samples were applied to polyacrylamide gels for electrophoresis and the hemolytic bands were detected by placing the gels on blood agar plates. For all the strains tested, the hemolysin migration appeared to be identical. From these results it seems that the 11 (toxigenic and non-toxigenic) strains of *Cl. tetani* have a hemolytic system which cannot be differentiated by their molecular weights, their isoelectric points or by polyacrylamide gel electrophoresis. The hemolytic system of each serotype tested can be resolved into three fractions by isoelectricfocusing.

Interaction of Tetanus Toxin with Cultured Neuroblastoma Cells: Analysis by Antitoxin Immunofluorescence

Jessie Zimmerman and J.-C. Piffaretti
Institut Universitaire de Microbiologie Médicale, quai E. Ansermet 22, CH–1211 Genève 4

The membrane binding of tetanus toxin and toxoid was studied with intact cells. 24 h monolayer cultures of mouse Neuroblastoma C1300 cell line clone NB2A (gift of Dr. D. MONARD, Friedrich-Miescher-Institut, Basel) were incubated for increasing time periods with 20 Binding Units of either toxin or toxoid. Each Binding Unit (BU) as defined by KRYZHANOVSKI (Bull. exp. Med. Micro., 1975, in Russian) is approximately equal to 1 L_f (RAMON, Rev. Immun., Paris 1940). Cells were grown in petri dishes containing glass cover slips and maintained at 37 °C in an 8% CO_2 atmosphere. Samples were fixed in acetone, cells stained by the Giemsa method and bound antigens visualized by indirect immunofluorescence with FITC- conjugated antiserum. Preparations were observed by phase contrast for cell morphology and with fluorescent microscopy for binding evidence. The toxin bound increased over a 24-h period, however no evidence of toxoid binding was demonstrated by immunofluorescence. Exposure to the toxin in growth (presence of serum) and 'differentiation' (absence of serum) cultures showed a marked difference in binding distribution. Observations of toxin treated cells pointed out a net difference in cell morphology in absence of serum, but no change was visible in growth cultures, either in cell morphology or population numbers. Chemical modulators of membranes were assayed for effect on toxin binding both by preincubation and coincubation with the agents: poly-D-glutamate, NH_4Cl, neuraminidase (*V. cholerae*) and β-galactosidase (*E. coli*). In these preliminary studies the technique has not only proven useful as a method to visualize and an immunofluorescent probe to evaluate the distribution of cell surface receptors and their character, but also as a qualitative back-up to [^{125}I]-toxin binding experiments (in preparation).

Production of Cholera Toxin by *Vibrio cholerae* Strain 569 B and B 1307

F. Grolimund and R. Germanier
Schweiz. Serum- und Impfinstitut, Forschungsabteilung, Rehhagstrasse 79, CH–3018 Bern

Strains 569 B and B 1307 of *Vibrio cholerae* synthesize in vitro an exo-enterotoxin. The rate of this synthesis was determined by immunochemical and biological methods. Measurements in the supernatants from cultures of strain 569 B revealed, that this toxin remained stable over a long period of time, whereas in supernatants from cultures of strain B 1307, a decrease in the specific biological activity was found although the immunochemical values remained constant. Strain B 1307 was also elaborating a proteolytic enzyme. The assumption, that this enzyme causes the loss of biological activity could not be confirmed. During cultivation of strain B 1307 a toxoid was spontaneously formed, which could not be differentiated immunochemically from the toxin. The decrease in biological toxin activity can partially be explained through the formation of this toxoid.

Analysis of PBSX⁻ and Cell Wall Turnover Reduced Mutants of *B. subtilis*

D. Karamata, M. Guex and H. M. Pooley
Institut de Microbiologie de l'Université de Lausanne, 19, rue César-Roux, CH–1000 Lausanne 17

MNNG mutagenized populations of *B. subtilis* 168, *thy*⁻, *trp*⁻, were successively treated with mitomycine C and UV and the survivors screened for mutants defective in production of active PBSX particles. 11 such mutants, designated Ni1–8, Ni10, Ni11 and Ni15 were isolated and back-crossed into a multiple auxotroph by co-transformation with *met* C. Derivatives of strain Ni1 were of 2 types designated 1 and 16 (see below). Strains Ni1–8, 11 and 15 did not lyse upon treatment with various phage inducing agents. Strains Ni10 and 16 showed a normal lysis curve upon induction and their lysates, as determined by electron microscopy, contained respectively phage heads (*xtl* mutation) and phage tails (*xhd* mutation). Using PBS1 mediated transduction all PBSX mutations were mapped by 3 point crosses between *arg C* and *met C*. The apparently reduced autolysis of stationary phase cultures of Ni strains prompted us to examine the turnover of their cell wall. It was found that all original strains have a reduced wall turnover. Genetic analysis has shown that this property of different PBSX⁻ non-inducible strains is due to different mutations all mapping in the PBSX region. Reduced turnover of the original strain Ni10 is due to an unlinked *gta* C mutation. PBSX inducible back-crossed strains 10 and 16 exhibit a wild-type wall turnover. It would appear that proteins involved in cell metabolism are under prophage PBSX control.

Cell Wall Turnover, Autolysin Levels, and Surface Expansion in *B. subtilis* and a Mutant, Ni 15

H. M. Pooley
Institut de Microbiologie de l'Université de Lausanne, 19, rue César-Roux, CH–1000 Lausanne 17

Ni15, a mutant of *B. subtilis* (see previous communication), growing on a casein hydrolysate supplemented medium at 45 °C (generation time = 20 min) has a markedly reduced cell wall turnover (POOLEY, J. Bact. *125*,

1127–1138, 1976). Chemical analysis of sodium lauryl sulfate inactivated cell walls isolated from Ni15 and parent cells revealed only minor differences. Lysis of several independent isolates of such walls by autolysate preparations of parent cell walls revealed no reproducibly significant differences in susceptibility between Ni15 and parent walls. Assay, in enzymically active cell wall preparations, for the two autolytic activities shown to be active in wall metabolism in vivo, revealed nearly parent levels of *N*-acetylmuramyl L-alanine amidase in Ni15, whereas the *N*-acetyl glucosaminidase activity was below 5% of that of the parent. 10 min after the addition of chloramphenicol (CAP) to the parent strain, the rate of release of turnover products to the medium became constant and remained so for at least 50 min. Thus CAP addition did not lead to an inhibition of wall degradation. CAP added to a culture 1 generation after a 3 min pulse label almost completely blocked release of labelled turnover products during the following 60 min. It is concluded that continued surface expansion, blocked by CAP, is required for new wall to reach the outer surface of the cell wall layer. These results support the model (see reference above) proposing a continuous enlargement or 'spreading' in the area occupied by old wall during surface expansion. I believe that this model offers a means of reconciling published work reporting a failure to observe segregation of old wall with the apparently contradictory evidence for a discrete segregation pattern which is presented in the following communication.

Autoradiographic Study of Segregation of Labelled Cell Wall in a Mutant of *B. subtilis* with Reduced Wall Turnover

J.-M. Schlaeppi, H. M. Pooley and D. Karamata
Institut de Microbiologie de l'Université de Lausanne, 19, rue César-Roux, CH–1000 Lausanne 17

An autoradiographic study of cell wall precursor insertion was attempted by use of strain Ni15 which has a considerably reduced wall turnover (see previous communications). This has allowed us to largely avoid complications due to wall turnover and follow wall segregation for over 5 generations. Ni15 cells were grown in casein hydrolysate supplemented medium containing *N*-acetyl-D-(1-^{3}H)glucosamine for over 5 generations (under these conditions cells grow in long chains and about 90% of ^{3}H-NAG enters the cell wall fraction (POOLEY, J. Bact. *125*, 1127–1138, 1976). Cells were filtered, washed and resuspended in fresh unlabelled medium. Samples were withdrawn at 0, 2, 4 and 5 generations after chasing, prepared for autoradiography and the grain distribution was determined. At the time of chase, grain distribution was Poissonian (50–70% probability). At 4 and 5 generations, about 10–15% of all the grains were distributed in dense clusters, situated at the ends of the chains, whose number corresponded accurately to the number of septa at time 0. The rest of the grains was distributed in a clearly non-Poissonian fashion (probability lower than 0,1%). Similar results were obtained using ^{3}H-glycerol as wall marker, suggesting comparable behaviour of teichoic acid and mucopeptide. These results are in unambiguous agreement with a discrete mode of insertion of new cell wall materiel, most probably at 3 or more sites per cell under these conditions. In our view, there are at least 2 reasons why previous experiments failed to reveal 'segregation' in gram + rod-shaped bacteria within 2 generations. The resolution by autoradiography may have been insufficient to visualize 3 or more insertion sites per cell. The 'spreading' of the surface area, occupied by old wall, could also obscure segregation within several generations after chase.

Analysis of Thermo-Sensitive Mutants of *B. subtilis* which Lyse at the Non-Permissive Temperature

C. Brandt and D. Karamata
Institut de Microbiologie de l'Université de Lausanne, 19, rue César-Roux, CH–1000 Lausanne 17

Study of cell wall biosynthesis is attempted by isolation of *ts* mutants which lyse at the non-permissive temperature due to defects in wall biosynthesis. A screening aimed to identify such mutants among over 650 *ts* mutants of *B. subtilis* Ni10, *trp*$^-$, *thy*$^-$, *xtl*$^-$, isolated by indirect selection, was performed. In a preliminary screening mutants affected in incorporation of ^{14}C-leucine were identified and further examined. Liquid cultures at relatively low cell density (2×10^7/ml) were shifted to the non-permissive temperature and the O.D. was followed. 32 strains which showed clear lysis were identified, backcrossed into a multiple auxotroph by congression and, by pairwise crossing (transformation), distributed into 8 genetic linkage groups designated A to H. Group A consisted of 15 and the other ones of 1 to 4 mutants. Using PBS1 mediated transduction or transformation groups B to H were mapped on the *B. subtilis* chromosome by 3-points crosses. Their approximate positions are as follows: *xtl*, *met* C, *ts* B; *cys* C, *ura*, *fur*, *ts* C; *ura*, *fur*, *ts* D; *pur* A, *ts* DNA C, *ts* E; *ts* F, *gta* A, *his* A; *ts* G, *his* A, *cys B*; *his* A, *ts* H, *cys* B. Group A has not been mapped so far. It shows no linkage to any of the following markers: *pur A*, *cys* A, *str* A, *pur* B, *arg* C, *met* C, *ura*, *thy* A, *cit* B, *ilv* A, *thy* B, *met* B, *trp*, *lys*, *phe* A, *leu*, *arg* A, *thr*, *cys* B, *his* A. Cell wall biosynthesis of one representative of each group was examined at the non-permissive temperature by ^{14}C-*N*-acetyl-D-glucosamine incorporation (POOLEY, J. Bact. *125*, 1127–1138, 1976). It appears that in groups A to G deficiencies of ^{14}C-NAG incorporation preceed the lysis.

Properties of Lipids in Membranes of the Stable Protoplast L-Form of *Proteus mirabilis*

H. H. Martin, H. P. Kroll and J. Gmeiner
Institut für Mikrobiologie, Technische Hochschule, Schnittspahnstrasse 9, D–6100 Darmstadt

Membranes of the cell wall-less stable protoplast L-form of *P. mirabilis* correspond to the cytoplasmic membrane of their parent bacteria. In membranes of L-form strains LVI and LD52 phosphatidyl ethanolamine (PE, 78–80%), phosphatidyl glycerol (PG, 12%), diphosphatidyl glycerol (DPG, 4.5–6%) and lyso-phospholipid (LP, 1%) were found as major phospholipid components. The fatty acid composition of the L-form phospholipids differed from that of the bacterial form by almost complete absence of octadecanoic acid and by a 50% increase of tetradecanoic acid. When horse serum was added to the L-form medium for growth stimulation the protoplasts contained more DPG and LP and less PG. Also, 'foreign' lipids from the serum supplement, phosphatidyl choline and cholesterol were taken up into L-form membranes. However, cholesterol containing L-form cells exhibited no sensitivity to sterol-specific inhibitors, amphotericin B and digitonin. Thus, unlike the sterol-requiring mycoplasmas the *Proteus* protoplast L-form does not incor-