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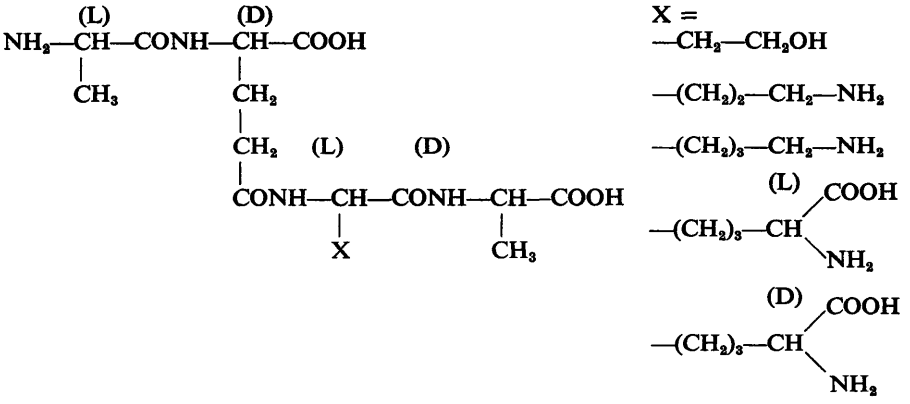
SYMPOSIUM: THE CELL WALL AND THE CYTOPLASMIC MEMBRANE OF BACTERIA

Introduction. By M. R. J. SALTON (*Department of Microbiology, New York University School of Medicine, New York, U.S.A.*)

The Primary Structure of Bacterial Wall Peptidoglycans. By JEAN-MARIE GHUYSEN and MELINA LEYH-BOUILLE. (*Service de Bactériologie, 32 Bvd de la Constitution, Université de Liège, Belgium*)

The bacterial wall peptidoglycan is an insoluble network composed of: (i) glycan chains of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues, i.e. a chitin-like structure except that every other sugar is substituted by a 3-*O*-D-lactyl group and that the average chain length is small (20 to 140 Hexosamine residues, depending upon the bacterial species). Variations so far encountered include the possible presence of *O*-acteyl substituents on C-6 of some of the *N*-acetylmuramic acid residues (*Staphylococcus aureus*; some strains of *Lactobacillus acidophilus* (unpublished) and of *Micrococcus lysodeikticus*), and the replacement of the *N*-acetylmuramic acid residues by another derivative of muramic acid, possibly *N*-glycolylmuramic acid (*Mycobacterium smegmatis*) (ii) tetrapeptide subunits which substitute through their *N*-termini the D-lactic acid groups of the glycan chains. (iii) peptide bridges which cross-link tetrapeptide subunits of adjacent glycan chains (average size of the peptide moieties: 1·5 to 10 cross-linked peptide subunits).

The tetrapeptide subunits have the general sequence R_1-γ-D-glutamyl-R_3-D-alanine. The R_3 residue is sometimes a neutral amino acid such as homoserine (Perkins, H. R. (1965), *Biochem. J.* **95**, 876), most often a diamino acid such as L-ornithine, L-lysine LL-diaminopimelic acid, *meso*-diaminopimelic acid. Irrespective of the nature of the R_3 residue, however, both the amino group and the carboxyl group engaged in linkages to the γ-COOH of D-glutamic acid and to the NH_2 of D-alanine, respectively, are located on the same carbon which has always an L configuration. It follows that, with the possible exceptions that the R_1 residue is not always L-alanine, but sometimes L-serine or glycine, and that glutamic acid may be replaced by 3-hydroxy-glutamic acid (Schleifer, K. H., Plapp, R. & Kandler, O. (1967), *Biochem. biophys. Res. Commun.* **28**, 566), the tetrapeptide units have the following general structure:



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The main variations thus so far encountered lie in the nature of the side chain of the third amino acid in the sequence. Depending upon the bacterial species, the general tetrapeptide unit may have additional substituents. Amide ammonia, glycine or D-serine are possible substituents of the α-COOH group of D-glutamic acid. Similarly, the carboxyl group of LL or *meso* diaminopimelic acid which is not engaged in peptide linkage, may also be substituted by an amide group.

The following peptide subunits have been fully characterized: N^{α}-[L-Ala-γ-(α-D-Glu-amide)]-L-Lys-D-Ala (*Micrococcus, Staphylococcus, Gaffkya, Streptococcus, Lactobacillus* sp.); N^{α}-[L-Ala-γ(α-D-Glu-Gly)]-L-Lys-D-Ala (*Micrococcus lysodeikticus* and related *Micrococci*); L-Ala-γ-D-Glu-(L)-*meso*-DAP-(L)-D-Ala (*Escherichia coli*; probably all the Gram-negative bacteria; *Bacillus* sp.); L-Ala-γ-(α-D-Glu-amide)-(L)-LL'DAP-(L)-D-Ala (*Clostridium welchii*; *Streptomyces* sp.) (unpublished experiments); N^{α}-(L-Ser-γ-D-Glu)-L-Orn-D-Ala (*Butyribacterium rettgeri*); Gly-γ-D-Glu-homo-Ser-D-Ala (plant pathogenic *Corynebacteria*).

The peptide bridges always involve the carboxyl group of the terminal D-alanine residue of one peptide subunit and either the free amino group of the diamino acid (peptidoglycans of type I, II and III) or the α carboxyl group of the D-glutamic acid (peptidoglycan of type IV) of another peptide subunit. The cross-linking between two peptide subunits may consist: (i) in a direct bond such as a D-alanyl-(D)-*meso*-diaminopimelic acid linkage, that is to say a peptide bond which extends from the amino group located on the D carbon of *meso*-diaminopimelic acid of one peptide subunit, to the C terminal D-alanine of another peptide subunit (van Heijenoort, J., Elberz, L., Dezélée, Ph., Petit, J. F., Bricas, E. & Ghuysen, J. M. (1969), *Biochemistry*, **8**, 200. (Gram negative bacteria; *Bacillus* sp.) (Peptidoglycan of type I). (ii) in a single additional amino acid (a glycine, a neutral L-amino acid, or a D-isoasparagine residue) or an intervening peptide chain composed of glycine and/or neutral amino acid residues and containing up to 5 residués. This bridging frequently occurs between N^{α}-[L-Ala-γ-(α-D-Glu-amide)]-L-Orn or L-Lys or LL-DAP)-D-Ala peptide subunits. It extends from the free amino group of the diamino acid of one peptide subunit to the C terminal D-alanine of another peptide subunit (Peptidoglycan of type II). (iii) in one or several peptides, each having the same amino acid sequence as the peptide subunit (Peptidoglycan of type III). The location of the bridging is identical to that of type II peptidoglycan. When the bridge is composed of several peptide subunits, these subunits are linked to each other through 'head to tail' D-alanyl-L-alanine linkages. One peculiarity of type III peptidoglycans (*Micrococcus lysodeikticus* and related *Micrococci*) is that the number of peptide subunits may be greater than the number of disaccharide units of the glycan moiety and that not all the *N*-acetylmuramic acid residues are peptide substituted (Campbell, J. M., Leyh-Bouille, M. & Ghuysen, J. M. (1969), *Biochemistry*, **8**, 193). (iiii) in a diamino acid residue, D-ornithine or D-lysine, extending between the α-COOH group of D-glutamic acid and C terminal D-alanine (Peptidoglycan of type IV) (some plant pathogenic *Corynebacteria* (2) and *Butyribacterium rettgeri* (Guinand, M., Ghuysen, J. M., Schleifer, K. H. & Kandler, O. (1969), *Biochemistry*, **8**, 207)). The bond to the C terminal D-alanine always involves the α-amino group of the intervening diamino acid.

The peptidoglycans of the Gram-positive bacteria are at least 100 Å thick and, thus, probably occur as three-dimensional multilayered structures. It may be that some of the bridges which characterize the peptidoglycans of type II, III and IV, are used to interconnect several superimposed monolayers. The peptidoglycans of type I have no additional amino acids to insure the bridging between the peptide subunits. This might be related to the fact that the peptidoglycan in the Gram-negative bacteria probably occurs as a two-dimensional monolayer (20 Å thick). The 100 Å thick peptidoglycans in the Gram positive *Bacillaceae* might then be visualized as made up of several interlinked *E. coli*-like peptidoglycan monolayers. DD-diaminopimelic acid which has been characterized in walls of *Bacillus megaterium* KM could be involved in these cross-linkages. Similarly, the peptide cross-linking within the *Micrococcus varians meso*-diaminopimelic acid containing peptidoglycan might be mediated through a polyglutamic acid sequence (with the amino terminus linked to COOH group of D-alanine; unpublished).

Structure, Function and Biosynthesis of the Rigid Layer of the *Escherichia coli* Cell Wall.
By V. BRAUN and U. SCHWARZ (*Max-Planck-Institut für Biologie Tübingen, Germany.*)

The rigid layer of the cell wall of *Escherichia coli* consists of a macromolecule the size and the shape of the cell, which is held together exclusively by covalent bonds. The repeating unit (*N*-Acetylglucosamine-*N*-Acetylmuramic acid)$_n$ forms polysaccharide strands which are cross-linked by the peptide L-Ala-D-Glu-meso-DAP-(D-Ala)-D-Ala-meso-DAP-D-Glu-L-Ala. The resulting network is called murein (peptido-glycan, mucopeptide or glycopeptide). Data will be presented which show that in addition to the murein network (sacculus) the rigid layer contains a lipoprotein which is covalently bound to the murein. Enzymic degradation of the rigid layer with trypsin or pronase revealed that on the average there is one lipoprotein molecule bound to every tenth repeating unit of the murein. The lipoprotein is linked by its *N*-terminal lysine to the carboxyl group of 2·6 diaminopimelic acid of the murein and the following sequence has been established for the murein-lipoprotein link: DAP-Lys-Arg. The size of the protein is about 7000 and the absolute amino acid composition has been determined. There are strong indications that also a part of the lipid is covalently bound to the protein. A model of the rigid layer will be given in molecular dimensions in which about 10^5 lipoprotein molecules are evenly distributed over the entire surface of the rigid layer and where the lipoprotein molecules are spaced 96 Å apart along the polysaccharide chains of the murein.

The rigid layer essentially determines the shape of the cell. This function is not only due to its chemical structure but is also based on its interaction with the other components of the cell wall. Our results suggest that this interaction is primarily mediated by the lipoprotein. When cell walls are incubated with trypsin we found a rapid decrease in the optical density. The rate of the reaction is very high and can only be obtained with trypsin. The target of the rapid trypsin reaction is the above mentioned Lys-Arg bond. After a short trypsin incubation of cell walls (20 min., room temp., ratio of enzyme to total cell wall protein = 1:50) all the lipoprotein is removed except lysine for which one residue remains for every tenth murein building block. In ultra-thin sections of such trypsin treated cell walls, all cell walls are split into two well separated layers which otherwise are closely adjacent to one another. The fast decrease of the optical density and the parallel appearance of two layers in thin-sections are consistent with the view that the lipoprotein has an important function in stabilizing the cell wall.

The enlargement of the macromolecule during cell growth and division should depend on the action of at least two enzymic systems. The one should provide both space and acceptor sites for incoming material; this can be done only by hydrolysis of covalent bonds in the pre-existing murein. The second system would then insert the new precursors. The composition of these precursors is known. They are put together in the cytoplasm and transported by carrier molecules to the place where they are used. It is only partially known how these precursors are polymerized into the sacculus.

The essential participation of murein hydrolases in the process of murein synthesis has been proved. *In vivo* studies with a DAP-requiring mutant of *E. coli* have demonstrated the activity of at least five murein-degrading enzymes in growing cells. They seem to be pacemakers of murein biosynthesis. In a mutant with temperature dependent murein hydrolase activity, murein neosynthesis comes to a stop by preventing murein hydrolases from acting.

Furthermore, murein hydrolases play a key role in bacterial morphogenesis. Experiments dealing with morphogenetic aspects of murein synthesis have shown that the growth of the sacculus is accomplished by several functionally different systems which can be distinguished from each other by their differential sensitivity to penicillin. One is involved in cell elongation, the others in cell division. Penicillin was also used to localize the site of action of murein hydrolases. It was demonstrated that an exact splitting of the sacculus by such enzymes is a step preceding cell division, and the topology and timing of this splitting are correlated with DNA replication.

The Organisation of the Polymers in Gram-positive and Gram-negative bacteria. By H. J. ROGERS (*National Institute for Medical Research, Mill Hill, London, Great Britain*)

The structures of the mucopeptides, teichoic acids and polysaccharides in the walls of a number of Gram-positive bacteria are now known, and likewise in a number of Gram-negative species the structures of the mocupeptides and of much of the lipopolysaccharide molecules are now known. Among the Gram-negative species divalent cations appear to play a considerable role in linking together some of the components. A large proportion of the lipopolysaccharide can be removed from the walls of pseudomonads and by chelating agents such as EDTA (Gray, G. W. & Wilkinson, S. G. (1965), *J. gen. Microbiol.* **39**, 385; Asbell, M. A. & Eagon, R. G. (1966), *Biochem. Biophys. Res. Commun.* **22**, 664), whilst treatments involving EDTA, osmotic and cold shock, can release enzymes from Gram-negative cells that often appear as truly exo-cellular enzymes in Gram-positive species, e.g. ribonuclease, alkaline phosphatase, penicillinase (Neu, H. C. & Heppel, L. A. (1964*a*), *Biochem. Biophys. Res. Commun.* **17**, 215; (1964*b*), *Proc. natn. Acad. Sci. U.S.A.* **51**, 1267). Likewise penetration into the cytoplasmic membrane by substances which inhibit mucopeptide synthesis such as the penicillins meet diffusion barriers in Gram-negative bacteria which are not present in Gram-positive species and which can be damaged by chelating agents (Hamilton-Miller, J. M. T. (1966), *Biochem. J.* **100**, 675). Thus the outermost layers of Gram-negative cells possess some of the properties usually associated with membranes rather than with walls. The functional wall behaves as if it were a region between two membranes rather than a region existing outside a membrane and which is freely permeable to smaller molecules as with Gram-positive species.

In a number of examples the polymers making up the walls of Gram-positive species have been shown to be linked together by firm covalent linkages which need relatively drastic treatment such as long exposure to low pH or treatment with hot formamide to break them. Recent work with *Bacillus licheniformis* and *B. subtilis*, for example, shows that both the teichoic acid and in the former, the teichuronic acid are linked by covalent linkages to the mucopeptide. When the walls are incubated with an autolytic enzyme hydrolysing the linkage between the terminal L-alanine of the peptide and the polysaccharide backbone (amidase), relatively small fragments can be isolated which contain teichoic acid, and teichuronic acid attached to the polysaccharide backbones of mucopeptide (Hughes, R. C., Pavlik, J. G., Rogers, J. H. & Tanner, P. J. (1968), *Nature. Lond.* **219**, 642). The teichoic acid can be removed by treatment under very mild alkaline conditions leaving a teichuronic acid molecule joined by its terminal reducing *N*-acetylgalactosamine molecule probably through a phosphate group to the mucopeptide backbone (Hughes, R. C. & Tanner, P. J. (1968), *Biochem. Biophys. Res. Commun.* **33**, 22).

The teichoic acid linked to a mucopeptide fraction containing an excess of glucosamine can be isolated and separated from the teichuronic acid-mucopeptide compound after the combined action of lysozyme, amidase and a ribonuclease preparation. Thus it appears that the teichuronic acid-mucopeptide, and the teichoic acid-*N*-acetyl glucosamine-mucopeptide may be linked together by a bond sensitive either to ribonuclease or to an enzyme present in preparations of crystalline ribonuclease.

The question arises as to how far the structure and organization of the various polymers in the walls defines the morphology and cell division of the micro-organisms. We have recently been examining morphological mutants of *Bacillus subtilis* 168 try$^-$ which grow in high NaCl concentration as rods, but in minimal medium of low tonicity as round cocci, these have been called rod$^-$. (Rogers, H. J., McConnell, M. & Burdett, I. D. J. (1968), *Nature, Lond.* **219**, 285). The shape of the mutants can also be corrected from spherical to rod form by including a sufficient concentration of casein hydrolysate in the growth medium. The casein hydrolysate can be replaced by 0·2 % glutamate, or by 0·2 % of the amino acids, L-proline, L-arginine or DL-ornithine. The mutants in both morphological forms contain a higher proportion of wall per unit weight of micro-organism than the wild type. The mucopeptide in the round forms, in one class of rod$^-$ mutant, is much less cross-linked than that in the rod-shaped cells grown in the presence of either high salt concentration or with casein hydrolysate. Grown in the presence of salt the walls contain much less ester-alanine in the phosphate containing polymers and more

N-acetylgalactosamine. Another class of rod⁻ mutants has also been isolated for which salt or casein hydrolysate are ineffective in correcting the shape back to a rod. In these the walls have the same degree of cross-linking as is present in the parent. This second class of mutant has nutritional requirements not shared by the parent.

Sructural Studies on some Salmonella lipopolysaccharides. By BENGT LINDBERG (*Institutionen för organisk kemi, Stockholms Universitet, Stockholm, Sweden*)

Lipopolysaccharides (LPS) from Gram-negative bacteria consist of a lipid part, an O-specific side chain and a 'basal core', linking the O-specific side chain to the lipid part. The O-specific side chains, which carry the O-antigens of the bacteria, are composed of oligosaccharide repeating units, containing three or more sugar residues. Structural studies on the O-specific side chains of LPS from different Salmonella bacteria, belonging to groups A, B, C, D and E are presented.

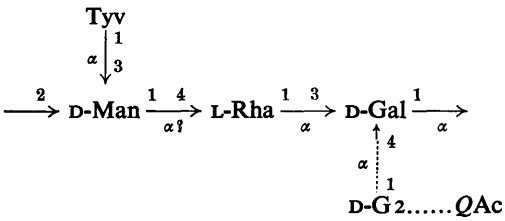
Methylation analysis has provided the essential informaion in these studies and has been performed by a new technique, whereby the mixture of methylated sugars, obtained on hydrolysis of the fully methylated polysaccharide, is qualitatively and quantitatively analysed GLC-mass spectrometry of their alditol acetates (Björndal, H., Lindberg, B. & Svensson, S. (1968), *Acta chem. scand.* **21**, 1801; (1968), *Carbohyd. Res.* **5**, 433).

By methylation analysis of the original LPS and of the partially hydrolysed LPS, it has been possible to determine the structures of the 'biological repeating unit' and the average number of repeating units in the O-specific side chains of several LPS.

Methylation analysis does not provide any information on the anomeric nature of the sugar residues. For some sugar residues, these have been determined by following the change in optical rotation on acid hydrolysis. For other sugar residues, this has been done, mostly by other investigators, by partial hydrolysis and structural determination of the resulting oligosaccharides.

Several LPS contain *O*-acetyl groups, which may be immunologically significant. The positions of such groups have been determined by protecting all hydroxyl groups as acetals, removing the *O*-acetyl groups by alkaline hydrolysis and subjecting the derived polysaccharide to methylation analysis (de Belder, A. N. & Norrman, B. (1968), *Carbohyd. Res.* **8**, 1).

As a result of these studies, complete or partial structures of O-specific side chains in several LPS have been determined. (Hellerqvist, C. G., Lindberg, B., Svensson, S., Holme, T. & Lindberg, A. A. (1968), *Carbohyd. Res.* **8**, 43; (1969) **9**, 237). Some results from previous studies have been confirmed, others have been revised and new structural features have been demonstrated. As an example, the structure of the repeating unit in a strain of *S. typhi* (9, 12_2) is given below. A dotted line



connecting a residue to the repeating unit indicates that only some of the repeating units contain this residue. The terminal α-tyvelose residue, linked to the 3-position in D-mannose, and the terminal α-D-glucose residue, linked to the 4-position in D-galactose, are associated with the presence of O-factors 9 and 12_2, respectively. The immunological significance of the 2-*O*-acetyl groups, present in some of the D-glucose residues, is not known.

How Genes Determine the Structure of the Salmonella Lipopolysaccharide By P. HELENA MÄKELÄ and B. A. D. STOCKER (*State Serum Institute, Helsinki, Finland, and Department of Medical Microbiology, Stanford University, California*)

The genetic determination of the polysaccharide part of the Salmonella lipopolysaccharide (LPS) has been studied over a period of 6–7 years. Mutants affected in various steps of LPS synthesis as well as representatives of several Salmonella species belonging to different O antigen groups have been used to identify the genes involved.

The polysaccharide consists of a *core*, common to all Salmonella serogroups and containing ketodeoxyoctonate, heptose, phosphate, glucose, galactose and *N*-acetylglucosamine, and of O *side-chains* attached to the second glucose of the core and differing in structure between different O groups. Corresponding to these two parts, two gene clusters, *rfa* and *rfb*, were identified.

The core is synthesized by sequential addition of monosaccharides from their nucleotide donors to the unfinished core, each addition requiring the action of a specific transferase. The *rfa* cluster, located on the bacterial chromosome between the loci *cysE* and *pyrE* and cotransducible with these, contains genes determining the synthesis of most of these transferases, including the transferase of the phosphate to heptose. The gene *rfaH* required for the transfer of one of the sugars (the first galactose) in the main chain of the core does, however, not appear to be in the same cluster.

The O side-chains are synthesized separately, on an intermediate lipid carrier called ACL. An oligosaccharide, the *repeating unit*, is first assembled by sequential addition of its component monosaccharides, and then polymerized still on the ACL. The polymer is then 'translocated' onto the core; if the core is not complete, this step cannot be performed and the polymer-ACL accumulates as O-specific 'hapten'. The *rfb* cluster, co-transducible with the histidine operon, contains several (at least ten in *Salmonella typhimurium*) genes for enzymes required in the synthesis of the monosaccharide-nucleotides, and the genes specifying the transferases needed for the assembly of the repeating unit. Hybrids of two Salmonella species belonging to different O groups have established that the *rfb* cluster contains all the information necessary to specify the structure of the repeating unit.

The polymerization of the repeating units requires a polymerase, which in groups B and D is specified by *rfc* gene(s) between *gal* and *trp*. The translocation of the polymer onto the core appears to require the cooperation of two genes, one in the *rfa* cluster, the other in the *rfb* cluster—each one perhaps specifying a subunit of the ligase enzyme. Finally, a *rfe* gene or genes close to *ilv* are required for the synthesis of O-specific material at least in O groups C_1 and L. It seems possible that their products might participate in the synthesis or preparation of ACL for LPS synthesis. T 1-specific side-chains are found attached to the core in the same manner as are O chains. Their synthesis is dependent on gene(s) in a separate cluster, called *rft*[1].

Mutations in any one of the genes mentioned result in the synthesis of a very deficient LPS without long O-specific side-chains, and leading to a rough (*R*) phenotype of the bacteria. A basic structure of the repeating units (and of the core) is therefore necessary for their utilization in the synthesis. Modifications of the basic structure are, however, permissible, and are recognized as O antigen factors such as 5 or 10 (O-acetylation of certain sugars in the O chain), 1 or 12_2 (glucose side-branches). Variants with or without these factors are found in nature or as laboratory mutants. Many of them are variable even within one clone, and many are determined by temperate bacteriophages. The *oaf* genes responsible for these factors are found outside the *rf.* clusters, and several of them are located in the upper right-hand quadrant of the chromosome.

Structure of the Cytoplasmic Membrane and Mesosomes. By D. J. ELLAR (*University of Cambridge, Great Britain*)

In the search for an understanding of the principles governing the morphogenesis and function of cellular structures, the cytoplasmic membrane of bacteria continues to provide valuable insights. Early electron microscope studies revealed an apparent absence in bacteria

of those organelles typically found in animal and plant cells and supported the classification of bacteria as prokaryotic cells. This classification elaborated by Stanier and van Niel in their classic paper ((1960), *Archiv. Mikrobiol.* **42**, 17) included as one of the distinguishing features of the prokaryotic cell a lack of internal membranes separating the cytoplasm from both the nucleus and the machinery of photosynthesis and respiration. While subsequent refinements in cytological techniques have confirmed the absence of a nuclear membrane in bacteria, the situation is not so unequivocal with regard to photosynthetic and other functional organelles. There is in fact considerable evidence that bacteria may contain units of cellular function enclosed within a membrane which forms a barrier of sorts between these structures and other regions of the cell. A careful study of the photosynthetic bacterium *Ectothiorhodospira mobilis* (Remsen, C. C. *et al.* (1968), *J. Bact.* **95**, 2374) revealed a photosynthetic lamellar system very similar in appearance to the aukaryotic chloroplast. Nevertheless, no simple analogy can be drawn between these two photosynthetic structures, since it is clear that in *E. mobilis* the lamellar stack originates from one or more plasma membrane invaginations. In the fully developed stack these invaginations persist to constitute an opening into the special environment which lies between the plasma membrane and the external layers of the cell envelope. This persistent connexion with the extracellular space also appears to occur in the vesicular membrane network found in other photosynthetic bacteria, but it is not a feature of eukaryotic chloroplasts. Such connexions have been repeatedly demonstrated in the case of bacterial mesosomes (Ellar, D. J. *et al.* (1967), *J. Bact.* **94**, 1189), although the function of these structures is still not clear. It appears therefore that the prokaryotic bacteria may indeed contain organelles isolated from the surrounding cytoplasm in the rather special sense that their interior, together with the outer surface of the plasma membrane is in direct communication with the extracytoplasmic space. The elimination of this communication by a fusion of apposed membranes at the base of the original invagination, could create a second type of specialized environment isolated now from both the cytoplasm and the extracellular space. In prokaryotic cells these phenomena may fulfil a particular evolutionary requirement for spatial organization, or for transport of certain molecules to and from the external environment.

Unfortunately these connexions between the plasma membrane and intracellular bacterial membrane structures have frequently hampered attempts to separate and purify them. Recently, however, by exploiting this connexion it has been possible to isolate the membranes of the mesosome in pure form using the techniques of plasmolysis and protoplast formation. (Ellar, D. J. & Freer, J. H. (1969), *J. gen. Microbiol.* (in press); Ferrandes, B. *et al.* (1966), *C. r. Acad. Sci., Paris* **263**, 1632); Reavely, D. A. (1968), *Biochem. biophys. Res. Commun.* **30**, 649). In this procedure the membrane bounding the mesosome evaginates, becoming part of the protoplast membrane and expelling its contents into the suspending medium. The influence of cation concentration on this expulsion will be discussed. Mesosomal membranes isolated in this way can now be compared chemically and functionally with the plasma membrane freed from mesosomal material. Furthermore, suggestions as to a mesosomal role in membrane synthesis, the uptake of transforming DNA, transverse septum synthesis and the partitioning of the bacterial genome are now more amenable to experimental study.

The results of comparisons of mesosomal and plasma membranes from *Micrococcus lysodeikticus* and *Bacillus megaterium* will be presented. These studies reveal significant differences in protein and lipid content which are in turn reflected in density differences upon sucrose gradient centrifugation. Mesosomes and plasma membranes have also been compared with respect to their content of enzymes and respiratory pigments, their ability to synthesise cell wall peptidoglycan *in vitro* and their ability to incorporate radioactivity into membrane lipids. The results of an electron microscope examination of these fractions will be reported. Experiments will be discussed which indicate considerable structural similarity between the plasma membrane of *M. lysodeikticus* and the inner mitochondrial membrane of eukaryotic cells. A number of enzymes including ATP'ase, $NADH_2$'ase, succinic dehydrogenase and cytochromes have been shown to be associated with the *M. lysodeikticus* membrane. Of these, the ATP'ase and $NADH_2$'ase can be selectively dissociated by washing with buffers and appear to represent 'detachable' components existing as granular substructure on the membrane (Muñoz, E. *et al.* (1968), *Biochem. biophys. Res. Commun.* **32**, 539; Ellar, D. J. *et al.* (in preparation)). Extensive washing to remove these components results in the production of

a smooth-surfaced residue resistant to further washing which contains the components of the electron transport system including the total succinic dehydrogenase activity. A markedly similar residue can be obtained by controlled washing of the freshly isolated plasma membrane with deoxycholate, (Salton, M. R. J. *et al.* (1968), *Biochem. biophys. Res. Commun.* **36**, 909). Evidence to be discussed suggests that certain components of the electron transport chain in *M. lysodeikticus* exist in a relatively rigid membrane fraction which resists various procedures designed to remove 'detachable' proteins. The relatively mild washing techniques required to produce this fraction favours the view that it constitutes an important functional and structural component of the native membrane. The use of low glutaraldehyde concentrations to modify the strength of attachment of membrane associated enzymes will be discussed.

Organization of Protein and Lipid in the Mycoplasma Membrane. By SHMUEL RAZIN (*Department of Clinical Microbiology, The Hebrew Universtiy-Hadassah Medical School, Jerusalem, Israel*)

Devoid of a cell wall and intracytoplasmic membranes, the mycoplasmas have only one type of membrane—the plasma membrane. This makes them excellent models for membrane study (Razin, S. (1969), *The Mycoplasmatales and L-Phase of Bacteria*, ed. L. Hayflick, Appelton-Century-Crofts: New York). Highly purified membranes have been isolated from several mycoplasmas by osmotic lysis, and were shown to consist of 50 to 60 % protein and 30 to 40 % lipid (Razin, S. (1967), *Ann. N.Y.Acad. Sci.* **143**, 115). Some of the biophysical and catalytic properties of the membrane proteins have recently been characterized (Rodwell, A. W. *et al.* (1967), *Archs Biochem. Biophys.* **122**, 621; Rottem, S. & Razin, S. (1967), *J. Bact.* **94**, 359; Engelman, D. M. ; Morowitz, H. J. (1968), *Biochim. biophys. Acta.* **150**, 385), and considerable information on the chemical nature of the membrane lipids is available (Smith, P. F. (1967), *Ann. N.Y. Acad. Sci.* **143**, 139). However, the organization of the protein and lipid in the membrane is not yet clear. Its investigation is likely to be of special interest as it might contribute to the solution of the highly disputed problem whether the biological membrane is built of a bimolecular leaflet of lipid coated with protein on both sides, of repeating lipoprotein subunits, or of some combination of both.

Morphological evidence for a subunit architecture is scanty and inconclusive. The subunits observed in negatively-stained membranes of *Mycoplasma pulmonis* (Hummeler, K. *et al.* (1965), *J.. Bact.* **90**, 517) and *M. gallisepticum* (Chu, H. P. & Horne, R. W. (1967), *Ann. N.Y. Acad. Sci.* **143**, 190) probably are surface projections, while the structure of the membrane *per se* may still conform to the leaflet model. On the other hand, the fact that the triple-layered structure is preserved in sectioned *M. laidlawii* membranes after about 95 % of the membrane lipids have been removed with aqueous acetone (Terry, T. M. (1966), Ph.D. Thesis, Yale Univ.) does not favour the leaflet theory.

Solubilization of mycoplasma membranes by sodium dodecyl sulphate (SDS) has yielded a clear solution exhibiting a single symmetrical schlieren peak of about 3 *S*. The initial interpretation that this represented lipoprotein subunits (Razin, S. *et al.* (1965), *Proc. natn. Acad. Sci. U.S.A.* **54**, 219) was disproven by showing that by ultracentrifugation or electrophoresis the protein could be separated from the lipid in the SDS-solubilized material (Engelman, D. M. *et al.* (1967), *Biochim. biophys. Acta* **135**, 381; Rottem, S. *et al.* (1968), *Arch. Biochem. biophys.* **125**, 46). Nevertheless, on removal of the detergent by dialysis, in the presence of Mg^{2+} or some other divalent or polyvalent cation, the lipid-SDS and protein-SDS micelles reaggregated spontaneously to structures morphologically and chemically resembling the original membranes (Razin, S. *et al.* (1965), *Proc. natn. Acad. Sci. U.S.A.* **54**, 219; Terry, T. M. *et al.* (1967), *Biochim. Biophys. Acta* **135**, 391). At a low Mg^{2+} concentration (5×10^{-3}M) the membraneous structures formed had a higher lipid to protein ratio than at the optimal Mg^{2+} concentration (2×10^{-2}M; Rottem, S. *et al.* (1968), *Arch. Biochem. Biophys.* **125**, 46). Electrophoretic and enzymic analyses tend to support the conclusion that the incorporation of different proteins into the reformed membranes at various Mg^{2+} concentrations is selective. Study of the reaggregation kinetics during dialysis against 2×10^{-2}M–Mg^{2+} has shown that initially the lipid-rich membranes are formed, which upon further dialysis are apparently transformed into membranes whose lipid to protein ratio resembles that of the original

membranes (Razin, S. *et al.* to be published). The reformation phenomenon may perhaps be visualized as a multi-step process which involves the formation of a primary lipid-rich membraneous structure containing only part of the membrane protein species. More protein can be bound to this structure when enough Mg^{2+} ions become available to neutralize electrostatic repulsive forces interfering with this binding. Though it is still doubtful whether such a mechanism also operates in membrane formation *in vivo*, it may be safely concluded that membrane protein and lipid molecules contain sufficient structure-determining information to interact spontaneously to form membrane-like structures in the absence of a pre-existing membrane template.

Recent studies (Kahane, I. & Razin, S. (1969), *Biochim, biophys. Acta.* in the Press) have shown that membrane protein and lipid biosynthesis in *Mycoplasma laidlawii* are not necessarily synchronized, so that plasma membranes having a different lipid to protein ratio can be formed. This finding speaks against the hypothesis that the membrane is built of lipoprotein subunits having a constant lipid to protein ratio.

SYMPOSIUM: ANIMAL DNA VIRUSES

A memorial review of the work of the late Dr Robin Valentine.

Internal Components of Adenovirus By W. C. RUSSELL (*National Institute for Medical Research, Mill Hill, London, Great Britain*)

At least 25% of the total protein of adenovirus resides within the viral capsid in close association with the nucleic acid. By acrylamide gel electrophoresis it can be shown that this 'core' protein consists of five components two of which are rich in arginine. These components are soluble in mineral acids and possess some of the characteristics of the arginine-rich histones; in some other respects they bear similarities to the protamines. Amino acid analyses have been carried out on the acid-soluble components and these have been compared with the amino acid patterns characteristic of histones and protamines. A number of methods of preparing viral 'cores' have been investigated, the primary purpose being to obtain the structures in their original configuration and free of contaminating capsid components. Attempts have also been made to obtain viral 'cores' from infected cells but these have not been successful. Some physical and biological properties of the 'cores' obtained by disruption of the capsid will be described.

The relationship of the core components to events in the infected cells have been investigated by 'pulse' labelling and serological techniques. Thus, immunofluorescence can detect an antigen whose production depends on the presence of exogenous arginine and which is probably related to the 'core' components. Early in infection cellular DNA synthesis is inhibited but this event does not appear to be related to the production of the antigen requiring exogenous arginine. The possibility that the arginine-rich core components are concerned essentially with the packaging of the viral nucleic acid has also been investigated and the results of some preliminary experiments will be described.

Capsid Mosaics of Human Adenoviruses. BY ERLING NORRBY (*Department of Virology, Karolinska institutet, Faculty of Medicine, Stockholm University, Stockholm, Sweden*)

Two distinct antigen specifications of type-specific nature are available at the surface of adenovirus virions. One is carried by fibres and can be demonstrated in hemagglutination-inhibition (HI) tests by use of soluble components as antigen and the other is carried by hexons. Antibodies against the latter can not inhibit the hemagglutinin (HA) activity of soluble components, but still in most cases (some members of Rosen's subgroup III form an exception) effectively inhibit the HA activity carried by homotypic virions. Antibodies against hexons play the major role as concerns neutralization of adenovirus infectivity.

Prototype strains can be specified by reference to the two type-specific antigens mentioned, viz. by neutralization tests (or virion-HI tests in most cases) and by HI tests with soluble components. A number of adenovirus strains have been identified in which

(*a*) only one of the two type-specific antigens are related to that of a prototype strain, or (*b*) the two antigen specificities are related to two *different* prototype strains. The latter type of strains have been called intermediate strains. By use of different immunological techniques including immune electron microscopy, attempts have been made to characterize, on the level of structural components, the relationship between prototype strains and strains displaying immunological characteristics of the kind mentioned above. As an example of strains sharing only one of the two type-specific antigens, the prototype strains 4 and 16 were chosen. These two strains which display a relationship (more pronounced in one direction) in neutralization tests were found to share a unique antigen specificity carried by hexons. Similarly intermediate strains (types 3–16 and 15–9 were studied) were found to carry hexons similar to, but not identical with hexons of the prototype strains to which they were related in neutralization tests. They furthermore carried fibres (alternatively pentons) immunologically related to those of prototype strains, with which they cross-reacted in HI tests.

Phenotype mixing experiments have revealed that mixing between structural components can be readily established both within the capsid of virions and within polymers of soluble components. Phenotypic mixing occurred even between distantly related serotypes, e.g. representative members of different subgroups. The occurrence of capsids including pentons of different prototype origin was readily demonstrated by identification of fibres of different lengths in isolated particles and of the occurrence of different HA activities carried by them. Phenotypic mixing between hexons was analysed by immune electron microscopy. Examples of mixing between structural components of different serotype origin into soluble polymers were found. For example, components of type 16, which normally form a soluble HA represented by dimers of pentons or dimers of fibres, were found to mix with type 4 pentons into dodecons.

Fate of Adenovirus During the Early Phase of Infection. By L. PHILIPSON and K. LONBERG-HOLM (*Department of Microbiology, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden*)

Adenovirion type 2 are uncoated by KB or HeLa cells to essentially free DNA. About half of this DNA is located within the nucleus. At least 3 intermediates can be resolved by appropriate gradient centrifugation techniques. The schematic overall sequence is:

A free virus
↓
B virus-receptor complex
↓
C partly uncoated intracellular virus
↓
D lipid complex with virus core
↓
E viral DNA

The virion (A) attaches to specific receptors on the plasma membrane via the fibre antigen to form component B. There are about 10^4 receptor sites per cell and these are mainly on the cell surface where they can be digested by subtilisin. B can be formed in cell free systems and this is blocked by pure fibre antigen.

The breakdown of B to C appears to be first order with a half life of the order of 10 min. Component C is partly DNase sensitive, has a buoyant density about 0·01 g./c.c. greater than A and probably lacks some or all penton units. There is no A within the cell. About half of component C is rapidly distributed into the cell nucleus.

The third virion product behaves in sucrose and CsCl gradients as to suggest that it is the virion core bound to a membrane component.

The fourth step involves the weakening of binding, or removal, of virion core proteins from the DNA. This goes to completion in about two hours in the nucleus but is slower in the cytoplasm. Even during the last step there is no major degradation of the DNA as is shown by alkaline-rate-zonal centrifugation. There is also no evidence for the formation of covalent circles or integrated forms with cellular DNA.

The proposed steps during the first 2 hr of infection are thought to have biological significance. It cannot yet, however, be proven that they are obligatory in the infective process because the ratio of infective to physical particles is only about 0·03 in this system.

Synthesis and Morphogenesis of Adenovirus Capsid Proteins. By H. S. GINSBERG and L. F. VELICER (*Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.*)

The icosahedral adenovirus capsid is composed of 240 face capsomers, the hexons, and 12 corner units, the pentons. Each penton consists of a base unit and a fibre of varying length. Although viral DNA is synthesized in the nucleus, the capsid proteins are only detected in the nucleus by immunofluorescent techniques, and the virions are assembled in the nucleus, autoradiography demonstrates that the viral proteins are synthesized in the cytoplasm and rapidly transported into the nucleus (Velicer, L. F. & Ginsberg, H. S. (1968), *Proc. natn. Acad. Sci. U.S.A.* **61**, 1264). The viral proteins are synthesized on polyribosomes with an average sedimentation coefficient of 200S, and the polypeptide chains synthesized have an average sedimentation coefficient of about 3·4S.

The viral polypeptide chains are synthesized within 1 min. after which they are rapidly released from the polyribosomes. After release from the site of their biosynthesis the nascent polypeptide chains acquire immunological reactivity of a rate similar to that at which they are transported from the cytoplasm into the nucleus. Utilizing isotope labeling pulse-chase techniques, the newly made polypeptide chains, which are of relatively uniform size, can be followed into 4 species of multimeric proteins having sedimentation coefficients of 6S, 9S, 10·5S, and 12S. When purified I^{125}-labelled fibres and hexons are used as markers the 6S and 12S proteins are seen to sediment in sucrose gradients as fibre and hexon proteins, respectively. The 6S and 12S proteins can also be identified immunologically as fibre and hexon respectively. The 9S protein is trypsin sensitive and in pulse-chase experiments it appears to be a precursor of the 10·5S macromolecule which on reaction with trypsin yields a 6S fibre protein and acid-soluble material. Hence, the 10·5S protein may be identified as the penton capsid structure.

It is striking that the hexon, which has a molecular weight of 300,000 daltons, and the fibre, which is about 65,000 daltons, can be disrupted into polypeptide chains with sedimentation coefficients of 3–3·5S. The hexon consists of 12 identical subunits of 25,400 daltons, and preliminary data indicate that the fibre is made from 3 polypeptide chains each with a molecular weight of about 22,000 daltons. Thus, the adenovirus capsid proteins are polymers composed of similar sized polypeptide chains which are synthesized on cytoplasmic polyribosomes after which they rapidly move into the nucleus and undergo immunological and morphological maturation before assembly into the virion.

Interactions Between γ-Globulins and Adenovirus Virions. By L. KJELLÉN (*University of Lund, Sweden*)

Reactions between adenovirus type 5 and separated fractions of rabbit IgC will be described.

Heavy chain fractions of rabbit IgC, rendered soluble in glycine-HCl buffer at pH 3·0, retain antiviral activity. No activity is found in the light chain fractions. The power to inactivate adenovirus is not limited to H chains, separated from anti adenovirus IgC molecules. H-chains separated from IgG's of rabbits, immunized with unrelated antigens or rabbits not immunized at all, show activity as well. The 'specificity' of the reactions will be discussed. A detailed report will be published elsewhere.

***In vitro* Transformation by Human Adenoviruses.** By J. VAN DER NOORDAA (*University of Amsterdam, Holland*)

Oncogenic human adenoviruses can be classified in three subgroups: A, B, and C. The viruses of subgroup A (types 12, 18,31) are highly oncogenic in hamsters, those of subgroup B (types 3, 7, 14, 16, 21) are weakly oncogenic and the 'non-oncogenic' subgroup C viruses (types 1, 2, 5) have the ability to transform rat cells *in vitro*. A number of adenoviruses of these

three subgroups have been shown to transform several types of cells derived from various species of animals.

Of the subgroup A, adenovirus type 12 transforms hamster, rat, and rabbit cells. The *in vitro* transformation studies with adenovirus type 12 and other types of adenovirus have been facilitated by the use of media containing low concentrations of calcium (0·1 mM). The adeno-12 transformed cells contain virus specific nuclear antigens (T, or tumour antigen) and virus specific messenger RNA. The cells transformed by adenovirus type 12 are oncogenic when introduced into the appropriate host system. Recently it has been demonstrated that adenovirus type 12 is able to induce foci of altered cells in certain susceptible human cell cultures. As yet it has not been possible to establish lines of transformed cells from these altered foci.

Of subgroup B, adenovirus type 3 transforms rat embryo and rat kidney cells. The adeno-3 transformed rat cells are oncogenic; they contain a subgroup B specific tumour antigen. Transformation of rat embryo cells by adenovirus type 7 has been mentioned.

The so-called non-oncogenic subgroup C adenoviruses types 1, 2 and 5 share the ability to induce morphological transformation of both rat embryo and rat kidney cells. By employing sera from hamsters carrying tumours induced by the adeno-1-SV40 and adeno-2-SV40 hybrid viruses a common T antigen has been detected in rat cells transformed by adenoviruses types 1, 2, and 5. The rat cells, morphologically transformed by subgroup C viruses, have not yet been proved to be oncogenic.

Recent studies in our laboratory have revealed the ability of the non-oncogenic adenovirus type 4 to transform rat kidney cells. Of interest was the enhancing effect of u.v.-irradiation of the virus. A decline of infectivity, caused by u.v.-irradiation of the virus, was accompanied by an enhanced transforming ability of the virus. The antigenic and oncogenic properties of the adeno-4 transformed cells are currently studied. Preliminary CF tests seem to indicate the presence of a T antigen in the adeno-4 transformed cells which is related to the T antigen of adeno-3 transformed cells.

Infectious adenovirus has not yet been recovered from any of the transformed or tumour cells.

The Molecular Structure of SV_{40} Virions. By M. A. KOCH and F. A. ANDERER (*Justus Liebig-Universität, Giessen, Germany*)

The simian virus 40 (SV_{40}) is a naked DNA containing virus with a diameter of 42 mμ. The molecular weight of the particle is $17{\cdot}3 \times 10^6$. Purified virus consists only of DNA and protein.

The DNA of SV_{40} is doublestranded, forming circular, super-coiled molecules with a S_{20} of 21S. The molecular weight of the DNA was found to be $2{\cdot}3 \times 10^6 \pm 15\,\%$.

Under conditions known to dissociate an assembly of polypeptide chains into monomers the protein moeity of SV_{40} consists of three different species of polypeptide chains, which can be separated by electrophoresis and by chromatography. These polypeptide chains were designated A, B and C. The polypeptides A and B are present in equimolar amounts and constitute together 90 % of the protein moiety. The polypeptide chain C is a basic protein containing more than 20 % basic amino acid residues. The molecular weights of the polypeptide chains were determined: M = 16, 800 ± 10 % for C, M = 16,900 ± 10 % for B and M = 16,400 for type A polypeptide chains.

Exposure of purified SV_{40} to pH 10·5 at low ionic strength results in degradation of the virion. The resulting degradation products can be separated in two fractions by sucrose density gradient centrifugation. One fraction contains material sedimenting with 30–40S, while the second fraction sediments with 2·5–5·0S. This slower sedimenting fraction contains no DNA and consists only of the A- and B-polypeptide chains. The faster sedimenting fraction contains the viral DNA together with the C-polypeptide chains. The molecular weight of the alkali-stable DNA-protein complex was determined to be $3{\cdot}7 \times 10^6$. Assuming that all C-polypeptide chains of the virion are bound to the viral DNA in this complex, a value of $3{\cdot}65 \times 10^6$ can be calculated for the molecular weight. The conclusion that the polypeptide chains type C are fixed to the viral DNA is supported by the finding that on tryptic digestion of virions the A and B chains are more rapidly degraded than the C-chains.

Electron microscopic investigation of the SV_{40} virion revealed that 72 morphological units compose the icosahedral viral particle. The symmetry of the icosahedral surface lattice is $T = 7d$.

To form the $T = 7$ icosahedral surface lattice 420 structural units are required. If one assumes that one A and one B polypeptide chain form together one structure unit, then the weight of the particle shell can be calculated to be $420 \times (16{,}400 + 16{,}900) = 13{\cdot}98 \times 10^6$. Together with the DNA C-polypeptide complex the calculated molecular weight is $17{\cdot}6 \times 10^6$.

Purified preparations of SV_{40} particles lacking DNA (= empty particles) were also investigated. Such particles were composed, as the SV_{40} virions, only of the three known polypeptides. As in full particles the polypeptide C constitutes 10% of the viral protein. From this observation it is concluded that the C polypeptide chains interact specifically with the particle shell. These interactions are broken during alkaline degradation.

On the basis of these findings it is proposed that the structure units consist each of one A- and one B-polypeptide chain. 420 structure units are arranged to form a particle with a $T = 7d$ icosahedral surface lattice. The C-polypeptide chains, being affixed to shell and DNA, orient the DNA within the particle.

Pox Viruses. K. DUMBELL (*Wright Fleming Institute of Microbiology, London, Great Britain*)

No abstract.

ORIGINAL PAPERS

SESSION A

Three-dimensional Molecular Models of the Mucopeptide from *Staphylococcus aureus*. By MARIANNE V. KELEMEN and H. J. ROGERS (*Department of Pharmaceutics, The School of Pharmacy, and The National Institute for Medical Research, Mill Hill, London, Great Britain*).

The composition and the chemical fine structure of some mucopeptides have been well established. However, no detailed study has yet been made of the secondary structures involved. As a preliminary step before undertaking physical measurements on mucopeptides, and in the light of the difficulties likely to be met in the interpretation of results of examining undergraded material, models of the mucopeptides from *Staphylococcus aureus* and Bacillus species have been constructed to see whether it is possible to distinguish the most likely secondary structures.

For *Staphylococcus aureus* the model consists of three polysaccharide chains, each containing eight monosaccharides, *N*-acetylglucosamine being placed at the non-reducing end of the chain. The peptide attached to the *N*-acetylmuramic acid residue contains one molecule of L-alanine, D-isoglutamate, L-lysine and D-alanine. The α-carboxyl group of D-isoglutamate is amidated, cross-linking between peptide chains is obtained by linking the carboxyl end of the pentaglycine to the ϵ-amino group of L-lysine in one chain and the amino group of the pentaglycine to the carboxyl group of D-alanine in another chain.

The polysaccharide chains run head to tail and can be hydrogen bonded as in cellulose or chitin. Two spatial configurations of the peptide chain have been constructed, a helical and a pleated (β-configuration) sheet structure. The helical structure forms few hydrogen bonds, and the required repeating unit for an α-helix is absent as within the chain peptide bonds are formed from the γ-carboxyl of D-glutamate, and the ϵ-amino group of L-lysine. The pleated sheet or β-configuration containing trans carboxyl groups provides a regular net-like structure in which 60% of the amino groups are hydrogen bonded.

In mucopeptide from various species of bacilli, the L-lysine in the peptide chains is replaced by DL2,6 diaminopimelic acid (DAP) and no D-alanine exists with a free carboxyl group. (Hughes, R. C., Pavlick, J. G., Rogers, H. J. & Tanner, P. J. (1968), *Nature, Lond*, **219**, 642). The cross-linking is directly between the DAP 6-amino group and the carboxyl group

of the D-alanine in the next peptide chain. Again, when extended in the β configuration extensive hydrogen bonding is possible, but only between pairs of heptapeptide chains.

It is hoped that speculations such as these will lead to further experiments which could test how mocopeptides provide both shape and rigidity to the bacterial cell wall.

Erythrocyte-sensitizing Activity of Staphylococcal Mucopeptide. By ARNE GROV (*The University of Bergen, School of Medicine, The Gade Institute, Department of Microbiology, Bergen, Norway*)

Teichoic acid-mucopeptide (T–M) complexes of staphylococci sensitize tanned sheep erythrocytes (TSE) for agglutination by antisera to staphylococci. A ^{32}P-labelled T–M complex from *Staphylococcus aureus* strain WOOD 46 sensitized TSE, but no radioactivity was detectable in the sensitized and washed TSE. Radioactivity was detected in dilutions of the ^{32}P-labelled complex corresponding to a content of 0·05 μg. The minimal sensitizing dose of the ^{32}P-labelled complex was 10 μg., and the minimal inhibitory dose was 0·5 μg. These results indicate that the teichoic acid is not attached to the TSE and that the mucopeptide moiety is responsible for the present serological activity. This conclusion is supported by the lack of precipitins against teichoic acid in antiserum to TSE sensitized with T–M complex, the inability of sensitized TSE to absorb teichoic acid precipitins, and by the ability of TSE to remove haemagglutinogens but not precipitinogens from a solution of T-M complex.

A TSE-sensitizing peptide, shown to be a mucopeptide subunit, has been isolated from *S. aureus*. This substance cross-reacted serologically with the T-M complex in indirect haemagglutination. Absorption and inhibition experiments revealed that the mucopeptide moiety of the T-M complex exhibits two antigenic specificities: one is due to the peptide subunit and cross-reacts with the isolated TSE-sensitizing substance, the other is due to the amino sugars.

Substrate Dependent Modifications of the Amino Acid Sequence of the Murein of Staphylococci. By K. H. SCHLEIFER (*Botanisches Institut der Universität München, Germany*)

The interpeptide chains in the murein (peptidoglycan) of staphylococci depend qualitatively and quantitatively on the amino acid content of the medium.

When *Staphylococcus aureus*, strain COPENHAGEN, is grown in the presence of an unfavourable ratio of glycine to alanine only one-third of the ϵ-amino groups of lysine is substituted by a pentaglycine, while one-third remains free and one-third is substituted by L-alanine. These L-alanine residues remain *N*-terminal and do not contribute to cross-linkage. Even on a defined medium which contains 50 mg. glycine/l. (enough for optimal growth) but no alanine, some ϵ-amino groups of lysine carry an L-alanine. When the glycine content is increased to 0·1 % almost all ϵ-amino groups of lysine are substituted by pentaglycine. The same is true when D,L-serine is added, however, about 0·5 mole of serine per mole of peptide subunit are incorporated into the interpeptide chains in this case. The morphology of the cell remains unchanged under the various conditions, although the percentage of cross-linkages differs greatly. A similar influence of the amino acid content of the medium on the cross-linkage of the murein was found in *S. epidermidis* strain 24. The murein of this strain contains about 0·5 mole of serine per mole peptide subunit even when no serine is added. In addition less L-alanine is bound to the ϵ-amino groups of lysine as compared to *S. aureus* when grown under identical conditions.

S. epidermidis strain 66 represents a different type. Here the interpeptide chain is Gly-Gly-Gly-Gly-L-Ala and, therefore, the ϵ-amino group of lysine is obligatory substituted by L-alanine. Even when a large excess of glycine is added the amount of L-alanine remains constant and the interpeptide chain is not changed to a pentaglycine. When serine is added to the medium, it can replace some glycine (about 0·3 mole per mole of peptide subunit) similar to the other strains mentioned above.

Biosynthesis of Mucopeptide by *Pediococcus cerevisiae* and by a Substrain requiring Methicillin for Growth. By B. J. WILKINSON and P. J. WHITE (*Department of Microbiology, The University, Sheffield, Great Britain*)

From the parent strain *P. cerevisiae* ATCC 8081 a substrain (8081 CRD) was developed that required methicillin (or some other penicillins) for growth in a partly defined medium, and comparisons were made of cell walls from the two strains (White, P. J. (1968), *J. gen. Microbiol.* **50**, 85 and 107).

Organisms of each strain were harvested during exponential growth, washed and incubated at 37° in a solution containing: potassium phosphate buffer, pH 6·5; glucose; $(NH_4)_2SO_4$; $MgSO_4$; L-alanine; L-glutamic acid; L-lysine and L-aspartic acid (derived from work of Shockman, G. D., Conover, M. J., Kolb, J. J., Riley, L. S. & Toennies, G. (1961), *J. Bact.* **81**, 44). Little protein synthesis occurred in this solution, but mucopeptide was synthesized as shown by increase of turbidity of the suspended organisms or increase of bound hexosamine and ninhydrin-positive material in mucopeptide isolated by the method of Park J. T. & Hancock, R. (1960), *J. gen. Microbiol.* **22**, 249.

Similar rates of mucopeptide synthesis (about 10 % increase of turbidity/hr) occurred with both strains, but parent organisms formed mucopeptide for only 2–3 hr whereas organisms of strain 8081 CRD continued for about 2 hr longer. With both strains addition of other amino acids or acetate was not stimulatory, and chloramphenicol, streptomycin and aminopterin were not inhibitory, but synthesis was considerably reduced if any one component (except aspartate) were omitted from the suspending solution. Methicillin was not needed for mucopeptide formation by strain 8081 CRD whether organisms had been grown in presence of drug or without it (in medium without acetate supplemented with yeast extract). Benzyl penicillin, cloxacillin and cycloserine inhibited mucopeptide synthesis; strain 8081 CRD was not resistant to these compounds. Methicillin (above 250 μg./ml.) also inhibited synthesis by parent organisms but strain 8081 CRD was relatively resistant (4 mg. of methicillin/ml. caused only partial inhibition).

Influence of Growth Environment on the Cell Wall Anionic Polymers in some Gram-positive Bacteria. By D. C. ELLWOOD and D. W. TEMPEST (*Microbiological Research Establishment Porton, nr Salisbury, Great Britain*)

When cultured in a simple salts medium, in a chemostat, the cell wall composition of Bacillus species varied with the chemical nature of the environment. In particular, cell wall teichoic acid, which was present in substantial amounts when the organisms were limited in their growth by the availability of magnesium, was totally replaced by a teichuronic acid-type compound when growth was limited by the supply of phosphate. Other interesting Gram-positive organisms (Staphylococcus, Micrococcus) would not grow in a simple salts medium; nevertheless, it was possible to arrange chemostat conditions in which growth in a complex medium was limited by the availability of either magnesium or phosphate.

When *Staphylococcus aureus* H organisms were cultured in a Mg-limited environment in a chemostat (D = 0·1 hr^{-1}, 35°, pH 7·0) their walls (3·0 % phosphorus) contained a ribitol teichoic acid which accounted for over 30 % of the wall weight. But as with cultures of Bacillus species, when *S. aureus* H was cultured in a phosphate-limited medium, the wall phosphorus content fell to a low level (0·4 %) and the teichoic acid was totally replaced by a teichuronic acid.

The walls of *Micrococcus lysodeikticus* are known to contain small amounts of a teichuronic acid but not teichoic acid. As expected *M. lysodeikticus* walls contained little phosphorus (0·1 %) when the organisms were grown in a phosphate-limited medium in a chemostat (D = 0·1 hr^{-1}, 35°, pH 7·0); these walls contained an anionic polymer (30 % of wall weight) which was composed of glucose and a amino hexuronic acid. Most surprisingly, however, when grown in a Mg-limited medium, *M. lysodeikticus* synthesised a wall which contained 3·2 % phosphorus; extraction of these walls with 10 % trichloroacetic acid (4°, 24 hr.) yielded a polymer which was characterised as a glycerol teichoic acid.

Action of Uranyl Acetate on the Murein of *Spirillum serpens*. By H. J. PREUSSER and H. H. MARTIN (*Institut für Mikrobiologie, Technische Hochschule, Darmstadt, Germany*)

In cell walls of Gram-negative bacteria murein (peptidoglycan) is present as a thin, sheet-like polymer forming the cell-shaped 'murein sacculus'. Sectioned murein sheets of isolated sacculi from enteric bacteria and *Spirillum serpens* have a thickness of only 20–25 Å, suggesting that they may be monolayer complexes of polysaccharide and peptide moieties covalently cross-linked in two dimensions (Frank, H. & Dekegel, D. (1967), *Fol. Microbiol. (Prague)*, **12**, 227; Hofschneider, P. H. & Martin, H. H. (1968), *J. gen. Microbiol.* **51**, 23; Frank, H. & Martin, H. H., unpublished). Precise geometrical order and orientation patterns with respect to the overall shape of the cell can be expected from such a polymer and invite electron microscopical investigation. Untreated isolated sacculi of *Spirillum serpens* were highly transparent and virtually invisible in the electron microscope. 'Staining' with a variety of heavy metal salts revealed that uranyl acetate has a special affinity for murein. Treatment with 0·5 % aqueous uranyl acetate at pH 4·5 caused the following effects: (1) strong increase in electron density of the sacculi, (2) specific shrinkage in longitudinal direction, (3) cleavage into hoops of varying breadth, (4) complete disintegration. The significance of these observations for the interpretation of murein ultrastructure and the use of uranyl acetate as contrasting agent in the electron microscopy of bacterial cell walls will be discussed.

The Action of Penicillin on the Mucopeptide of *Escherichia coli*. By W. ROBERTS, A. DAVIES and M. K. R. BURNHAM (*Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, Great Britain*)

Gram negative organisms are, in general, less susceptible than Gram-positive organisms to penicillin although low concentrations of penicillin reduce the cross-linking of mucopeptide synthesised by a particulate enzyme system from *Escherichia coli* (Izaki, K., Matsuhashi, M. & Strominger, J. L. (1968), *J. biol. Chem.* **243**, 3180). In *Staphylococcus aureus* penicillin reduces both the cross-linking and the rate of synthesis of mucopeptide (Tipper, D. J. & Strominger, J. L. (1968), *J. biol. Chem.* **243**, 3169). The low penicillin sensitivity of *E. coli* could be due to enzymes being less affected by penicillin, to permeability barriers or a decreased requirement for cross-linking. *E. coli* ATCC 11229 was grown in glucose (uniformly labelled with ^{14}C) ammonium salts medium without and with sodium penicillin G 10 (30) μg./ml. for 90 min. at 37°: viabilities were then 20 (1·0) % of the control. Relative radioactivities with respect to the control were: whole bacteria 1·23 (0·86); whole walls 1·44 (0·80) mucopeptide 0·64 (0·24). The ratio alanine: glutamic acid (1·84 in control) was 2·28 (2·63) and the percentage of diaminopimelic acid found as the dinitrophenyl derivative following dinitrophenylation of the mucopeptide was 26·5 (26·0) against 15·7 in the control. The percentage of mucopeptide hydrolysed to monomer by lysozyme increased from 10 % to about 25 % following penicillin treatment.

These results indicate that penicillin although at less than the minimal inhibitory concentration (at 10 μg./ml.) causes some killing during the experiment, reduces both the cross-linking and the rate of synthesis of mucopeptide while at above the minimal inhibitory concentration (which was rapidly lethal) has only little more effect on cross-linking but greatly reduces the rate of synthesis of mucopeptide.

Bacterial N-Acetyl-β-Glucosaminidases. By R. C. W. BERKELEY, J. W. DROZD and J. M. BROUGHALL (*Department of Bacteriology, The Medical School, University Walk, Bristol Great Britain*)

N-acetyl-β-glucosaminidases have been detected by Woollen, J. W., Walker, P. G. & Heyworth, R. (1961), *Biochem. J.* **79**, 294) in cells of several bacteria, but with preparations of several others no activity was found under the test conditions which involved incubation of the enzyme preparation with *p*-nitrophenyl-*N*-acetylglucosaminide at pH 4·5. The pH optimum of some bacterial *N*-acetyl-β-glucosaminidases is, however, more alkaline than this ranging from pH 6·3 to pH 7·5. The higher value is the optimum of the *E. coli* B enzyme which

exhibited no activity at pH 4·5 (Maass, D., Pelzer, H. & Weidel, W. (1964), *Z. Natuff.* **19***b*, 413). Some strains of certain of the organisms in which Woollen *et al.* (1961) failed to find *N*-acetyl-β-glucosaminidase are known to hydrolyse chitin. Almost invariably two enzymes are involved in this hydrolysis—a chitinase and a chitobiase which hydrolyses other β-*N*-acetylglucosaminides in addition to *N,N'*-diacetylchitobiose.

We have grown several bacteria in a chitin-salts medium supplemented where necessary by 0·5 % casein hydrolysate and tested for *N*-acetyl-β-glucosaminidase under conditions allowing detection of enzymes with pH optima of both about pH 4·3 and about pH 7·3. Many, including some of those previously regarded as lacking this enzyme, were found to exhibit a high level of activity towards *p*-nitrophenyl-*N*-acetyl-β-glucosaminide. The pH optima of crude enzyme preparations from 5-day-old cultures of several Bacillus species and one *Escherichea coli* strain were found to be between pH 7·0 and pH 7·9. *N*-acetyl-β-glucosaminidase from *B. subtilis* has been examined in more detail and there is evidence that more than one enzyme is produced. The role of these enzymes and the possible involvement of *N-acetyl-β*-glucosaminidases in cell wall metabolism will be discussed.

Behaviour of some Enzymes of the Cell Envelope of Baker's Yeast During the Digestion of the Cell Wall. By T. NURMINEN and H. SUOMALAINEN (*Research Laboratories of the State Alcohol Monopoly, Alko, Helsinki, Finland*)

The carefully isolated cell wall of baker's yeast contains fragments of plasma membrane (Suomalainen, H., Nurminen, T. & Oura, E. (1967), Federation of European Biochemical Societies, Fourth Meeting, Oslo, *Abstr. Commun.*, p. 111, and *Suomen Kemistilehti* B **40**, 323; Nurminen, T., Oura, E. & Suomalainen, H. (1968), Federation of European Biochemical Societies, Prague, *Abstr. Commun.*, p. 115). The isolated cell walls showed no activities of intracellular enzymes investigated. The isolated cell walls were found to contain considerable amounts of saccharase and phosphatases hydrolysing *p*-nitrophenyl phosphate, ATP, ADP, thiamine pyrophosphate and inorganic pyrophosphate with optimum activity at pH 3–4, and an activity of Mg^{2+}-dependent ATPase at neutral pH. The activities appearing in the cell walls were mostly released into the medium during enzymic digestion, as has become apparent in the preparation of yeast protoplasts. The bulk of Mg^{2+}-dependent ATPase remained in the plasma membrane preparation. Accordingly, it may be assumed that the enzymes released into the medium are located in the cell wall outside the plasma membrane, whereas the Mg^{2+}-dependent ATPase is an enzyme of the plasma membrane.

The Chemical Characteristics of Endotoxin from *Bacteroides fragilis* By T. HOFSTAD (*Laboratory of Oral Microbiology, The Gade Institute, The University of Bergen, Schools of Medicine and Dentistry, Bergen, Norway*)

Endotoxin has been prepared from *Bacteroides fragilis*, strain NCTC 9343, by phenol–water extraction of acetone-dried cells, followed by purification by ultracentrifugation and subsequent treatment with ribonuclease and deoxyribonuclease.

The lyophilized endotoxin contains 6 % nitrogen, 40 % protein (Folin), 33 % neutral sugar (orcinol), 15 % lipid as fatty acid esters, and 7 % hexosamine. Paper chromatography of acid hydrolysates revealed glucose, galactose, rhamnose, fucose, trace amounts of mannose, glucosamine and galactosamine, of which glucose was the major sugar component. In addition, a fast-moving compound has been detected on paper chromatography of acid hydrolysates. The compound, which possibly is a novel amino sugar, reduces silver nitrate and gives a positive ninhydrin reaction. In the Elson–Morgan reaction it produces an absorption spectrum similar to that of glucosamine. On Dowex 50 (H^+ form) the compound has a chromatographic mobility of 1·37 with respect to glucosamine when eluted with 0·33 N-HCl.

Heptose and 2-keto-3-deoxy-octonate (KDO) has not been demonstrated. The absence of these sugars, together with the presence of the novel sugar compound, suggest that *B. fragilis* endotoxin has a structure quite different from that of endotoxins of aerobic Gram-negative bacilli. In this connection it is of great interest that heptose and KDO are lacking also in

endotoxin from *B. melaninogenicus* (Hofstad, T.) 1968, *Arch. oral Biol.* **13**, 1149), In contrast, endotoxin from oral strains of *Fusobacterium* contains both KDO and heptose (Kristoffersen, T. 1969. *Acta path. microbiol. scand.* in Press).

The Structure of Colanic Acid. By I. W. SUTHERLAND (*Department of General Microbiology, University of Edinburgh, Great Britain*)

The formation of a polysaccharide slime common to many species of Enterobacteriaceae has been noted by several workers (Kauffmann, F. (1954), *Enterobacteriaceae.* Copenhagen: Munksgaard; Anderson, E. S. & Rogers, A. H. (1963), *Nature, Lond.* **198**, 714). Although the control of synthesis of this polysaccharide has been studied, there has been little reported on its structure other than identification of the component sugars and the aldobiuronic acid 3-*O*-β-D-glucuronosyl-galactose (Roden, L. H. & Markovitz, A. (1966) *Biochim. biophys. Acta*, **127**, 252). We have confirmed the presence of D-glucose, D-galactose, D-glucuronic acid and L-fucose. In addition an *O*-acyl group has been identified as acetyl and Rees and his colleagues (unpublished) have isolated a complex of pyruvate and galactose.

The colanic acid from representative strains of *Escherichia coli, Aerobacter cloacae* and *Salmonella typhimurium* has been isolated, purified and found to be identical in all respects tested. By partial acid hydrolysis a number of neutral and charged oligosaccharides have been obtained from all three preparations. The structure of these fragments has been determined and several were found to contain pyruvate. No acetylated fractions were obtained. Oligosaccharides were also isolated from partial acid hydrolysates of carboxyl reduced colonic acid. The glucose emanating from glucuronic acid being identified by tritiation. On the basis of these results, together with periodate oxidation and other tests, a hexasaccharide repeating unit containing 2 moles fucose, 2 moles galactose and 1 mole each of glucose, glucuronic acid, acetyl and pyruvate is proposed.

The significance of this structure in a biosynthetic system for colanic acid will be discussed.

The Relation of K Antigens to the Complement Sensitivity of Strains of *Escherichia coli* By A. A. GLYNN and C. J. HOWARD (*Wright-Fleming Institute, St Mary's Hospital Medical School, London, Great Britain*)

The structure of the bacterial cell wall is of great importance in the analysis of the bactericidal and bacteriolytic action of fresh serum. Previous work (Glynn (1969), *Immunology*, **16**, in Press) suggests that the probable sequence of events in *Escherichia coli* is damage to the lipopolysaccharide lipoprotein layer by complement followed by lysozyme action on the mucopeptide. However, some smooth strains of *E. coli* are resistant to complement and antibody and Sjostedt (1946, *Acta. path. Scand.* Suppl. 63) suggested that this was due to K antigens. Later work by Muschel (1960, *Proc. Soc. exp. Biol. Med.* **103**, 632) confirmed this.

Complement resistance in *E. coli* is not related to any particular antigenic type of K, but resistant strains in general contain more K than do sensitive strains. This has been shown most clearly using 4 strains of O6 K 13 differing widely in complement resistance. However, qualitative differences are also important. A significant property of K antigens is their ability to suppress agglutination in heterologous systems. Pure K antigen from a complement resistant strain of *E. coli* possessed more agglutination inhibiting activity weight for weight than purified K from a sensitive strain. Using ^{125}I labelled antibody it has been shown that K antigens decrease the amount of antibody bound by red cells and also affect complement action directly. Chromatographic analysis and chemical modifications suggest that both molecular size and charge may be involved.

SESSION B

Egg Production and Disease: Adenovirus. By D. M. BERRY (*Virus Vaccines Department, Glaxo Laboratories, Ltd., Greenford, Middlesex, Great Britain*)

The finding that adenoviruses were frequently isolated, together with infectious bronchitis (I.B.), virus in laying flocks apparently showing typical I.B. infection (Berry, D. M. & Stokes, K. J. (1968), *Vet. Rec.* **82**, 157) prompted this study to investigate the effect of adenovirus on egg production in chicken. Attempts were made to isolate virus from laying flocks with severe depression of egg production. Adenovirus isolates were compared with G.A.L. virus supplied by I. A. Macpherson, Institute of Virology, Glasgow, and C.E.L.O. virus (E.V. 89) supplied by D. A. McMartin, Veterinary Laboratory, Lasswade, in cross-neutralization tests, but no antigenic differences were demonstrable.

Pullets from a flock free from I.B. virus, adenovirus and *Mycoplasma gallisepticum* were raised in isolation and transferred to individual laying cages at point of lay. Individual egg production records were kept from the point of lay until the end of each experiment. Birds laying less than 5 eggs/week before challenge were excluded from the experiments. Groups of twenty pullets were exposed either to intranasal infection and intramuscular inoculation of 10^9 EID_{50} adenovirus/bird alone, or together with the inoculation of 10^9 ID_{50} *M. gallisepticum* strain S6 into the thoracic air sacs, or together with intranasal and intramuscular inoculation of 10^6 EID_{50} I.B. virus Massachusetts strain/bird and 10^9 ID_{50} *M. gallisepticum* into the thoracic air sac of each bird.

In susceptible laying hens, adenovirus infection by itself showed a 10% depression of egg production which lasted 3 weeks.

The egg production of birds infected with both adenovirus and *M. gallisepticum* was reduced for a longer period, and this trend was accentuated further in the group infected with adenovirus, I.B. virus and *M. gallisepticum*. The egg production of birds infected with the three agents was more severely depressed than the egg production of birds infected with I.B. virus and *M. gallisepticum*, but not with adenovirus. This is important in view of our finding adenovirus in three out of twelve infections of laying hens characterised by depressed egg production, in which I.B. virus had also been isolated.

It is clear that adenovirus infection affects egg production in a way indistinguishable from I.B. infection, particularly when such infection is enhanced by infection with *M. gallisepticum*. It is seen that the depth and duration of any depression of egg production due to infection is dependent on the number of agents present.

Comparison of Two Canine Adenoviruses. By R. MARUSYK and E. NORRBY (*Department of Virology, Karolinska Institutet, Stockholm, Sweden*)

Morphological and biophysical studies have revealed a unique relationship between infectious canine hepatitis (ICH) virus and the lesser known canine adenovirus, designated infectious canine laryngotracheitis (ICL) virus. Serological testing has shown the two virus types to be very closely related with regards to neutralization and haemagglutination-inhibition tests. However, anion-exchange chromatography has revealed a distinct difference in the elution pattern of the soluble components of each virus type. Electron micoscopic examination of the virions and components has also shown a substantial difference in the length of the fibre components. Preliminary data from further studies with these types will also be presented.

Structural Aspects of the Adenovirus type 2 Penton Antigen. By U. PETTERSSON and S. HÖGLUND (*The Wallenberg Laboratory, University of Uppsala, Sweden*)

The penton antigen from adenovirus type 2 has been purified by DEAE-chromatography, agarose chromatography and preparative polyacrylamide electrophoresis. The final product is homogeneous by electronmicroscopy, immunoelectrophoresis, polyacrylamide electrophoresis and analytical ultracentrifugation.

Penton antigen so prepared has a sedimentation coefficient of 10·5 and a molecular weight around 400,000. A minimum of three antigenic specificities can be demonstrated with immunodiffusion.

Electron microscopy shows a complex structure with a prismatic head, sometimes showing five-fold symmetry, and a threadlike tail. Low concentrations of trypsin selectively inactivates the cytopathic effect of the type 2 penton. At high trypsin concentrations the main part of the vertex capsomer antigenicity and morphology is also lost. Rabbit antisera to pure pentons contain low titres of neutralizing antibodies when assayed by inhibition of plaque formation. The use of the fluorescent focus assay reveals, in contrast, high neutralizing activity in these sera. Treatment of penton antigen with pyridine releases free vertex capsomers which are immunologically and morphologically intact and have the ability to induce cytopathic changes in KB-cell cultures.

Aspects on the Neutralization of Adenoviruses. By GÖRAN WADELL (*Department of Virology Karolinska Institutet, Stockholm, Sweden*)

Non-vertex capsomeres, hexons, have been considered to contain the antigenic specificity, which can induce the production of antibodies capable of neutralizing the infectivity of adenoviruses (Wilcox & Ginsberg (1963), *Proc. Soc. expt. Biol. Med.* **114**, 37–42; Kjellen & Pereira (1968), *J. gen. Virol.* **2**, 117–185; Norrby, (1969), *Virology*, in Press). Hexons of adenovirus type 2 have been separated into two different populations by anion exchange chromatography and by iso-electric focusing techniques. The immunological properties of these two populations of hexons have been characterized in complement-fixation and neutralization tests.

In further studies the enhancing capacity of sheep anti-rabbit sera on the neutralizing capacity of adenovirus specific rabbit-antisera was examined (Ashe & Notkins (1966), *Proc. nat. Acad. Sci. U.S.A.* **56**, 447). The relative effect on antisera against different structural components of various representative members of Rosen's three subgroups was evaluated.

Adsorption and Fate of Adenovirus type 5 Hexon antigen in HeLa cells. By P. J. SANDERSON (*National Institute for Medical Research, Mill Hill, London, Great Britain*)

Crystalline Hexon antigen (kindly supplied by Dr H. G. Pereira) was labelled with ^{131}I by the method of Greenwood, Hunter & Glover (1963, *Biochem. J.* **89**, 114) and mixed with suspensions of HeLa cells under different conditions. Adsorption of the labelled antigen was determined by the loss of radioactivity in the supernatants of test and control mixtures. Break down and elution of adsorbed antigen by cells was determined by changes in the amount of antigen precipitated by 10 % Trichloracetic acid in supernatants and cells.

It was found that up to 3 % of the labelled antigen added to a HeLa cell suspension, equilibriated to a temperature of 4°C, was adsorbed after 1 h by $1{\cdot}0 \times 10^6$ cells and that adsorption was dose dependant over the range of antigen concentrations tested. Adsorption was increased by mixing the antigen with specific antibody and by pre-exposing cells to partially purified preparation of adenovirus type 5 Penton antigen.

In experiments where the antigen was adsorbed to cells at a temperature of 4° and the cells washed and transferred to a temperature of 37°, it was found that a significant proportion of the adsorbed antigen was converted to acid soluble products, some of which appeared in the suspending medium. Thus adsorption studies carried out at a temperature of 37° may underestimate or fail to show adsorption unless the extent of cellular break down of the adsorbed antigen is determined also.

Cellular break down of the antigen was temperature dependant and inhibited by sodium fluoride. Break down was found in HeLa cell monolayers, in suspensions of chick fibroblasts, monkey kidney cells and mouse peritoneal exudate cells. It did not occur in medium from which cells shown to be capable of breaking down antigen had been removed. When different quantities of antigen were adsorbed to cells a similar proportion of adsorbed antigen was broken down, indicating that the amount of break down was dependant upon the quantity of antigen adsorbed over the range of antigen concentrations tested.

In experiments where adsorption and break down were measured concurrently in cell suspensions equilibriated to a temperature of 37° it was found that antigen adsorption and break down both occurred rapidly and it was concluded that these events are closely associated.

These results and those obtained with partially purified preparations of the other adenovirus type 5 capsid antigens will be discussed.

Cross-reaction Between TSTAs of Tumors Induced by Adenoviruses of Groups A and B. By J. ANKERST and H. O. SJÖGREN (*Department of Medical Microbiology, University of Lund, Sweden*)

Viral neoplasms are characterized by tumor specific transplantation antigens (TSTA) which are identical, or have at least some identical components in all the neoplasms induced by the same virus but are usually different in tumors induced by different viruses. Adeno 12 tumors possess this type of cross-reacting TSTA. The adeno viruses might be divided in three groups with regard to oncogenicity: group A (including type 12), group B (including weakly oncogenic virus types), and finally the non-oncogenic virus types. It has been studied whether the TSTAs of tumors induced by adenovirus types belonging to groups A and B might cross-react or whether the TSTA are 'subgroup' specific. It was demonstrated by isograft rejection tests that hamster adenovirus group B tumor cells are immunogenic with regard to the TSTA adenovirus group A tumors. Furthermore, by use of ^{51}Cr-cytotoxic tests *in vitro* it was shown that sera of animals immunized against adenovirus group A tumors were cytotoxic to adenovirus group B tumor cells.

Factors Affecting Replication of the Lactate Dehydrogenase-elevating Virus (LDH virus) in Peritoneal Macrophage. By R. EVANS (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surrey*)

Replication of the LDH-virus in peritoneal macrophage cultures to high levels comparable to those seen in the infected mouse has been previously reported (Evans, R. (1967), *J. gen. Virology* **1**, 363). Further experiments have shown that a consistent response to a standard of virus, $10^{6\cdot0}$ ID_{50} ml. was obtained when cultures were inoculated 24 h after preparation. Peak titres up to 10^{9} ID_{50}/ml. were reached by 3 days, followed by a decline to below the ID_{50} within 21 days. This decline could not be explained by the production of interferon or DNA-directed protein inhibitors since actinomycin-D was without effect on LDH-virus replication in these cultures. Lymphocytes, frequently found in macrophage cultures during the first few days, were shown not to be involved in the replicative process. Peak virus titres were reached more quickly when doses of virus greater than 10^{6} ID_{50}/ml. were inoculated into cultures, though the yield of virus was no greater. Doses less than 10^{3} ID_{50}/ml. failed to infect cultures. The possibility that susceptibility is related to the length of the time the cells are maintained *in vitro*, and the presence of an apparently small percentage of susceptible cells will be discussed.

Inhibition by Frog Virus 3 of Vaccinia Virus and Host Cell DNA Replication in KB Cells. By ANDRÉ KIRN and ANNE-MARIE AUBERTIN (*Groupe de Recherches de l'I.N.S.E.R.M. sur la Pathogénie des Infections Virales-3, rue Koeberlé 67-Strasbourg, France*)

Frog virus 3 (FV_3) a desoxyribovirus which replicates in the cytoplasm of amphibian and mammalian cells at low temperature (Granoff, A. *et al.* (1966), *Virology*, **29**, 133) produces an inhibition of host cell DNA synthesis (Macauslan, B. & Smith, W. R. (1968), *J. Virol.* **2**, 1006).

It was of interest to know if FV_3 had also an effect on viral DNA replication. We have undertaken a study on the action of FV_3 on host cell and vaccinia virus DNA replication in a non-permissive system (KB cells) at non permissive temperature (37°) for FV_3 development.

When monolayers of KB cells were preinfected during 4 hours with FV_3 (10 p.f.u./cell) and then infected with vaccinia virus and labelled, ^{3}H thymidine uptake in the nuclei as well as in the cytoplasmic fraction of the cells was completely inhibited.

However, vaccinia virus was uncoated as it could be demonstrated by electron microscope examinations. A similar inhibition of cellular and viral DNA synthesis was noted when both viruses were mixed and put on KB cells. Moreover, in suspended KB cells infected with vaccinia virus, host cell and vaccinia virus DNA replication were completely blocked by superinfecting the cells 1 h later with FV_3. We have shown that the fraction of the virus responsible for inhibition is thermolabile.

Compositional Heterogeneity of the DNA of Phage P2. By R. B. INMAN (*Department of Biophysics, University of Wisconsin, Madison, U.S.A.*) and G. BERTANI (*Department of Microbiology, Karolinska Institutet, Stockholm, Sweden*

The DNA of bacteriophage P2 (which is double stranded, linear, approximately 13 μ long, and able to form rings reversibly *in vitro* through terminal cohesive sites) showed at least three well-defined steps in optical density with progressive heat denaturation (melting curves). This suggested heterogeneity of composition in respect to nucleobase ratio.

Electron microscope studies of partially denatured P2 DNA showed localized zones, clearly identifiable by the temperature at which they start denaturing. These observations indicate that the heterogeneity of composition is intramolecular, and yield highly specific denaturation maps for the P2 chromosome.

P2 *Hy dis*, a phage believed to be genetically identical to P2 except for a small segment of its chromosome near one end of the genetic map, gives multistep denaturation curves, by optical density, and denaturation maps, by electron microscopy, which are almost superimposable to those of P2. A small, but probably significant difference exists, however, and this leads to a tentative orientation of the physical (electron microscopy) map in respect to the genetic map of these phages.

Further Studies of P2 Associated Eduction in *Escherichia coli* K12. By M. G. SUNSHINE (*Department of Microbiology, Karolinska Institutet*) *and* B. L. KELLY (*Department of Microbiology, San Diego State College, California, U.S.A.*)

Escherichia coli K-12 strains lysogenic for phage P2 in chromosite H, closely linked to histidine, can spontaneously lose several adjacent genes when being cured of their prophage. This phenomenon, termed eduction, also occurs upon infection of sensitive K-12 strains with P2. Eduction has not been observed to occur with K-12 strains lysogenic for P2 in other chromosites or with *E. coli* C (Kelly, B. L. T. & Sunshine, M. G. (1967), *Biochem. biophys. Res. Comm.* **28**, 237). The deletions have now been shown to include the closely linked genes for histidine biosynthesis, 6-phospho-gluconic dehydrogenase (*gnd*), and part or all of the *rfb* locus governing the biosynthesis of nucleotide sugars. Eduction occurs with several different mutants of P2 which are capable of lysogenization, but not with P2 mutants unable to lysogenize. Specifically, it does not occur upon infection with P2 *int* mutants, i.e. mutants which have lost the ability to integrate as prophages in the host chromosome. All *E. coli* K-12 strains so far tested, including one recombination deficient strain, give rise to eductants. The mechanism favored for eduction is a recombination event, promoted by the *int* function of the phage, involving not only the normal chromosite but also another homology region on the host chromosome which defines the end of the deletion.

Control of Prophage Integration in Bacteriophage P2. By L. ELIZABETH BERTANI (*Institutionen för Mikrobiologi, Karolinska Institutet, Stockholm, Sweden*)

Following superinfection of an immune host, bacteriophage P2 is able to attach with great efficiency to an unoccupied attachment site. The attachment depends on the activity of a gene that presumably determines synthesis of P2 integrating enzyme (integrase). This result suggests that, unlike the case for phage λ, the synthesis of P2 integrase is not under the control of the ordinary phage repressor. However, no integrase activity can be found in P2 lysogens. Models to explain this paradoxical situation will be discussed.

SESSION C

The Location of Chemical Components on Ultra-thin Sections of *Bacillus cereus* Embedded in Glycol Methacrylate By P. D. WALKER (*Wellcome Research Laboratories, Beckenham, Kent, Great Britain*)

The details of sporulation and germination in bacteria have been extensively investigated using the technique of ultra-thin sectioning. These investigations have invariably used osmic acid as a fixative and epoxy resins for embedding. The structure of *Bacillus cereus* has been investigated during various stages of sporulation and germination after aldehyde fixation and embedding in a water soluble medium, Granboulan & Leduc (1967). In addition the susceptibility of ribosomes to digestion by ribonuclease has been investigated by floating ultra-thin sections of the organism on to the enzyme. Ribosomes were readily removed from ultra-thin sections of young vegetative cells by digestion of ribonuclease. Increased resistance to digestion was observed during development of the spore coat which was associated with the beginning of refractability under phase contrast. Similar differences in susceptibility were observed during germination of spores.

Polysaccharides were located on the ultra-thin sections of the organisms using the silver-methenamine staining technique, Walker & Short (1968). Their location corresponds to that previously described using osmic acid fixed material in epoxy resins.

Structural Changes in *Escherichia coli* Cells Infected with ØX-174 type Bacteriophages. By D. E. BRADLEY (*Department of Zoology, University of Edinburgh, Great Britain*)

The ØX-174 type *Escherichia coli* bacteriophages are morphologically identical, being 250 Å icosahedra with large apical capsomeres. They contain single-stranded DNA. Three serologically unrelated groups, represented by phages $\alpha 3$, ØX-174 and St/1, have been identified; each has a different host range. St/1 alone growing on male strains. The electron microscopy of infected bacteria shows that phage $\alpha 3$ produces somewhat different morphological changes compared to ØX-174 and St/1.

The cytological effects associated with infection by ØX-174 and St/1 are similar. First, intracellular phage is assembled there being no obvious changes in the structure of the cell envelope. After assembly, however, the plasma membrane begins to break up and small holes appear in the cell wall. The cell contents and phage progeny pass through the holes. Alternatively, a single hole may form in the cell wall and plasma membrane at the midpoint of the cell. In this case, the plasma membrane remains intact, but retracts from the poles.

With phage $\alpha 3$ the first signs of infection are a retraction of the plasma membrane from the poles of the cell and the appearance of a bulge at the midpoint. This swells up until a typical spheroplast is formed. Lysis occurs in two stages. First, the cell wall breaks and curls back from the spheroplast bulge, then the plasma membrane disintegrates releasing the contents. Spheroplast formation is in complete contrast to the production of excess cell wall and plasma membrane associated with infection by the single-stranded DNA filamentous phages.

The significance of these observations is that morphologically identical phages have a noticeably different effect on the host cell.

The Ultrastructure of the Protein-lipopolysaccharide Surface of *Spirillum serpens*. By R. G. E. MURRAY and F. L. A. BUCKMIRE. (*Statens Seruminstitut, Copenhagen, Denmark and Department of Bacteriology, University of Western Ontario, London, Canada*)

The outer surface of Spirillum species presents a hexagonal array of macromolecules (Houwink, A. L. (1953), *Biochim. biophys. Acta*, 10, 360); details of this regular structure (RS) have been described for *Spirillum serpens* VH (Murray, R. G. E. (1963), *Can. J. Microbiol.* 9, 381). Ring-like units (*c.* 85 Å diameter) are arrayed with a repeat interval of *c.* 145 Å and have Y-form linkers. This type of surface has been identified on 6 of 12 strains of *S. serpens* and it has also been found that the integrity of the RS layer requires Ca^{2+} (Steed-Glaister, P. D., Maier, S. M. & Murray, R. G. E., unpublished). The structural description is

now amplified by micrographs of freeze etching preparations and of isolated and reaggregated components of the RS layer.

The RS layer, removed cleanly from growing cells by heat treatment (60° for 1 hr) in 0·01 M-$CaCl_2$, consisted of sheets of units or tubes of units arrayed on an amorphous backing material; the layers and components can be identified by freeze etching of intact or treated cells. RS material definitely lies outside the 'double track' profile of the cell wall. The units were removed and dissolved by M-guanidine-HCl without solution of LPS backing layer (apparently lipopolysaccharide). The solution of RS units (protein in nature) obtained by differential centrifugation and after dialysis was free of identifiable ring-like units and was monodisperse in the ultracentrifuge (4·1 S); yet units complete with Y-linkers of expected size and spacing were formed from this solution when interacting with the surface of the RS-free VHL mutant (which appears to have the LPS layer).

The concept of Y-linkers as a fabric on which the units sit (Murray, R. G. E. *op. cit.*) is untenable; they may be a consequence of assembly of the open V or short stemmed Y forms visible in preparations of the protein. Both hydrogen and salt (Ca^{2+}) bonds may unite RS and LPS components and Ca^{2+} is required for RS production.

Some Electron Microscopic Features of *Cardiobacterium hominis*. By ALICE REYN, R. G. E. MURRAY and A. BIRCH-ANDERSEN (*Neisseria and Biophysics Departments, Statens Seruminstitut, 2300 Copenhagen S, Denmark*)

Cardiobacterium hominis (Slotnick, I. J. and Dougherty, M. (1964), *Antonie van Leeuwenhoek* **30**, 261) was chosen for study because it was reported to be Gram-negative but 'with many cells retaining discrete Gram-positive areas'. In our hands, three strains (NCTC 10426 and 10427, SSI 1742) of these organisms were small pleomorphic but definitely Gram-negative organisms without any Gram-positive patches when grown on 10 % horse blood broth agar and on two kinds of 'chocolate agar'.

Twenty-hour cultures were harvested from these media with 0·1 % YAP medium (yeast extract-sodium acetate-peptone medium), the resulting suspensions fixed with osmium tetroxide (Ryter, A. & Kellenberger, E. J. (1957), *Z. Natur.* **13***b*, 597) or prefixed with 1 % glutaraldehyde for one hour preparatory to fixation with 1 % osmium tetroxide. Profiles from sections showed similar structures in each of the three strains. The cell wall was of a coherent Gram-negative type, each of the elements remaining closely apposed in sections. The distinctive features were as follows: The 'doublet' layer of the cell wall (unit membrane ~ 75 Å) was sandwiched between dense outer and inland layers, of which the former (~40–50 Å showed a hint of repeating structure, and the latter was very dense, of variable thickness (50–100 Å) and usually adherent to the plasma membrane. Intrusions of the plasma membrane formed irregular masses near the periphery or partially filled membrane-lined vesicles; one strain (NCTC 10426) showed numerous membranous lamellae at the periphery. Each strain showed caps of 2–300 Å thickness and irregular, tufted density restricted to the poles. Cytoplasm and nucleoplasm was not remarkable.

Cell wall fragments were obtained from ultrasonically disrupted cell suspensions by differential centrifugation and negatively stained with 1 % ammonium molybdate pH 6·5, 1 % lithium tungstate pH 7·5 and 1 % uranyl formate pH 4·5–5 with excellent and equivalent differentiation of surface structure. The outer layer of the wall consisted of a rectangular close packing array of 30–40 Å particles with a repeating distance of 50–60 Å. This represents a new type of surface structure on a Gram-negative bacterium. The distinctive polar caps were not found in negatively stained preparation of fragmented or whole cells.

A Bacteriolytic Hexosaminidase from *Staphylococcus aureus*. By T. WADSTRÖM and K. HISATSUNE (*Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden*)

The production of extracellular lysozyme-like enzyme(s) is a frequent property of staphylococcal strains and has been reported to be strongly correlated to pathogenicity. *Staphylococcus aureus* strain M 18 has been cultivated in CCY-medium in a batch and a continuous process.

The lytic activity was assayed on whole cells of *Micrococcus lysodeikticus*. Ethanol precipitation (35 %, v/v; 0–to −10°; pH 7·0), CM-Sephadex chromatography, isoelectric focusing, and Sephadex G-150 gel filtration were used in this order to purify an extracellular hexosaminidase (pI 9·5 ± 0·05). The purity of this enzyme was demonstrated by analytical acrylamide electrophoresis in several buffer systems and gel concentrations. The release of reducing sugar and *N*-acetylglucosamine from *M. lysodeikticus* cell walls by a partly purified enzyme has earlier been reported (Wadström and Hisatsune, FEBS congress, Prague, 1968, abstract 422). The purified enzyme has been used for investigation of the bacteriolytic spectrum. Degradation products of *M. lysodeikticus* cell walls and *S. aureus* strain 3528 mucopeptide are analysed after chromatography on Sephadex G-10 and Dowex 50 W-X8.

Different strains (v 8, Wood, 46, Foggie, r 1, 524 and Duncan) of *S. aureus* earlier investigated for the production of enzymes and toxins, were all found to produce an activity which was lytic for *M. lysodeikticus* and was isoelectric at pH 9·5. This was shown by the method of isoelectric focusing.

Some data will be presented on the autolytic system in some of the strains investigated.

The Production of Bacteriolytic Enzymes by *Staphylococcus aureus* in Batch and Continuous Culture. By S. Arvidsson, T. Holme and T. Wadström (*Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden*)

The production of extracellular bacteriolytic enzymes by *Staphylococcus aureus* strain m 18 has been studied. *Micrococcus lysodeikticus* was used as the test organism. One of the enzymes has been shown to be a hexosaminidase. Preliminary experiments showed that CCY medium gave a better enzyme yield than Brain Heart Infusion Broth (Difco) and Trypticase Soy Broth (BBL). Batch cultivations performed in a stirred fermenter increased the yield five-fold compared to that obtained in shaken flasks. A rapid increase in lytic activity was obtained during the last three hours of the logarithmic phase, followed by a rapid decrease. Less than 50 % of the activity remained two hours after the onset of the stationary phase. An increased aeration with formation of an excess of foam was found to speed up this process. Maximal lytic activity was produced at pH 6·5–7·5 and was also influenced by the carbon source. These and further recent results obtained in continuous culture experiments will be discussed. In continuous culture, grown under similar conditions as in batch cultivation, the yield of lytic activity was significantly increased. The dilution rate applied was 0·5 hp^{-1}. When the dilution rate was changed to 0·3 hp^{-1}, a rapid increase in bacterial density and lytic activity was obtained.

Effects of Antibiotics on Bacterial Lysis and Intracellular Ribosomes. By Margot Kogut (*Department of Biochemistry, King's College, Strand, London, Great Britain*)

Rapid release of ribosomes from bacteria by lytic methods has facilitated examination of ribosome patterns under different growth conditions and antibiotic treatment (Luzzatto *et al.* (1968), *Proc. natn. Acad. Sci.*, **60**, 873). However, interpretation of ribosome profiles obtained by these methods has assumed equal extent of lysis and release of ribosomes in treated and control cultures. By prelabelling treated cultures with ^{14}C-uracil and harvesting together with a ten-fold excess of unlabelled control cultures in exponential growth, and differences due to harvesting, lysis and extraction of ribosomes can be minimized. After lysis of the combined cultures (Godson & Sinsheimer (1967), *Biochim. biophys. Acta* **149**, 476, 489) centrifugation through sucrose gradients yields simultaneous ribosome profiles of controls and treated culture. Since treatment of sensitive cultures of *Escherichia coli* during growth on defined media with 20–30 μg./ml. of dihydrostreptomycin produces gradual decline of growth rates (Kogut, Lightbown & Isaacson, (1965), *J. gen. Microbiol.* **39**, 165; Kogut & Harris, (1969), *Europ. J. Biochem.* in Press) ribosome profiles were obtained as above at various times during streptomycin treatment. They showed, compared with controls, a gradual diminution of the larger polysomes concomitant with increasing reduction in growth rate. Complete disappearance of polysomes was only observed some time after growth had ceased. These patterns resembled those obtained during growth inhibition by chloramphenicol, but differed from those

produced by progressive exhaustion of nitrogen source. Quantitative estimation of the extent of lysis showed a progressive decrease during growth inhibition by dihydrostreptomycin; this was more extensive during chloramphenicol treatment, but amino acid exhaustion had a lesser effect. Sucrose gradient centrifugation of material re-extracted from the cell-wall-membrane fraction showed similar patterns to those obtained immediately after lysis. Thus no preferential retention of certain ribosome classes had occurred in antibiotic-treated cultures.

Effect of Light on Malate Oxidation by Membranes of a Carotenoidless Mutant of *Sarcina lutea*. By J. PREBBLE and S. HUDA (*Department of Biochemistry, Bedford College, University of London, Great Britain*)

Protection of bacteria by carotenoids against photodynamic action of light has been demonstrated in several organisms including *Sarcina lutea*. Illumination of carotenoidless cultures resulted in death at very high light intensities (sunlight) or at low light intensities in the presence of a sensitizer, toluidine blue. From experiments with whole cells, the site of light action was believed to be the cell membrane where carotenoids are located. (Mathews, M. M. & Sistrom, W. R. (1960), *Arch. Mikrobiol.* **35**, 139).

The light sensitivity of malate oxidase activity (demonstrated by a depressed oxidation rate) in membranes prepared by a lysozyme method from a strain of *S. lutea* and a u.v.-induced carotenoidless mutant have been compared manometrically at 28° and 660 lumens/sq. ft. The malate oxidase of whole cells and membranes of the pigmented strain is slightly sensitive to illumination in the presence of toluidine blue at concentrations used by Mathews and Sistrom but mutant cells and membranes both show a greater sensitivity. At toluidine blue concentrations between 10^{-6} and 10^{-5} M, the sensitivities of mutant and pigmented membranes increased with sensitizer concentration, but mutant membranes showed much greater sensitivity particularly at lower concentrations.

With 10^{-5} M toluidine blue, malate oxidation by mutant membranes, as compared with a dark control, declines after 30–40 min. irradiation. Short exposures to light show that up to 10 min. illumination has no effect while 20–30 min. shows a depressed oxidation rate which is reversible in the dark. Illumination for longer than 40 min. renders the effect irreversible.

It is suggested that an initial reversible effect on electron transport, normally inhibited by carotenoids in the wild type, could lead to secondary irreversible effects including membrane damage, increased permeability, inactivation of membrane enzymes and death.

The Preparation and Use of a New Type of Bacterial Immunoabsorbent Column. By R. O. THOMSON (*Wellcome Research Laboratories, Beckenham, Kent, Great Britain*).

A number of methods have been described for the preparation of columns containing bacteria for the absorption of specific agglutinins (Morrison, R. B. (1961), *Lancet*, 1288; Weetall, H. H. (1967), *J. Bact.* **93**, 1876). In general these columns have low absorptive capacities because of the difficulty of incorporating sufficiently high concentrations of organisms into them.

The type of column used in the present work was prepared from an immunoabsorbent which consisted of organisms dispersed in agar subsequently converted to a bead form by a method similar to that of Hjerten, S. (1964, *Biochem. biophys. Acta* **79**, 393). The concentration of organisms could be made extremely high and the columns prepared had sufficient capacity to enable them to be used (*a*) for the removal of cross-reacting agglutinins from production batches of type specific antisera, (*b*) for the large-scale isolation of specific agglutinins from crude sera by absorption-elution and (*c*) for the fractionation of such agglutinins. Columns were regenerated after absorption by removing the agglutinins by elution with acid buffers.

Although the organism used in this work was *Escherichia coli* it is felt that the technique could be applied with equal success in studies of other bacterial, viral and tissue antigens and antibodies.

Effect of azide on anaerobic respiratory enzymes in *Proteus mirabilis*. By G. N. DE GROOT and A. H. STOUTHAMER (*Microbiology Department, Botanical Laboratory, Free University, Amsterdam, The Netherlands*)

Proteus mirabilis can form four reductases after anaerobic growth, which are linked to the electron transparent chain of the cytoplasmic membrane. Chlorate-resistant mutants are blocked in the synthesis of nitrate reductase A and chlorate reductase C. Formate dehydrogenase, hydrogenase, thiosulphate reductase and tetrathionate reductase are formed by only some of the chlorate-resistant mutants. Azide (1 mM) fully inhibits nitrate reductase A, but it has no effect on the other reductases.

Anaerobic growth of the wild type in the presence of azide gives a strong derepression of cytochrome b_1 and nitrate reductase A. Simultaneously the formation of chlorate-reductase C is fully repressed. Azide has no influence on the formation of thiosulfate reductase and tetrathionate reductase. In chlorate-resistant mutants azide does not stimulate the formation of cytochrome b_1 (and nitrate reductase). Both in the wild type and some chlorate-resistant mutants the formation of cytochrome a_2 (and a_1) is repressed by azide. The oxidation of several metabolites is not influenced by azide after growth with or without azide. This suggests that the electron flow from these metabolites to oxygen is not mediated by those cytochromes which are influenced by azide.

Cells grown with azide also show a much higher formate-nitrate reductase activity, but it appears that this is not a consequence of increased amounts of formic dehydrogenase. On the other hand the formation of formic hydrogenlyase is diminished in cells grown with azide. Azide does not inhibit formic dehydrogenase, the reduction of cytochrome b_1 by formate, nor hydrogenase, but it inhibits completely formic hydrogenlyase. Possibly the formation of the cytochrome b_1-nitrate reductase complex is regulated by one of the components of the formic hydrogenlyase complex.

A Bacteriocinogenic Factor of *Enterobacter cloacae*. By G. A. TIEZE and A. H. STOUTHAMER (*Biological Laboratory, Microbiology Department, Free University, Amsterdam*), H. S. JANSZ (*Laboratory for Physiological Chemistry, State University, Utrecht*), *and* E. F. J. VAN BRUGGEN (*Laboratory of Structural Chemistry, The University, Groningen, The Netherlands*)

Enterobacter cloacae strain DE 13 produces a bacteriocin which is able to kill some strains of Enterobacter, Klebsiella, and Escherichia (Stouthamer, A. H. & Tieze, G. A. (1966). *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **32**, 171). Using the method of Mulczyk & Duguid (1966), this property can be transferred to another strain of *Ent. cloacae* (up to 90 % of the acceptor-population became bacteriocinogenic and to *Escherichia coli* K 12 (transfer frequency about 3×10^{-3}). Transfer of chromosomal material was never observed, suggesting that the production of the bacteriocin is determined by a plasmid, which is mostly in a repressed state (Graaf, F. K. De *et al.* (1968), *J. Bact.* **95**, 631). However, all attempts to eliminate this plasmid failed. The plasmid F'trp cys ColB ColV could be transferred from *E. coli* into *Ent. cloacae* strain DF 13, and subsequently it could be eliminated by acridine orange treatment. By CsCl gradient centrifugation in the presence of ethidium bromide (Jansz, H. S. *et al.* (1969), *Europ. J. Biochem.* in Press) closed circular DNA molecules were selected from native DNA isolated from these strains. Electron microscopic investigaton was done by a modified Kleinschmidt method. *Ent. cloacae* strain DF 13 harbours small closed circular DNA molecules of different length classes (0·65 μ, 1·35 μ, 2·65 μ, and 3·0 μ). One of these rings with a molecular weight of about $5{\cdot}2 \times 10^6$ daltons (length 2·65 μ, S_{20w} *c.* 26S) was transferred concomitantly with the ability to produce the bacteriocin DF 13 to *E. coli*. It is concluded that this DNA represents the bacteriocinogenic factor BacDF 13, of which there are at least six copies per cell. Similar results were obtained with *Ent. cloacae* strain 02, which also contains small closed circular DNA molecules (0·9 μ, 1·3 μ, 1·8 μ, and 2·5–3·0 μ). After this strain had become bacteriocinogenic, a significant increase was found in the amount of the DNA rings in the 2·5–3·0 range.

Germination of Bacterial Spores by High Hydrostatic Pressures. By G. W. GOULD and A. J. SALE (*Unilever Research Laboratory Colworth/Welwyn, Sharnbrook, Bedford*) and W. A. HAMILTON (*Department of Biological Chemistry, Marischal College, University of Aberdeen, Great Britain*)

Bacterial spores have generally been found to be more resistant hydrostatic pressure than vegetative forms. However, under certain conditions, low pressures (100 to 2000 atmos.) may heat-sensitize or inactivate spores even more than high pressures (e.g. 8000 atmos.). Inactivation at low pressures resulted from the effect of pressure in causing germination of spores (Clouston, J. G. & Wills, P. A. (1969). *J. Bact.* in Press), and subsequent loss of viability of the germinated forms. At temperatures below about 50° high pressures were generally less germinative, and therefore less lethal, than low pressures. In contrast, at temperatures above about 50° germination and inactivation of spores generally increased with pressure at least up to 8000 atmos.

Spores germinated by pressure became heat-sensitive, excreted dipicolinic acid, calcium and peptidoglycan components, and showed phase and electronmicroscopic changes similar to those typical of spores germinated by nutrient germinants at 1 atmos. pressure. Pressure germination showed well-defined highly temperature-dependent pressure optima, temperature optima and pH optima; was inhibited by metabolic poisons, high concentrations of ionic but not of non-ionic solutes, extreme pH values and low temperatures; but was strongly potentiated by certain nutrient germinants (in particular by L-alanine and some other amino acids) at concentrations well below those effective at 1 atmos. Pressure increased the rate of racemization of alanine by spores and thereby allowed D-alanine, which inhibits L-alanine-initiated germination at 1 atmos., to potentiate germination under pressure.

It was concluded that low pressures caused germination by (1) increasing the rate of a germination reaction which was negligibly slow at 1 atmos. and (2) increasing the permeability of a barrier within the spore to exogenous and endogenous L-alanine or other germinative L-amino acids.

Influence of O- and T1 Side-Chains on the attachment of Bacteriophages to the Somatic Antigen of Salmonella. By A. A. LINDBERG (*Department of Bacteriology, Statens Bakteriologiska Laboratorium, Stockholm, Sweden*), M. SARVAS (*Department of Bacteriology and Serology, University of Helsinki*) and P. HELENA MÄKELÄ, (*State Serum Institute, Helsinki, Finland*)

The phage adsorption ability and serological specificity of different Salmonella strains having incomplete, leaky blocks in their lipopolysaccharide (LPS) synthesis were compared with their genotype and sugar composition to provide a set of standards relating these parameters to LPS structure. Strains that had T1-specific side-chains in their LPS, both with or without O side-chains, were examined in the same way to learn more about the organization of these two side-chains in the LPS.

Phage P22 adsorption was found to be dependent on the presence of a nearly complete O side-chain complement. The adsorption of the FO (Felix 0–1), 6SR and Br2 bacteriophages, attaching to structures in the LPS core, was a sensitive indication of any defect in O side-chain synthesis. Strains with T1 side-chains adsorbed the FO and 6SR phages efficiently whereas the adsorption of the Br2 phage was blocked to a large extent. Strains with both O and T1 side-chains showed that as the amount of O side-chains increased there was a reduction of the 6SR, Br2 and FO attachment with a concomitant increase in Pss attachment. The results suggest that the apparent competition between O- and T1 side-chains may not be a competition for available sites in the LPS.

Virulence of Salmonella strains with a reduced amount of O-antigen By VILLE VALTONEN (*Department of Serology and Bacteriology, University of Helsinki, Finland*)

It is well known that when the lipopolysaccharide (LPS) of Salmonella loses its O-antigenic side-chains the bacteria lose their virulence. The present report concerns the effect of less

complete alterations of the O side-chains on virulence. Ten-fold dilutions of an overnight culture of bacteria were injected intraperitoneally into groups of ten adult SAW mice, and the LD_{50} values were calculated from the 10-day survival. All the strains were derivatives of SL 1027 of *S. typhimurium* line LT 2; this strain and its *rfb* and *rfc* mutants were obtained from Dr B. A. D. Stocker, Stanford University. The LD_{50} of the smooth virulent strain was 5×10^4, and the LD_{50} of its avirulent *rfb* mutant, which has no O side-chains, 5×10^7.

A *rfc* mutant, with only one repeating unit in each O side-chain (in contrast to the average ten units in the S form) had an intermediate virulence, $LD_{50} = 10^6$.

If smooth bacteria acquire a *rft*+ locus by genetic recombination they will produce both T 1-specific and O side chains. (Sarvas, M. (1967), *Ann. Med. exp. Fenn.* **45**, 447–471). These T 1, S forms produce about 10 times fewer O side-chains than the smooth parent. (Lindberg, A. A., Sarvas, M., Mäkelä, P. H. (1969), *J. Bact.* in Press). An F+, T 1 form of *S. paratyphi* B was used as a genetic donor to a *gal*- derivative of the smooth *S. typhimurium*, gal+ recombinants were selected. Some recombinants were T 1, S, having inherited the *rft*+ of the donor, others were S like the recipient. The S recombinants and the recipient parent were virulent ($LD_{50} = 10^5$), while the T 1, S, recombinants were almost avirulent ($LD_{50} = 10^7$). Thus the reduction in the O side-chain material had a deleterious effect on virulence, which could not be compensated for by the T 1 side-chains. Preliminary results indicate, however, that T 1 side-chains alone confer a slight degree of virulence on a *rfb* mutant.

Phage Receptor Development after the addition of Galactose to a Galactose-Epimeraseless Mutant of *Salmonella typhimurium*. By T. HOLME (*Department of Bacteriology, Karolinska Institutet*) and A. A. LINDBERG (*Department of Bacteriology, Statens Bakteriologiska Laboratorium, Stockholm, Sweden*)

A rapid incorporation of galactose and S-specific sugars in the cell wall polysaccharide was observed after addition of galactose to cultures of the UDP-Gal-4-epimeraseless mutant *Salmonella typhimurium* LT 2–M 1. The incorporation velocity was higher when galactose was added to bacteria in the log. phase of growth than when it was added to stationary phase bacteria. The adsorption rate constants for S-specific phages increased from zero to a maximum value in 20–40 min. after addition of galactose. Adsorption of phage C 21, which attacks only Rd mutants and Rc mutants grown without galactose, decreased to zero in 10 min. after the addition. The FO-1 phage, which adsorbs rapidly only to Ra mutants by slowly to the parent S-form, displayed low adsorption rate constant at all times after the addition of galactose. Passive hemagglutination inhibition tests revealed the rapid synthesis of the O-antigenic side-chains but also that the immunological specificity characteristic of the LT 2–M 1 cells was still present 60 min. after the addition of galactose. The LPS were further analysed by g.l.c.-mass spectrometry.

A Genetic Analysis of the O-antigenic Conversion by Phage 27 In Group B Salmonellas. By G. BAGDIAN and P. HELENA MÄKELÄ (*State Serum Institute, Helsinki, Finland*)

When the temperate bacteriophage P 27 lysogenises Salmonellas of group B, a new O-antigenic factor, 27_B, appears on the surface of the converted bacteria. Previous work had shown that the neo-antigen probably corresponds to an altered linkage between the repeating units of the lipopolysaccharide O side-chain. This linkage is in nonconverted bacteria formed by a polymerase coded by gene(s) at a *rfc* locus between *gal* and *trp*.

We could show that the site of integration of the prophage 27 (att*P*27) in the bacterial chromosome is far removed from the *rfc* locus, *attP*27 being contransducible with *purE* between *pro* and *gal*. This finding excludes a direct modification of the polymerase gene(s) through the integration of the prophage.

By means of F-mediated crosses we introduced the prophage 27 into group B Salmonella strains lacking the polymerase gene(s) because of a previous recombination event with a group C_1 Salmonellas and therefore having a semirough phenotype. We could show that recombinants which had the prophage 27 but no bacterial polymerase gene(s) were still semirough. The same result was obtained with two other strains that were lacking polymerase activity because

of a mutation in the *rfc* gene(s): they too remained semirough even after acquiring the prophage 27.

Transduction and Dominance Studies of the envA Gene Present in a Chain-Forming Mutant of *Escherichia coli* K 12. By STAFFAN NORMARK, (*Department of Microbiology, University of Umeå, Sweden*)

We have recently reported a genetical and physiological characterisation of a chain-forming mutant of *Escherichia coli* K 12. The gene responsible for chain formation *envA*, was located at 2–4 min. and besides the chain formation it also mediated a drastically decreased resistance to ampicillin and several other antibiotics (Normark, S., Boman, H. G. & Matsson, E. (1969), *J. Bact.* **97**, 1334.)

The *envA* gene has now been found to be contransducible with leucine requirement (*leu*) and with azide resistance (*azi*). A low number of cotransductants was also obtained with threonine requirement (*thr*) but these recombinants did not show the normal phenotypic properties of the *envA* strain D 22. The available frequencies of cotransduction indicate the following gene order: *the-leu-envA-azi.*

Using the method of Low with strains carrying the *recA*$^-$ gene episomes were isolated with different alleles of the *envA* gene. Studies of the morphology and antibiotic resistance of these partial diploids indicate that *envA* is recessive to its wild type allele. When the *envA* gene was integrated into a highly ampicillin resistant strain its ampicillin resistance was decreased by a 100-fold.

In addition to the antibiotics previously studied it has now been found that the *envA* gene also mediates sensitivity to actinomycin D and rifamycin. *EnvA* strains also show a drastically increased uptake of crystal violet. The results now reported are consistent with the previous hypothesis that *envA* strains carry a defect in their envelope formation which in term is responsible both for the chain formation and for a changed penetration of actinomycin D, rifamycin and crystal violet.

Cell Division and DNA Synthesis in a Chain-forming Derivative of *Escherichia coli* K 12. By HANS G. BOMAN, GUNNAR BLOOM, STAFFAN NORMARK, and EVA MATSSON. (*Departments of Microbiology and Histology, University of Umeå, Sweden*)

We have recently described a mutant of *Escherichia coli* K 12 carrying the *envA* gene which mediates chain-formation and decreased antibiotic resistance (Normark, S., Boman, H. G. & Matsson, E. (1969), *J. Bact.* **97**, 1334). Strain D 223 is a thymine requiring (*thy*) *envA*-strain obtained in a cross between KL 16 (*thy*) and D 22 (*ampA, envA, proB, trp, his*) followed by scoring for a *his*$^+$ and *thy* recombinant. Strain D 212, containing the wild type allele of *ampA* was obtained from strain D 21 by an analogous cross and similar scoring.

Strain D 223 can form chains consisting of more than ten cell units. Experimental conditions have been obtained during which D 223 can give less than one doubling of cell mass and DNA synthesis, but more than a 10-fold increase in the number of viable cells. This process of cell division was also followed by electron microscopy. Strain D 213 was used as a control both in the physiological and morphological work. The present results indicate that the *envA* gene, in some way, affects the association of the inner and outer layers of the cell envelope and that cell division follows after a constant period of DNA synthesis.

AmpB a Mutation which Affects the Cell Envelope and Ampicillin Resistance in *Escherichia col*
By KURT NORDSTRÖM, LARS G. BURMAN and KERSTIN G. GRENNBERG (*Department of Microbiology, University of Umeå, Sweden*)

Development of ampicillin resistance in *Escherichia coli* is a stepwise process involving at least two types of mutations. The first type was called *ampA* (Eriksson-Grennberg, K. G., (1968), *Genet. Res. Camb.* **12**, 147) and raises ampicillin resistance by a factor of 10 due to an increased synthesis of penicillinase.

Without affecting penicillinase production the second mutant type, *ampB*, doubles the resistance of *ampA*$^+$ and *ampA* cells as well as that of cells with R-factor mediated penicillinase in tests where resistance is defined as ability to form colonies on ampicillin plates. However, in liquid medium *ampB* reduces ampicillin resistance in all cases (Nordström, K. Eriksson-Grennberg, K. G. & Boman, H. G. (1968) *Genet. Res., Camb.* **12**, 158). Normally, both chromosome- and episome-mediated penicillinases are strictly cell-bound in *E. coli*, but *ampB* causes a considerable liberation of these enzymes. This leakage seems to be an advantage for cells on ampicillin plates, while in liquid medium resistance of the individual cell correlated only to cell-bound penicillinase activity.

AmpB cells are osmotically fragile, especially after EDTA-treatment or wash with NA^+ ions. Osmotic stability is restored by 10^{-3} M Mg^{2+}. Moreover, these cells produce mucoid colonies and show low plating efficiency of phage T4 due to impaired adsorption. They are also 'tolerant' to colicin E2 and E3 although these are adsorbed normally. Finally *ampB* cells are cholate sensitive and show changed resistance to different cell wall antibiotics as well as chloramphenicol and streptomycin.

These data indicate that *ampB* affects the cell envelope. It cannot be judged at present whether *ampB* is a structural or a regulatory mutation or which part of the cell envelope is primarily affected.

Preliminary mapping experiments indicate that mutations in several loci can give the *ampB* phenotype. One locus is located in the *gal* region and two loci in the *trp* region.

Use of Sex Specific Phages for Demonstration of Cell Surface Changes Mediated by Genes for Ampicillin Resistance in *Escherichia coli*. By DAVID MONNER, HANS G. BOMAN and STAFFAN JONSSON (*Department of Microbiology, University of Umeå, Sweden*)

The phages used were MS2, which is male specific, and ØII, which is female specific. The main experimental approaches were to study efficiency of plating (EOP) and adsorption of phage to strains of bacteria with known genetic constitution.

It was found that in Hfr strains the *ampA* gene improved adsorption of MS2 from 85 to 90 %. In strains, carrying both *ampA* and *ampB*, adsorption of MS2 was decreased to 75 %. The presence of the *ampAB* genotype reduced adsorption to 40 %. The corresponding variations in EOP range from 1 for *ampA* strains (0·8 for wild type) to 0·03 for *ampAB* mutants (for gene symbols see *Genet. Res.* (1968), **12**, 169).

Only the restricted form of phage ØII (see next abstract) was used in this study. This phage was not adsorbed to male or female cells with *ampA* or wild type genes. Female strains with both *ampA* and *ampB*, or *ampAB*, and male strains with *ampAB*, gave all three 80–85 % adsorption. A corresponding all-or-none pattern was found for EOP values, which varied from 1 to 10^{-7}. The insertion of F-factor in female strains did not alter the EOP values.

Taken together the male and the female specific phages show a minor-pattern behaviour with respect to *ampAB* strains. In addition the adsorption data show that all presently known genes for ampicillin resistance also mediate changes on the cell surface of the bacteria.

A Mutated R-factor Mediating Increased Resistance to Several Antibiotics. By KURT NORDSTRÖM (*Department of Microbiology, University of Umeå, Sweden*)

An F$^-$-strain of *Escherichia coli* (D1) carrying the R-factor R1 was treated with ethylmethane sulphonate. A number of clones wtih increased ampicillin resistance were isolated. Most of them were mutated in the R-factor since the increased resistance was infective. One mutated R-factor, R1 B1, was selected for further studies and was transferred to an Hfr strain (G11). All four resistancies mediated by R1 (ampicillin, chloramphenicol, streptomycin, sulphonamides) (Meynell, E. & Datta, N. (1966), *Genet. Res.* **7**, 134) were increased 2–3 times. R-factor mediated ampicillin resistance is due to the production of penicillinase (Datta, N. & Kontomichalou, P. (1965), *Nature, Lond.* **208**, 239). The activity of this enzyme was two times higher in G11–R1 B1 than in G11–R1. There was no indication of any difference in specific activity or in other properties of the pure penicillinases mediated by R1 B1 and R1. Chloramphenicol resistance is also due to metabolizing enzymes (Okamoto, S. & Suzuki, Y. (1964)

Nature, Lond. **208**, 1301) and indirect data showed that this activity was about two times higher in R 1 B 1 than in R 1 strains. R-factor transfer and transfer of an early chromosomal gene (*proB*) was increased about three times. These results indicate that more gene product is formed from RTF genes as well as from resistance genes of the R-factor. Several explanations for this result are possible. An increased transcription of the genes in R 1 B 1 is rather unlikely. It is more reasonable that the number of R 1 copies per cell is increased in R 1 B 1 strains either by changes in the regulation of the multiplication of the episome or by the formation of a dimer of R 1. The increased resistance was transduced as one block by phage P 1, favouring the former possibility but a definite conclusion about the character of the mutation in R 1 B 1 cannot be drawn at present. Mutants of this type may perhaps be valuable in studies of the regulation of episome multiplication.

Regulation of RNA Formation in T-even Phage Infected Bacteria: Shut-off of Early mRNA Synthesis. By OLA SKÖLD (*Department of Microbiology, Faculty of Pharmacy, University of Uppsala, Sweden*)

The formation of T-even phage specific, DNA-synthesizing enzymes in *Escherichia coli* B at 37° abruptly ceases 12–15 min. after phage infection in a rich medium. This regulation could act by a restriction either of the transcription of early genes or of the translation of early mRNA. The present investigation was undertaken with the aim of measuring the time course of early mRNA synthesis. Formation of phage RNA was followed either by the cumulative incorporation of ^{14}C-uracil into acid-insoluble RNA or by short time incorporation of pulses of ^{14}C-5-fluorouracil or 5-^{3}H-uracil. Wild type and several mutants of phage T4 was used to infect *E. coli* B. It could be seen with early gene, phage mutants, both of the amber and of the temperature sensitive type, none of which will produce late mRNA under non-permissive conditions, that early mRNA formation diminished rapidly with time after infection. This restriction in early mRNA synthesis was almost abolished if gene 55 was also damaged by mutation. This could be observed both by the use of a single mutant and of a double mutant, combining the gene 55 lesion with another early gene mutation. It was concluded that the T4 phage seems to have a transcriptional regulation mechanism for early mRNA synthesis, and that this mechanism is related to the gene 55 function.

Regulation of early mRNA formation in T-even Phage Infected Bacteria: Dependence on Protein Synthesis. By CHRISTINA BOLUND and OLA SKÖLD (*Department of Microbiology, Faculty of Pharmacy, University of Uppsala, Sweden*)

In the previous communication evidence was obtained for a transcriptional regulation mechanism controlling early mRNA synthesis in T4 phage infected bacteria. This regulatory phenomenon was studied by a different approach in the present investigation. The phenotypic reversion of amber mutations in early T4 phage genes by 5-fluorouracil, was studied as a function of time after infection. Several early gene mutants were used, which could not produce DNA under nonpermissive conditions. Reversion by 5-fluorouracil was measured as the rescue of DNA synthesis, determined as the incorporation of ^{3}H-thymidine into acid-insoluble product. The 5-fluorouracil was added at different times after the infection of *Escherichia coli* B cells with the early gene mutant. The rescuability of DNA synthesis was found to decrease rapidly with time after infection. This is interpreted as a reflexion of an mRNA synthesis regulation, because 5-fluorouracil rescue of early genes could take place only as long as the analog is actively incorporated into early mRNA under synthesis. This decrease in 5-fluorouracil rescuability was almost abolished by chloramphenicol. It was, furthermore, demonstrated that the restriction in early mRNA synthesis determined by pulse-labelling of RNA in early amber mutant infected *E. coli* B, was abolished by the presence of chloramphenicol.

In conclusion, the observed regulation mechanism for early mRNA synthesis in T4 phage infected bacteria seems to require an undisturbed protein synthesis.