Cell-wall Constituents of Rickettsiae and Psittacosis-Lymphogranuloma Organisms

By H. R. PERKINS* AND A. C. ALLISON

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 27 June 1962)

SUMMARY

The taxonomic position of organisms belonging to the rickettsiae and psittacosis-lymphogranuloma groups is controversial. Although, like viruses, all pathogenic forms of these organisms are obligate intracellular parasites, in other respects they resemble bacteria. Since a cell wall, deriving its rigidity from mucopeptide, which contains the amino sugar muramic acid as a key constituent, has so far only been found in bacteria and the closely related blue-green algae, the presence of muramic acid in an organism may be used as a taxonomic criterion. The mucopeptides of bacterial cell walls are also often sensitive to lysozyme, so that dissolution by this enzyme serves as an indication of the presence of mucopeptide. Organisms of the rickettsiae and psittacosis-lymphogranuloma groups have been examined for the presence of muramic acid. Critical chemical tests have shown that this substance is present in organisms of both groups. Cell walls of *Rickettsia burnetii* were dissolved by lysozyme. In the light of these and other results the taxonomic position of these groups of organisms is discussed.

INTRODUCTION

The taxonomic position of organisms belonging to the rickettsia and psittacosis-lymphogranuloma groups is controversial (Andrewes, 1952; Bedson, 1959). All pathogenic forms of these organisms appear to be obligate intracellular parasites; no unequivocal evidence has been obtained that they multiply in cell-free media. In this respect these organisms resemble viruses, but in other important properties they are quite distinct from them. Viruses may be regarded as biological entities which contain protein and either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) but not both (Allison & Burke, 1962), and they do not multiply by binary fission; instead, the virus nucleic acid and protein constituents replicate more or less independently and are later reassembled (see Cohen, 1957; Schafer, 1959). Furthermore, viruses, unlike bacteria, are insensitive to penicillin, chloramphenicol and tetracyclines; in contrast, the rickettsia and psittacosis-lymphogranuloma organisms contain both DNA and RNA (Allison & Burke, 1962), and are sensitive to these antibiotics (Hurst, 1953). As discussed below, it seems highly probable that at least some typical rickettsia and psittacosis-lymphogranuloma organisms replicate by binary fission, although there is uncertainty about the exact mode of replication of other members of the psittacosis-lymphogranuloma group. In view of these difficulties there is a need for independent evidence to help clarify the

* Present address: Twyford Laboratories, Ltd., Twyford Abbey Road, London, N.W. 10.

affinities of rickettsiae and psittacosis-lymphogranuloma organisms. A contribution might be made by study of their cell walls, the composition of which has come to be recognized as characteristic of different groups of organisms. All the bacteria so far studied (Eubacteriales and Actinomycetales, excluding permanent L-forms) have been found to possess cell walls which contain relatively rigid mucopeptide. An essential component of mucopeptide is muramic acid (Cummins & Harris, 1956; Work, 1961). Some, but not all, mucopeptides also contain diaminopimelic acid. These two components, typical of bacterial cell walls, have not been found in protozoa or fungi, although both have been shown to occur in some blue-green algae (Work & Dewey, 1953; Frank, Lefort & Martin, 1962).

Evidence is presented in this paper that bacterium-like cell walls are present in rickettsiae and psittacosis-lymphogranuloma organisms. A new and highly sensitive technique for detection of muramic acid is also presented. A preliminary account of the results with rickettsiae has already been published (Allison & Perkins, 1960). Jenkin (1960) mentioned the presence of muramic acid in the feline pneumonitis organism (a member of the psittacosis-lymphogranuloma group), although no technical details were given. The results of these and other studies suggest that rickettsiae and psittacosis-lymphogranuloma organisms have evolved from bacteria by adaptation to intracellular replication; this conclusion is discussed.

METHODS

Rickettsiae organisms. Q fever (*R. (Coxiella) burnetii*); the Nine Mile strain isolated by Davis & Cox (1938) in Montana; number of passages is unknown. Scrub typhus *R. tsutsugamushi*, Karp strain, isolated in 1943 from the blood of an American soldier in New Guinea (Derrick & Brown, 1949); material from 101st yolk-sac passage was used. Rocky Mountain Spotted Fever, *R. rickettsii* (Maxcy, 1929) Bitterroot strain after an unknown number of passages in guinea pigs and eggs. Epidemic typhus, *R. prowazekii*, Madrid π attenuated strain, isolated in 1941 during an epidemic in Madrid (Clavero & Perez-Gallardo, 1943); material in the 267th yolk-sac passage was used.

Psittacosis-lymphogranuloma organisms. Psittacosis (Ornithosis) P-4 pigeon strain isolated in 1942 from a sick pigeon in New York (Smadel, Wall & Gregg, 1943), material from a 42nd yolk-sac passage was used. Mouse pneumonitis (Nigg) Hampstead strain, isolated by Andrewes (see Andrewes & Horstmann, 1949) and passaged in mouse lungs. Feline pneumonitis, strain isolated by Baker (1944), used after an unknown number of yolk-sac passages. Trachoma, strain TE-55, isolated by T'ang, Chang, Huang & Wang (1957) and passaged in the yolk sac.

Preparations of organisms. All organisms except mouse pneumonitis were propagated in yolk sacs of embryonated eggs, inoculated at 6 days and harvested after a further 6 days of incubation at 35°. Tests for bacterial contamination were negative. The yolk sacs were homogenized in Ten Broeck grinders, centrifuged at 2000g for 10 min. and the supernatant fluids centrifuged at 25,800g for 2 hr. at 2°. The deposits were resuspended in Gey's saline solution and used for preparation of cell walls or for chemical analysis. Mouse pneumonitis organisms were grown in mouse embryo cell cultures prepared as described by Allison & Armstrong (1960). They were harvested after 5 days of incubation at 36°, homogenized and centrifuged as

described above. Control material was prepared in the same way from uninfected yolk sacs.

Viruses. Concentrated suspensions of vaccinia and influenza viruses were also examined. Vaccinia virus (Lister egg-adapted strain) was grown in the chorio-allantoic membranes of embryonated eggs (Westwood, Phipps & Boulter (1957). The membranes were homogenized and centrifuged as already described. The titre of the final preparation was 8×10^9 pock-forming units/ml., from which it is likely that the number of virus particles was of the order of 10^{11} /ml. (Kaplan & Valentine, 1959). Influenza virus (strain Mel 1953) was grown in the chick embryo. Allantoic fluid was harvested 40 hr. after inoculation of 0.05 ml. of seed virus suspension into the allantoic cavity of 10-day chick embryos and incubated at 35°. Centrifugation was at 38,000g for 30 min. The deposit was resuspended in 0.05 M-tris saline (pH 7.0). The number of virus particles in the final suspension, estimated from the haemagglutination end-point (Donald & Isaacs, 1954) was 8×10^{11} particles/ml.

Preparation of cell walls. These were prepared by a technique like that described by Schaechter *et al.* (1957b). Suspensions of *Rickettsia burnetii* were incubated at 45° with 1 % (w/v) sodium deoxycholate in 0.1 M-tris buffer (pH 7.0) for 4 hr. with constant stirring. The cell-wall preparations were centrifuged at 50,000g for 30 min. and resuspended in 0.1 M-tris buffer (pH 8.0). In some experiments the purified cell walls were incubated with lysozyme in the presence of ethylenediaminetetraacetate under the conditions described below.

Detection of muramic acid. After extraction of lipids, suspensions of organisms were precipitated with 5 % (w/v) trichloroacetic acid. Much protein was removed at this stage by incubation with pepsin (1 mg./ml. in 0.05 N-HCl) and then with trypsin (1 mg./ml. in 0.05 M-phosphate buffer, pH 7.6); these enzymes do not dissolve bacterial mucopeptides (Cummins & Harris, 1956). The residues were hydrolysed in sealed ampoules with 4N-hydrochloric acid at 105° for 4 hr. After removal of excess hydrochloric acid *in vacuo* hexosamines in the hydrolysates were concentrated by chromatography in a column of Dowex 50 (H⁺). The hexosamine peak was transferred to a charcoal+Celite column, and glucosamine was eluted with water (Perkins & Rogers, 1959). Substances eluted by 10 % (v/v) ethanol in water were subjected to paper chromatography in butanol+pyridine+water (6+4+3 by vol.). A sample of the material eluted from the expected position of muramic acid was submitted to the Elson-Morgan test as described by Rondle & Morgan (1955) except that, to increase sensitivity, the final volume in the reaction mixture was 1.5 ml. Absorption curves were drawn from measurements with the spectrophotometer (Unicam S.P. 500) using small cuvettes with a 2 cm. light path. Muramic acid gives a maximum absorption at 510 mμ or, on standing overnight, 505 mμ (Crumpton, 1959).

Identification of microgram quantities of muramic acid. When the sample of material tested by the Elson-Morgan reaction gave an absorption curve with a maximum well below 530 mμ, suggesting that muramic acid might be present, the remainder of the material was treated as follows. To a sample dissolved in 20 μl. water was added 2.5 μl. pyridine followed by 0.1 μl. 1-¹⁴C-acetic anhydride (specific activity 1.7 mc./mmole) from a graduated capillary tube (total capacity 0.7 μl.). The acetylation reaction was allowed to take place at room temperature for 1 hr. The sample was then dried *in vacuo* over soda-lime, and 0.2 ml. of 10 % aqueous

acetic anhydride (non-radioactive) was added and removed *in vacuo*; this step was repeated three times. At this stage 50 μg . *N*-acetylmuramic acid was added as a carrier, to the sample and also to a control tube which had been treated in the same way throughout. The samples were transferred to the origin of a paper chromatogram and run in butanol + acetic acid + water (68 + 10 + 27 by vol.). In this solvent *N*-acetyl-muramic acid runs twice as far as *N*-acetylglucosamine. The position of *N*-acetylmuramic acid was determined from a marker strip by using the spray system of Partridge (1948) except that the final heating after spraying with Ehrlich's reagent was omitted. Radioactivity in the corresponding region of the chromatogram was detected by radioautography. The radioactive region was eluted and counted on a planchette with an end-window counter.

Under these conditions samples of 0.2, 1 and 10 μg . (0.8, 4, and 40×10^{-3} μmole) of muramic acid originally present before the addition of carrier gave final counts of 61, 295, and 3022 counts/min., respectively. Since the counting efficiency was such that 1 μc . gave 2×10^5 counts/min., this corresponded to a 45% over-all yield of labelled *N*-acetylmuramic acid.

In some experiments the samples of radioactive *N*-acetylmuramic acid were converted to a Morgan-Elson chromogen, which retains the acetyl group, by heating in a sealed tube with aqueous triethylamine at 100° for 8 min. (Perkins, 1960*a*). The reagent was removed *in vacuo*, the sample transferred to a paper chromatogram (solvent *n*-butanol + pyridine + water; 6 + 4 + 3 by vol.) and run overnight. The site of radioactivity was found by radioautography; subsequently the position of the chromogen was determined by spraying with Ehrlich's reagent in butanol (Partridge, 1948). When the resulting purple spot coincided exactly with the area that contained radioactivity, it was concluded that the parent labelled-compound was indeed muramic acid.

Conversion of muramic acid to a substituted pentose. Samples of the material isolated from chromatograms and suspected to be muramic acid were heated at 100° for 30 min. in sealed ampoules with ninhydrin in aqueous pyridine (Stoffyn & Jeanloz, 1954). Authentic samples of muramic acid were treated in the same way. The reaction mixtures were dried and run on paper chromatograms in butanol + pyridine + water overnight, together with markers of the four pentoses. The dried paper was sprayed with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950).

Incubation of residues from cultures of Rickettsia burnetii with lysozyme. Samples (5 mg.) of the residue obtained from cultures by treatment with fat solvents, trichloroacetic acid and proteolytic enzymes, as described above, were incubated in tris buffer (pH 8.0, 0.02M), sodium chloride (0.02M) and ethylenediaminetetraacetate (0.002M) for 2 hr. at 37°, with or without lysozyme of final concentration 50 μg ./ml. The residue was removed by centrifugation at 11,000*g* and the samples concentrated *in vacuo* to 0.25 ml. The reaction for *N*-acetylhexosamines was then done as described by Aminoff, Morgan & Watkins (1952), except that the heating period was 12 min. since it is known that the lysozyme digestion products of mucopeptide give the highest yield of colour under these conditions (Perkins, 1960*b*). A purple colour with an absorption maximum at 585 m μ was considered to be indicative of the presence of liberated *N*-acetylhexosamine end-groups.

RESULTS

Incubation of Rickettsia burnetii with lysozyme

Purified cell-wall preparations of *Rickettsia burnetii* were digested with lysozyme in the presence of ethylenediaminetetra-acetate. This treatment led to the disappearance of cell walls recognizable as such in the electron microscope. The conditions used were like those under which lysozyme will digest the walls of some Gram-negative bacteria (Repaske, 1958). No precipitation was observed, so that it seems unlikely that aggregation in the presence of the highly basic protein lysozyme was responsible for the disappearance of the cell walls. Under suitable conditions the cell walls of some Gram-positive and some Gram-negative bacteria are attacked by egg-white lysozyme with liberation of material which gives a positive Morgan-Elson reaction for *N*-acetyl-hexosamines. This reaction is due to the liberation of reducing groups of the walls (Perkins, 1960*b*; Salton & Ghuysen, 1960). Since other evidence suggested that muramic acid was present in *R. burnetii*, we attempted to determine whether preparations from this organism could undergo a similar reaction with lysozyme. When these preparations were treated with lysozyme (as described in Methods), material giving an absorption maximum at 585 m μ in the Morgan-Elson reaction for *N*-acetylhexosamines was obtained. Although bacterial cell-wall mucopeptides containing muramic acid are not the only polymers hydrolysed by lysozyme (for instance chitin, a β (1-4)-linked poly *N*-acetylglucosamine, is attacked to some extent as Berger & Weiser (1957) showed), the result with *R. burnetii* is at least consistent with the other evidence for the presence of muramic acid described below.

Chemical evidence for the presence of muramic acid

So far as is known muramic acid is a compound which is specific to the mucopeptides found in bacterial cell walls. Thus, when an organism is shown to contain muramic acid it is reasonable to assume that it possesses a cell wall which contains a mucopeptide resembling that of bacteria. Preparations of rickettsiae and the psittacosis-lympho-granuloma group of organisms were therefore examined for the presence of muramic acid. Hydrolysed samples were fractionated as described, and the material likely to contain muramic acid was subjected to the Elson-Morgan reaction for amino sugars. After colour development the tubes were allowed to stand overnight, and the light absorption then measured over the range 490 to 540 m μ . Absorption curves typical of muramic acid were found in specimens derived from several of the organisms studied (see Table 1), as shown in the curves in Fig. 1; these results suggested that muramic acid was present in the preparations. The chemical identity of the isolated material was examined further.

The *N*-acetylated derivatives of hexosamines are compounds which yield the characteristic Morgan-Elson reaction; this reaction can be used to detect their presence on chromatograms. The material isolated from the rickettsiae and psittacosis-lymphogranuloma organisms and suspected of being muramic acid was therefore converted to its *N*-acetyl derivative. Because of the small quantities of substance available, the reaction was performed with 1-¹⁴C-acetic anhydride of high specific activity and the identity of the labelled material with non-radioactive

N-acetylmuramic acid added as carrier was traced chromatographically. The radioactivity due to the labelled acetyl group ran in the same position as the *N*-acetylmuramic acid detected by the Morgan–Elson reaction (Table 1). Further confirmation that radioactivity present in the region corresponding to *N*-acetylmuramic

Fig. 1. Absorption curves of substances in the Elson–Morgan reaction. Samples were treated by the procedure of Rondle & Morgan (1955) and the final reaction mixtures were allowed to stand overnight before preparation of the absorption curves. ●—●, Glucosamine; △—△, muramic acid; ○—○, material from *R. burnetii*; □—□, material from mouse pneumonitis (Nigg) organism.

acid was indeed due to this compound was obtained as follows. *N*-acetylhexosamines heated with alkali at 100° yield the chromogens of the Morgan–Elson reaction (1934), the chief of these being the compound:

(Kuhn & Kruger, 1957), which retains its acetyl group. Heating *N*-acetylmuramic acid with alkali produces the same chromogen (Perkins, 1960*a*). If, therefore, material containing radioactivity due to an acetyl group can be converted by heating in alkali to a Morgan–Elson chromogen which is also radioactive, it is

probable that the original compound was an *N*-acetylhexosamine. If, also, this material came from chromatographically isolated *N*-acetylmuramic acid, then it is almost certain that the compound originally acetylated with radioactive acetic anhydride was muramic acid. The radioactive spots of *N*-acetylmuramic acid described above were eluted from the paper and heated with aqueous triethylamine to produce the chromogen, which was then run on another chromatogram. In all instances this procedure gave a radioactive spot which coincided with the chromogen, thus confirming that the radioactive acetyl group apparently belonging to *N*-acetylmuramic acid on the first chromatogram was indeed combined in an acetylhexosamine. No other type of acetyl compound would be converted to Morgan-Elson chromogen in this way. The results of this test are shown in Table 1.

Table 1. *Tests for muramic acid applied to organisms of the rickettsia and psittacosis-lymphogranuloma groups*

R. burneti showed conversion to substituted pentose by ninhydrin.

Organism	Chemical test as described in the text	
	Conversion to radioactive	
	Elson-Morgan reaction for muramic acid	<i>N</i> -acetylmura- mic acid and chromogen
Rickettsia		
<i>R. burnetii</i>	+	+
<i>R. tsutsugamushi</i>	+	+
<i>R. rickettsii</i>	+	.
<i>R. typhi</i> (<i>mooseri</i>)	+	.
<i>R. prowazeki</i>	±	.
Psittacosis-lymphogranuloma group organisms		
Psittacosis	+	+
Mouse pneumonitis (Nigg)	+	.
Feline pneumonitis	+	+
Trachoma	±	+

+ indicates a positive reaction. A point indicates that the test was not applied, usually because of lack of total material. ± indicates a weak reaction only.

Since the validity of this work largely depends upon the positive chemical identification of small quantities of substance isolated from the organisms in question, with authentic muramic acid, when sufficient material was available a further critical reaction was performed. When 2-amino-2-deoxyhexoses are heated with ninhydrin they lose their reducing groups; the $-\text{CHNH}_2-$ group at C_2 is converted to an aldehyde group, so that the corresponding pentose results (Stoffyn & Jeanloz, 1954). Similarly, muramic acid yields a product which is presumably 2-*O*-carboxyethylarabinose. Samples of the substance suspected to be muramic acid and samples of the authentic compound were treated in this way, and the final products run on a paper chromatogram. Spraying with *p*-anisidine hydrochloride revealed that muramic acid and the test material both yielded slow running spots of the same colour in the same position ($R_{\text{arabinose}} = 0.46$). This

result gave additional chemical evidence for the identification of muramic acid isolated from rickettsiae. The results obtained in the chemical tests for muramic acid applied to the organisms examined are given in Table 1. They show that muramic acid is present in the rickettsiae and in the members of the psittacosis-lymphogranuloma group of organisms, as in bacteria. The same tests applied to yolk-sac preparations, uninfected tissue cultures and vaccinia and influenza viruses all gave negative results.

DISCUSSION

The observations presented show that a bacterium-like cell wall consisting of mucopeptide containing muramic acid is present in rickettsiae and psittacosis-lymphogranuloma organisms. Preparations of cell walls of *Rickettsia typhi* (*mooseri*) have been shown to contain amino acids, glucose, glucuronic acid and galactose (Schaechter *et al.* 1957*b*), although these components are not confined to the mucopeptides of bacterial cell walls. Jenkin (1960) reported the presence of muramic acid in cell-wall preparations of meningopneumonitis virus. The presence of a cell wall of bacterial type is also evident in electron micrographs (Plotz, Smadel, Anderson & Chambers, 1943; Wissig, Caro, Jackson & Smadel, 1956; Stoker, Smith & Fiset, 1956; Armstrong, Valentine & Fildes, 1963). In air-dried preparations of suspensions of psittacosis-lymphogranuloma organisms the cell wall has a characteristic appearance like the brim of a hat lying flattened around the shrivelled central mass of the organism. In ultra-thin sections the cell wall appears as a distinct layer outside the delicate cytoplasmic membrane of rickettsiae and psittacosis-lymphogranuloma organisms. These cell walls resemble closely those seen in ultra-thin sections of bacteria (Glauert, Brieger & Allen, 1961). The free acidic groups in the cell wall might account, at least in part, for the well-known basophilia of the organisms when studied by conventional staining techniques. The fate of the cell wall during intracellular replication of the organisms is not yet known. It is widely accepted that rickettsiae undergo binary fission in the cytoplasm of infected cells, as shown by the time-lapse cinematographic studies of Schaechter, Bozeman & Smadel (1957*a*). If this be so, it is reasonable to suppose that a typical and intact cell wall is present throughout the cycle of replication of rickettsiae.

The situation is less certain in the psittacosis-lymphogranuloma group. Bedson (1959) concluded that the organisms pass through a series of developmental forms larger than the 0.2μ elementary bodies when multiplying by binary fission. On the other hand, electron micrographs of thin sections of cells infected with high multiplicities of psittacosis organisms (Tajima, Nomura & Kubota, 1957) and trachoma organisms (Armstrong *et al.* 1963) indicate that an early stage of development is the formation of comparatively large bodies or plaques. In the first 24 hr. after infection no definite cell walls surrounding the small invading organisms are seen, and although the photographs are difficult to interpret unambiguously, the appearances suggest that there may be an intimate association of the organisms with one another and even with the host cell cytoplasm. Later newly formed small bodies with discrete cell walls appear in large numbers and seem to undergo typical binary fission. One interpretation of these findings is that the relatively rigid cell walls are temporarily lost during the early phases of intracellular replication, the organisms being analogous to the L-forms of bacteria, and correspondingly pleo-

morphic. With the re-formation of cell walls later would come the regular appearance of the 'elementary bodies' which are stable and highly infectious.

Another argument has been presented to support the view that the psittacosis organism undergoes a virus-like replication. Tanami, Pollard & Starr (1961) showed that multiplication of psittacosis organisms in tissue-culture cells was inhibited by 5-fluoro-2-deoxyuridine (FUDR) and FU (5-fluorouracil). From the effects of adding FUDR and FU at different times during the course of development they concluded that synthesis of psittacosis DNA preceded synthesis of psittacosis protein by about 7 hr. They therefore suggested that psittacosis DNA replicates independently and is later incorporated into newly formed organisms. However, several points about this investigation make these conclusions unconvincing. In the first place, the inhibitory effect of FUDR was annulled by uracil in only slightly higher concentrations than by thymidine. This is difficult to reconcile with a specific FUDR inhibition of thymidylate synthetase (see Rich, Saslaw & Eidinhoff, 1960). Furthermore, the conclusion of Tanami *et al.* (1961) that psittacosis DNA synthesis occurs relatively early is at variance with the observations of Becker, Mashiah & Bernkopf (1962) that most DNA synthesis (incorporation of labelled thymidine) occurred late in the intracellular developmental cycle of the related trachoma organism.

There is another quite different inhibitory effect of these drugs which may account for such results. FU powerfully inhibits not only RNA and protein synthesis (Tanami *et al.* 1961), but also the synthesis of the mucopeptide component of bacterial cell walls, which takes place through uridine intermediates (Rogers & Perkins, 1960). Thus the inhibition by FU of the formation of small infectious elementary bodies of the psittacosis organism, despite accumulation of large amounts of RNA (Starr, Pollard, Tanami & Moore, 1960; Pollard, Moore, Tanami & Starr, 1961), may well be because mucopeptide cell walls are required for this process.

Penicillin is another compound known to act on bacteria by inhibiting the synthesis of cell-wall mucopeptides (see Rogers, 1962). It has been suggested that this action is specifically due to a structural relationship between penicillin and *N*-acetylmuramic acid (Collins & Richmond, 1962). Thus the observation that penicillin also prevents the formation of infectious elementary bodies of the psittacosis organism (Starr *et al.* 1960; Pollard *et al.* 1961) may be due to its specific action on the synthesis of mucopeptide structures containing muramic acid like those of bacteria.

In view of these and all the other findings mentioned above it seems difficult to escape the conclusion that rickettsiae and psittacosis-lymphogranuloma organisms have closer biochemical affinities with Gram-negative bacteria than with the animal viruses. Perhaps they have evolved from bacteria by loss of certain enzymes necessary for independent replication, and so have become obligate intracellular parasites. Bacteria such as *Mycobacterium lepraemurium* are also restricted to intracellular replication. The apparent inability of suspensions of rickettsiae to utilize glucose, even though they metabolize tricarboxylic acid cycle intermediates (Bovarnick & Snyder, 1949; Price, 1953), supports this suggestion. There is a substantial body of opinion that all rickettsiae have evolved from insect parasites, and among the rickettsiae which infect insects there seems to be no sharp line of distinction between intracellular and free-living forms (Steinhaus, 1946).

We are indebted to Drs P. Bradstreet, E. B. Jackson and J. Marshall, and to Lederle Laboratories, Inc., for providing suspensions of some of the organisms studied, and for electron micrographs of preparations.

REFERENCES

- ALLISON, A. C. & ARMSTRONG, J. A. (1960). Abnormal distribution of nucleic acids in tissue culture cells infected with polyoma virus. *Brit. J. Cancer*, **14**, 313.
- ALLISON, A. C. & BURKE, D. C. (1962). The nucleic acid contents of viruses. *J. gen. Microbiol.* **27**, 181.
- ALLISON, A. C. & PERKINS, H. R. (1960). The presence of cell walls like those of bacteria in rickettsiae. *Nature, Lond.* **188**, 796.
- AMINOFF, D., MORGAN, W. T. J. & WATKINS, W. M. (1952). The action of dilute alkali on the N-acetylhexosamines and the specific blood-group mucoids. *Biochem. J.* **51**, 379.
- ANDREWES, C. H. (1952). Classification and nomenclature of viruses. *Annu. Rev. Microbiol.* **6**, 119.
- ANDREWES, C. H. & HORSTMANN, D. M. (1949). The susceptibility of viruses to ethyl ether. *J. gen. Microbiol.* **3**, 290.
- ARMSTRONG, J. A., VALENTINE, R. C. & FILDES, F. C. (1963). Structure and replication of the trachoma agent in cell cultures as shown by electron microscopy. *J. gen. Microbiol.* **30**, 59.
- BAKER, J. A. (1944). A virus causing pneumonia in cats and producing elementary bodies. *J. exp. Med.* **79**, 159.
- BECKER, Y., MASHIAH, P. & BERNKOPF, H. (1962). Biochemical changes in FL cell cultures infected with a trachoma agent. *Nature, Lond.* **193**, 271.
- BEDSON, S. P. (1959). The psittacosis-lymphogranuloma group of infective agents. *J. roy. Soc. publ. Hlth*, **22**, 67.
- BERGER, L. R. & WEISER, R. S. (1957). The β -glucosaminidase activity of egg-white lysozyme. *Biochim. biophys. Acta*, **26**, 517.
- BOVARNICK, M. R. & SNYDER, J. C. (1949). Respiration of typhus rickettsiae. *J. exp. Med.* **89**, 561.
- CLAVERO, G. & PEREZ-GALLARDO, F. (1943). Estudio experimental de una cepa apatógena e inmunizante de *Rickettsia prowazeki*. *Cepa E. Rev. Sanid Hig. Publ., Madr.* **17**, 1.
- COHEN, S. S. (1957). The biosynthesis of nucleic acids in some microbial systems. In *The Chemical Basis of Heredity*, p. 651. Ed. W. D. McElroy & B. Glass. Baltimore: Johns Hopkins Press.
- COLLINS, J. F. & RICHMOND, M. H. (1962). A structural similarity between N-acetylmuramic acid and penicillin as a basis for antibiotic action. *Nature, Lond.* **195**, 142.
- CRUMPTON, M. J. (1959). Identification of amino-sugars. *Biochem. J.* **72**, 479.
- CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell walls in some Gram-positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.
- DAVIS, G. E. & COX, H. R. (1938). A filter-passing infectious agent isolated from ticks. *Publ. Hlth Rep., Wash.* **53**, 2259.
- DERRICK, E. K. & BROWN, H. G. (1949). Isolation of the Karp strain of *Rickettsia tsutsugamushi*. *Lancet*, **ii**, 150.
- DONALD, H. B. & ISAACS, A. (1954). Counts of influenza virus particles. *J. gen. Microbiol.* **10**, 457.
- FRANK, H., LEFORT, M. & MARTIN, H. H. (1962). Chemical analysis of a mucopolymer component in cell walls of the blue and green alga *Phormidium uncinatum*. *Biochem. Biophys. Res. Comm.* **7**, 322.
- GLAUERT, A. M., BRIEGER, E. M. & ALLEN, J. M. (1961). The fine structure of vegetative cells of *Bacillus subtilis*. *Exp. Cell Res.* **22**, 73.
- HOUGH, L., JONES, J. K. N. & WADMAN, W. H. (1950). Quantitative analysis of mixtures of sugars by the method of partition chromatography. V. Improved methods for the separation and detection of their methylated derivatives on the paper chromatogram. *J. chem. Soc.* p. 1702.

- HURST, E. W. (1953). Chemotherapy of virus diseases. *Brit. med. Bull.* **9**, 180.
- JENKIN, H. M. (1960). Preparation and properties of cell walls of the agent of meningo-pneumonitis. *J. Bact.* **80**, 639.
- KAPLAN, C. & VALENTINE, R. C. (1959). The infectivity of purified and partially purified preparations of vaccinia and cowpox viruses. *J. gen. Microbiol.* **20**, 612.
- KUHN, R. & KRÜGER, G. (1957). Das Chromogen III der Morgan-Elson-reaktion. *Chem. Ber.* **90**, 264.
- MAXCY, K. F. (1929). Endemic typhus fever of southeastern United States; reaction of guinea pig. *Publ. Hlth Rep., Wash.* **44**, 589.
- MORGAN, W. T. J. & ELSON, L. A. (1934). A colorimetric method for the determination of *N*-acetylglucosamine and *N*-acetylchondrosamine. *Biochem. J.* **28**, 988.
- PARTIDGE, S. M. (1948). Filter-paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42**, 238.
- PERKINS, H. R. (1960*a*). The structure of a disaccharide liberated by lysozyme from the cell walls of *Micrococcus lysodeikticus*. *Biochem. J.* **74**, 182.
- PERKINS, H. R. (1960*b*). Substances reacting as hexosamine and as *N*-acetylhexosamine liberated from bacterial cell walls by lysozyme. *Biochem. J.* **74**, 186.
- PERKINS, H. R. & ROGERS, H. J. (1959). The products of the partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus*. *Biochem. J.* **72**, 647.
- PLOTZ, H., SMADEL, J. G., ANDERSON, T. F. & CHAMBERS, L. A. (1943). Morphological structure of rickettsiae. *J. exp. Med.* **77**, 355.
- POLLARD, M., MOORE, R. W., TANAMI, Y. & STARR, J. J. (1961). Cytochemical studies with psittacosis virus by fluorescence microscopy. *Texas Rep. Biol. Med.* **19**, 343.
- PRICE, W. H. (1953). The epidemiology of Rocky Mountain Spotted Fever I. The characterization of strain virulence of *Rickettsia rickettsii*. *Amer. J. Hyg.* **58**, 248.
- REPASKE, R. (1958). Lysis of Gram-negative organisms and the role of versene. *Biochim. biophys. Acta*, **30**, 225.
- RICH, M. A., SASLAW, L. & EIDINHOFF, M. L. (1960). Some differences among various mammalian cell lines in reversal of growth inhibition by 5-fluorouracil. *Proc. Soc. exp. Biol., N.Y.* **103**, 791.
- ROGERS, H. J. (1962). The mode of action of the penicillins. *Ciba Study Group, no. 13: The Resistance of Bacteria to the Penicillins*, p. 25. London: J. & A. Churchill.
- ROGERS, H. J. & PERKINS, H. R. (1960). 5-Fluorouracil and mucopeptide biosynthesis by *Staphylococcus aureus*. *Biochem. J.* **77**, 448.
- RONDLE, C. J. M. & MORGAN, W. T. J. (1955). The determination of glucosamine and galactosamine. *Biochem. J.* **61**, 586.
- SALTON, M. R. J. & GHUYSEN, J. M. (1960). Acetylhexosamine compounds enzymically released from *Micrococcus lysodeikticus* cell walls. III. The structure of di- and tetrasaccharides released from cell walls by lysozyme and streptomycetes F_1 enzyme. *Biochim. biophys. Acta*, **45**, 355.
- SCHAECHTER, M., BOZEMAN, F. M. & SMADEL, J. G. (1957*a*). Study on the growth of rickettsiae. II. Morphologic observations on living rickettsiae in tissue culture cells. *Virology* **3**, 160.
- SCHAECHTER, M., TOUSIMIS, A. J., COHN, Z. A., ROSEN, H., CAMPBELL, J. & HAHN, F. E. (1957*b*). Morphological, chemical and serological studies of the cell walls of *Rickettsia mooseri* (R. typhi). *J. Bact.* **74**, 822.
- SCHAFER, W. (1959). Some observations concerning the reproduction of RNA-containing animal viruses. *Symp. Soc. gen. Microbiol.* **9**, 61.
- SMADEL, J. E., WALL, M. J. & GREGG, A. (1943). An outbreak of psittacosis in pigeons, involving the production of inclusion bodies, and transfer of the disease to man. *J. exp. Med.* **78**, 189.
- STARR, J. J., POLLARD, M., TANAMI, Y. & MOORE, R. W. (1960). Cytochemical studies with psittacosis virus by fluorescence microscopy. *Texas Rep. Biol. Med.* **18**, 501.
- STEINHAUS, E. A. (1946). *Insect microbiology*. Ithaca: Comstock.
- STOFFYN, J. & JEANLOZ, R. W. (1954). Identification of amino sugars by paper chromatography. *Arch. Biochem. Biophys.* **52**, 373.

- STOKER, M. R. P., SMITH, K. M. & Fiset, P. (1956). Internal structure of *Rickettsia burnetii* as shown by electron microscopy of thin sections. *J. gen. Microbiol.* **15**, 632.
- TAJIMA, M., NOMURA, Y. & KUBOTA, Y. (1957). Structure and development of the psittacosis-lymphogranuloma group observed in the electron microscope. *J. Bact.* **74**, 605.
- TANAMI, Y., POLLARD, M. & STARR, J. J. (1961). Replication pattern of psittacosis virus in a tissue culture system. *Virology*, **15**, 22.
- T'ANG, F. F., CHANG, H. C., HUANG, Y. T. & WANG, K. C. (1957). Studies on the etiology of trachoma with special reference to the isolation of the virus in the chick embryo. *Chinese Med. J.* **75**, 429.
- WESTWOOD, J. C. N., PHIPPS, P. H. & BOULTER, E. A. (1957). The titration of vaccinia virus on the chorio-allantoic membrane of the developing chick embryo. *J. Hyg., Camb.* **55**, 123.
- WISSIG, S. L., CARO, L. G., JACKSON, G. B. & SMADEL, J. E. (1956). Electron microscopic observations on intracellular rickettsiae. *Amer. J. Path.* **32**, 1117.
- WORK, E. (1961). The mucopeptides of bacterial cell walls. A review. *J. gen. Microbiol.* **25**, 167.
- WORK, E. & DEWEY, D. L. (1953). The distribution of $\alpha\epsilon$ -diaminopimelic acid among various micro-organisms. *J. gen. Microbiol.* **9**, 394.