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SCANNING ELECTRON MICROSCOPIC OBSERVATIONS OF THE SURFACE OF THE INITIAL LESION IN EXPERIMENTAL STREPTOCOCCAL ENDOCARDITIS IN THE RABBIT

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Summary—The surfaces of vegetations in the first 24 h after initiation of experimental streptococcal endocarditis in rabbits were examined systematically using the scanning electron microscope (SEM). Most of the observed fields were occupied by amorphous material but with the SEM very small features of interest could be identified. Erythrocytes were more frequent before 6 h and leukocytes were rarely seen. Bacteria were significantly more frequent at 10/15 min and at 20 h. It is suggested that bacteria lodge on the vegetation surface following inoculation and that after a lag phase colonies are formed within the vegetation which reach the surface again at about 20 h.

THE RABBIT MODEL of infective endocarditis devised by Garrison and Freedman (1970) and modified by Durack, Beeson and Petersdorf (1973) has gained wide acceptance as a method of study applicable to the problems of prophylaxis and treatment of infective endocarditis in man (Kaye, 1976).

In the experimental system described by Durack and Beeson (1972*a*) a plastic cannula is inserted into a rabbit's heart and the endocardial trauma produces vegetations of non-bacterial thrombotic endocarditis. When the vegetations are infected by exposure to an experimental bacteraemia a disease is produced which mimics human infective endocarditis in its clinical course. The infected lesions closely resemble those found in the human disease by all means of examination (McGowan, 1977), and as the time of infection is known it is possible to observe the very earliest events in their development. Durack and Beeson (1972*a*,*b*) examined the colonization and progress of the experimental lesion over the first few days of infection. Observations of the first 24h period were limited to a few animals but they described the presence of organisms on the vegetation surface 6 h after infection and an abrupt change at 20 h when the developing colonies became covered with fibrin. As the progress of the lesion was monitored by counting the number of colony-forming units (c.f.u.) per g of vegetation, the distribution of organisms within the vegetation was not measured.

Infection in experimental endocarditis is produced by lodgement of bacteria from the circulating blood on the surface of the vegetation and it therefore seemed logical to look at this process using the scanning electron microscope (SEM) which uniquely enables the examination of minute surface structure. The fixation and preparation of a soft-tissue specimen for scanning electron microscopy inevitably involves the possibility of distortion or damage and these difficulties are compounded when the specimen is taken from a lesion the surface of which is exposed to deposits from the

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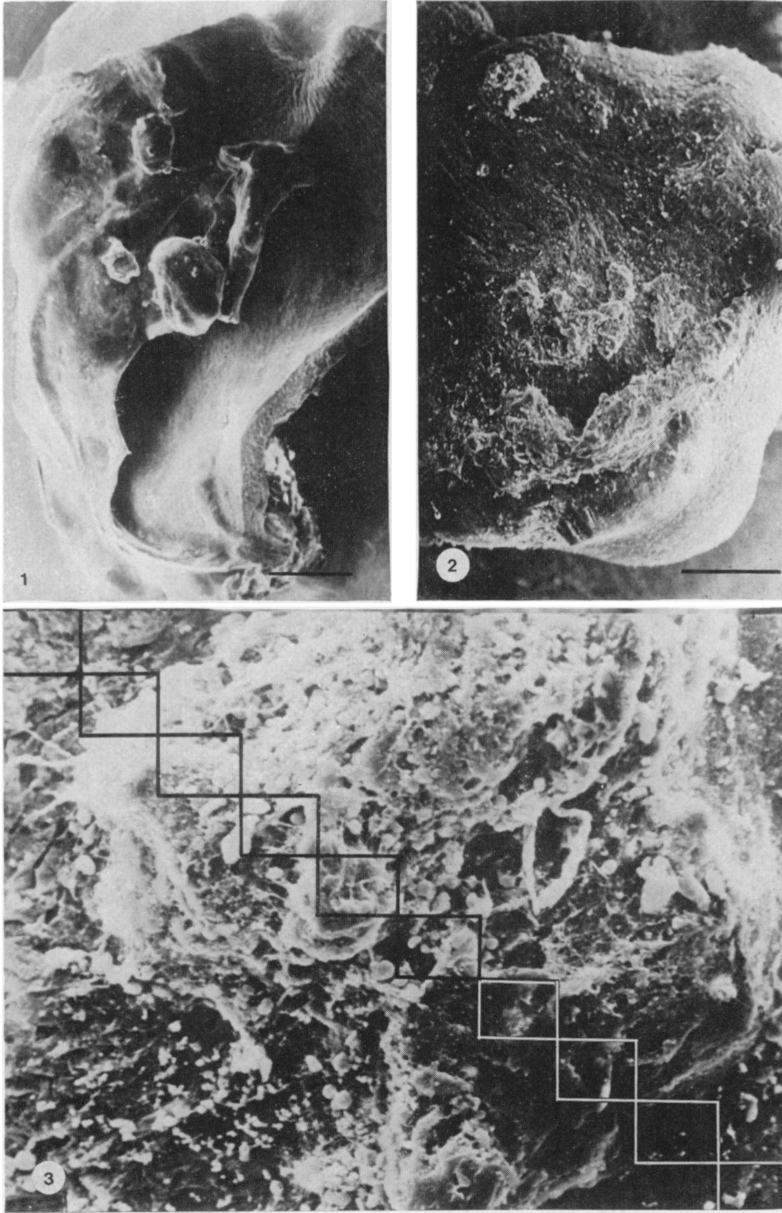

FIG. 1.—Scanning electron micrograph of rabbit heart valve showing prominent vegetations. Scale bar = 1 mm.

FIG. 2.—Higher-power scanning electron micrograph of one vegetation from Fig. 1. Scale bar = 200 μm.

FIG. 3.—One 250μm × 180μm field from Fig. 2, selected for analysis at this magnification. Superimposed are the 10 fields, 25 μm × 18 μm, from this area which were analysed.

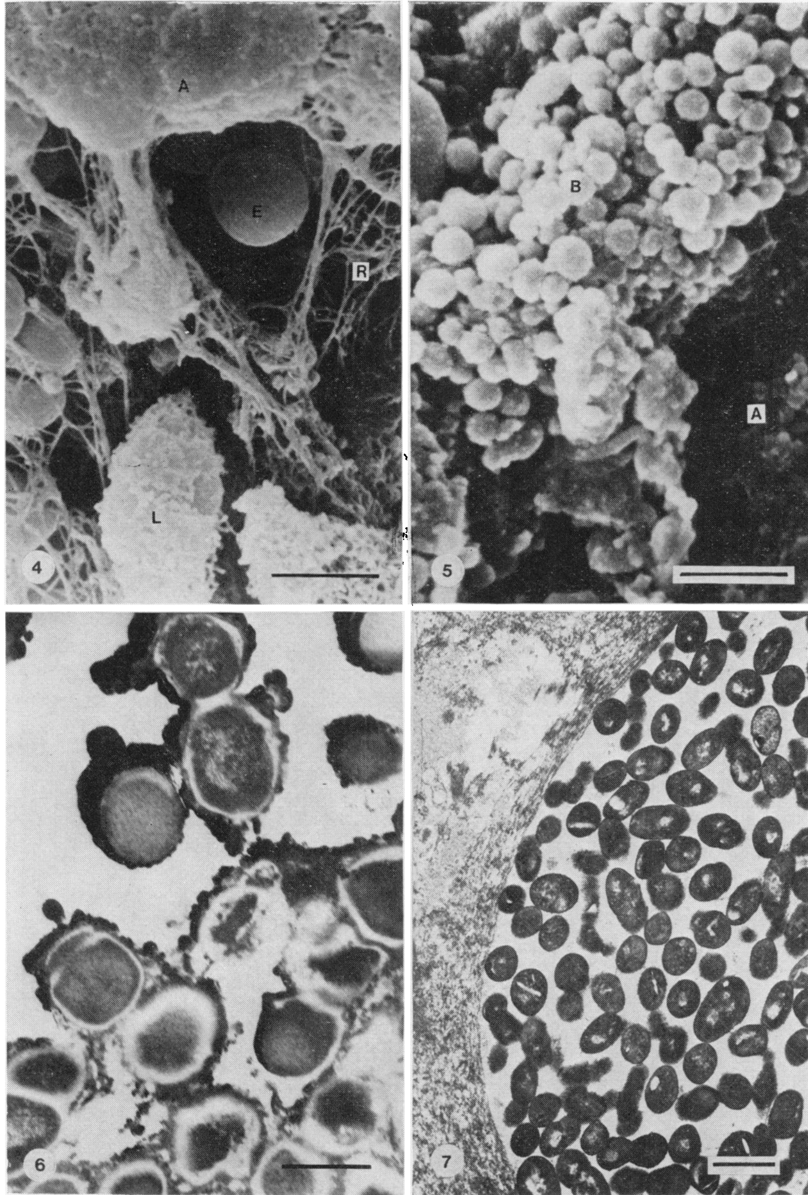

FIG. 4.—A typical 25μm × 18μm field (the fourth in the diagonal sequence shown in Fig. 3) at the magnification at which counting was performed. Note the amorphous (A) and reticular (R) appearances of the underlying vegetation, and the erythrocyte (E) and leucocyte (L) included in this field. Scale bar = 4 μm.

FIG. 5.—A similar high-power field showing a collection of bacteria (B) on an amorphous area of vegetation (A). Scale bar = 2 μm.

FIG. 6.—Transmission electron micrograph from reprocessed SEM material, confirming that the 0·8–1μm spheres seen in Fig. 5 are bacteria. Note the gold coating applied for scanning electron microscopy. Scale bar = 1 μm.

FIG. 7.—Transmission electron micrograph from a deeper area of a vegetation, 22 h after inoculation, showing an active bacterial colony of moderate size walled off with fibrin. Scale bar = 1 μm.

circulating blood, and which has to be harvested aseptically to avoid extraneous bacterial contamination. There are few published quantitative studies of soft-tissue material using the scanning electron microscope so that methods are not well established. Despite these problems a series of experiments was planned to produce material for a systematic study of the vegetation surface in the first 24 h after initiation of experimental streptococcal endocarditis.

METHOD

Twenty-three 2–4 kg New Zealand white rabbits were cannulated, *via* the external carotid artery, with lengths of plastic tubing (PP25, Portex Limited, Hythe, Kent) and 2 or 3 days later inoculated with 1ml doses of a broth suspension (Hardie and Bowden, 1974) of *Streptococcus mitior*. The strain used was obtained from Dr D. T. Durack and was the one used in his earlier experiments (Durack and Beeson, 1972*a*, *b*).

Doses were made up from fresh overnight broth cultures which were centrifuged at 1,000 *g* for 15 min. The supernatant was discarded and the organisms resuspended in fresh broth to make up a turbid solution. The optical density of the solution was compared in a spectrophotometer with a predetermined standard to ensure that the number of organisms contained was at least 10^7 c.f.u. and more accurate quantification was achieved subsequently by serial dilution and colony counts.

Animals were heparinized with 5,000 u i.v. and killed with i.v. Nembutal at 10–15 min (2 animals), 1 h (2), 6 (2), 12 (2), 16 (2), 18 (3), 20 (4), 22 (3) and 24 (2) h after inoculation. Typical vegetations from the left ventricle and aortic valve were harvested aseptically and prepared for examination. A limited quantity of material was prepared by conventional methods for light (LM) and transmission electron microscopy (TEM) (McGowan, 1978) but priority was given to samples for SEM. Specimens of bacteria in pure culture, clotted rabbit blood, and endocardium and vegetation from uninfected animals were also prepared for SEM comparison with the experimental material.

The pieces of tissue were dropped immediately into bottles of 2·5% distilled glutaraldehyde in 0·1M phosphate buffer (pH 7·3). The specimens usually consisted of a portion of aortic or ventricular wall with a vegetation on the surface or a section including an aortic valve cusp and a related vegetation. They were shaken vigorously for 10–15 sec in the buffered glutaraldehyde and then left in it for 3 h. At this time the liquid was poured away and replaced with a fresh quantity of the same solution. The specimens were then left for a further 20 h when they were washed in 0·1M phosphate buffer (pH 7·3) and slowly dehydrated by passage through graded ethanol solutions—30% initially, followed by 50%, 70%, 80%, 90%, 95% and finally 3 changes of dry absolute ethanol. After 45 min the ethanol was replaced by dry acetone and left for 30 min. This last procedure was repeated once. Specimens were critical-point-dried from the dry acetone *via* liquid CO_2 in a Polaron E3000 critical-point dryer (Polaron Equipment Ltd., Watford) and then mounted on stubs with Silver "Dag" 915 (Acheson Colloids Co., Prince Rock, Plymouth) and coated with gold in a Polaron E 5000 sputter coater. Some of these specimens were later rehydrated and processed for TEM in order to verify interpretation of surface detail and gain additional sub-surface information (Fig. 6). Specimens were viewed in a Cambridge Stereoscan 600 scanning electron microscope at an accelerating voltage of 15 kv. Photographs were obtained on Ilford 35mm negative film using the Cambridge Record Console attached to the Stereoscan 600 fitted with a Nikon Nikomat Camera and lens. They were taken at a frame speed of 50 sec using the smallest spot size available.

By naked eye examination and at the lowest SEM magnifications ($\times 20$ and $\times 100$) (Figs 1, 2) areas of vegetations were selected for more detailed examination. At a magnification of $\times 500$ (Fig. 3) the topography of $250\mu m \times 180\mu m$ areas of vegetation likely to harbour leucocytes and bacteria could be recognised, though individual cells could not be seen. A standardized sampling procedure was adopted whereby 10 fields measuring $25\mu m \times 18\ \mu m$ were observed at a magnification of $\times 5000$ along a diagonal of the selected $250\mu m \times 180\mu m$ field (Fig. 3). At least 4 sets of observations were made of vegetations from at least 2 animals at each time interval. The counts of the $25\mu m \times 18\mu m$ fields were transferred to 80-column punch cards and analysed statistically by non-parametric methods using the "Statistical Package for the Social Sciences" (Nie *et al.*, 1974) on a CDC.600 computer at the London University Computer Centre.

RESULTS

Background areas, either amorphous or reticular in pattern, made up the major part of the observed field at all time periods. The mean proportion of amorphous areas ranged from 54% to 81% and tended to be higher at times later than

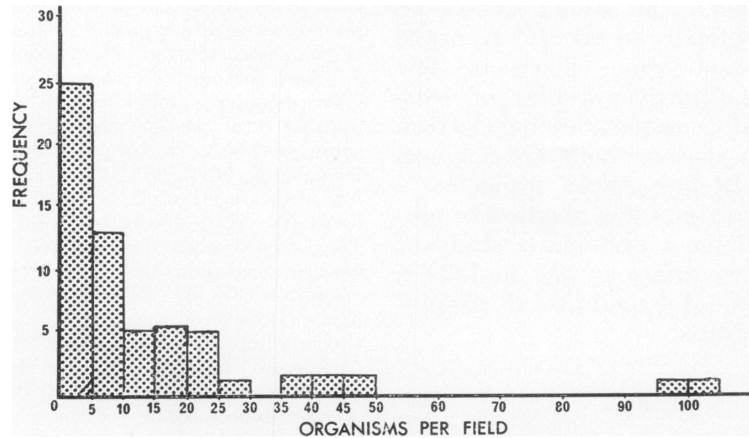

FIG. 8.—Distribution of bacteria at 15 min.

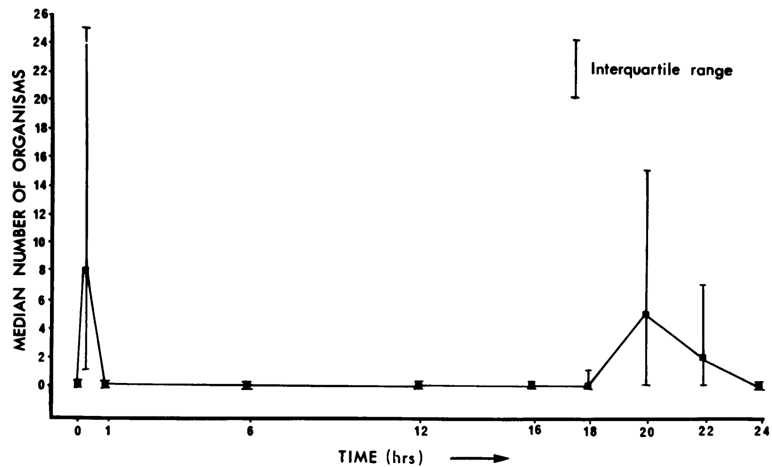

FIG. 9.—Median number of bacteria per field.

12 h, a difference which was statistically significant ($P < 0{\cdot}05$). The mean proportion of reticular areas varied from 4% to 23% but there was no significant variation between times ($P > 0{\cdot}05$). Specimens from animals killed 3 days after inoculation in other experiments (McGowan, 1978) showed the surface to be completely covered by an amorphous fibrin layer. The mean number of erythrocytes per field varied from 1·4 to 10·3 and they were seen in very significantly larger numbers before 6 h than after ($P < 0{\cdot}001$). Leucocytes were very rare at all time periods. The largest number was seen at 20 h, when the mean was just under 4 cells in 10 fields, but this difference was not significant ($P > 0{\cdot}05$). Median rather than mean numbers of bacteria per field were calculated, since the distribution was grossly skewed (Fig. 8) and the results are shown graphically (Fig. 9). In view of this distribution the data were analysed nonparametrically by Kruskal-Wallis one way analysis (Siegel, 1956) and the differences between certain time periods were highly significant ($P < 0{\cdot}001$). The data for the different samples taken at the 2 time periods corresponding to the peaks on the graph, *i.e.* 10/15 min and 20 h, were

analysed by the same method and there were no significant differences between animals in these groups ($P > 0{\cdot}05$). Specimens from another group of animals which had died from experimental streptococcal endocarditis (McGowan, 1978) were almost completely covered in masses of bacteria derived from the terminal septicaemia.

DISCUSSION

The main purpose of this study was the examination of the events on the vegetation surface during the first 24 h following inoculation. The comprehensive series of SEM observations were made for this purpose. The TEM and LM material, which is more relevant to subsurface events, was less complete. Nevertheless, almost all sections included a portion of vegetation surface and some information was gained from this complementary material. The expected advantages of scanning electron microscopy in examination of the surface of recently infected vegetations were fully borne out in practice. It proved possible to pick out areas of interest for detailed examination and to avoid lengthy analysis of the featureless areas which predominated at all time intervals. This predominance means that examination by light microscopy or transmission electron microscopy must be by means of multiple sections, the vast majority of which will be of no interest.

There was some suggestion of a change in vegetation surface structure during the period of observation. The increase in the mean proportion of amorphous appearance after 12 h was slight but statistically significant and could indicate predominance of platelet rather than fibrin material at later times. This gains some support from the marked predominance of platelets in one of the 20-h TEM specimens. However, this change in proportion may simply reflect a fall in the numbers of erythrocytes as the proportion of reticular appearance was relatively constant and the proportions occupied by bacteria and leucocytes was negligible. The figures for erythrocyte numbers, which of course are independent, show the fall after 6 h to be a real phenomenon. Since no differences were noted in the erythrocytes themselves it might indicate a reduction in vegetation thickness surface "stickiness" which would allow the cells to be washed off during preparation.

The most clear-cut results were those for the numbers of bacteria seen on the lesion surface in the SEM (Fig. 9). There were 2 peak times at 10/15 min and at 20 h, and these were statistically highly significant. The peak at 10/15 min is perhaps not unexpected, since large numbers of organisms are likely to stick to the surface after such a high dose inoculation. There would not be time for any process other than dispersal and lodgement to occur. The observations at this time showed cocci in groups or sheets dispersed over the lesion surface in an apparently random fashion but tending to be more numerous where surface roughness or minute projections or defects might facilitate lodgement. There was no clear pattern suggesting a preferential attachment to any particular type of surface other than by these purely mechanical aspects. In LM sections of 1-h vegetations, bacteria could be seen in the substance of the vegetation. They appeared to be randomly distributed and, while staining deeply, showed little other evidence of activity and in particular no colony nor even chain formation. Bacteria were not found again by any method of examination till the 16-h stage, when small colonies were seen in LM sections. In the only previous study of the earliest lesion in experimental endocarditis (Durack and Beeson, 1972*a*; Durack, 1974), it was found that in 6 rabbits killed 30–90 min after inoculation the number of streptococci/g was similar to that found in spleen or liver, whereas by 6 h specimens from 2 rabbits showed a marked difference with over a 1000-fold increase in the vegetation against a fall in the other tissues. This finding implies a lag phase of between $1\frac{1}{2}$

and 6 h. The present experiments do not help to define this time any more accurately but are confirmatory in that no evidence of bacterial multiplication between 15 min and 12 h was found by any means of examination. This point requires further study, particularly since antibiotic prophylaxis may be ineffective while organisms are quiescent.

A dramatic reappearance of bacteria on some areas of the surface, seen in the SEM specimens and confirmed in TEM sections (Figs 5, 6) occurred at about 20 h after inoculation. Durack and Beeson (1972*a*) suggested that at about 20 h after infection a layer of fibrin was quite suddenly deposited on the surface of the growing colonies at the vegetation surface and that this layer protected them from phagocytosis. Though the SEM specimens examined show peak numbers of surface bacteria at this time, the two sets of observations are compatible, in that fibrin deposition was observed immediately after the 20-h point, the surface becoming completely recovered by 24 h. The difference of a few hours between the two sets of observations need not have any real significance. There was evidence of phagocytosis on TEM examination at 20 and at 22 h but leucocytes were hardly ever seen on the SEM specimens. The streptococci themselves or breakdown products from their necrosis could conceivably stimulate the thrombotic process. Durack (1972) found that streptococci did cause aggregation of platelets by a mechanism not involving adenosine diphosphate, but this aspect remains to be fully explored. There was no evidence of dissolution of fibrin in relation to the colonies; indeed, TEM observations suggests condensation of the material as the colonies increase in size (Fig. 7). If the fibrin deposition rate is constant, then explanation of the failure of coverage could lie in the growth rate of the colonies, which may reach a maximum at the 18–20-h stage and outstrip the capacity of the covering mechanism. The period of observation was too short to find out if this suggested sequence of events might be repeated at later times on a cyclical pattern. Sections of older vegetations certainly show a layered appearance which could be explained in this way, though "reseeding" of the vegetation surface by bacteria from the bloodstream is also likely to produce such an appearance. Whatever the explanation, it seems clear that at 18–24 h after infection there is a relatively high concentration of streptococci on some parts of the surface of the infected vegetations. The suggestion of a bacterial lag phase occurring in the depths of the vegetation, followed by a phase of multiplication in the depths of the vegetation, followed in turn by a phase of multiplication in the superficial layers, could explain the need for prolonged antibiotic exposure which Pelletier, Durack and Petersdorf (1975) found was necessary for effective prevention of the experimental infection. The ideal pattern of antibiotic serum level which they advocate matches quite well with the fluctuation in numbers of bacteria on the vegetation surface.

Few leucocytes were seen on the vegetation surface at any time period and the proportion of leucocytes to erythrocytes was of the order normally found in blood. Two slight increases at 15 min and at 20 h were not statistically significant. Concentrations of leucocytes, both polymorphs and monocytes, were found in TEM preparations in the subsurface and deep parts of the vegetations but they were only numerous in sections obtained from 20 h onwards. There was no evidence to support the suggestion of Durack (1974) that phagocytes may actually carry bacteria on to the vegetation surface. A few leucocytes and bacteria were found even on 15min specimens but despite an extensive search by TEM and light microscopy there was no evidence of phagocytosis until well after substantial colony formation had been established. It is quite likely that leucocytes which have phagocytosed bacteria in the circulation would come to lodge on the vegetation surface but this does not necessarily imply any significant role in the process of initiation of the

infection. The lack of observed leucocyte activity is in accordance with most observations on the mature lesions in human endocarditis. Cell-mediated immune reactions seem to be of minor importance but humoral responses may play a role which was not indicated by the methods of observation used.

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REFERENCES

DURACK, D. T. (1972) Experimental Endocarditis. D.Phil. Thesis, University of Oxford.

DURACK, D. T. (1974) Experimental Bacterial Endocarditis. IV Structure and Evolution of Very Early Lesions. *J. Path.*, **115**, 81.

DURACK, D. T. & BEESON, P. B. (1972*a*) Experimental Bacterial Endocarditis. I: Colonization of a Sterile Vegetation. *Br. J. exp. Path.*, **53**, 44.

DURACK, D. T. & BEESON, P. B. (1972*b*) Experimental Bacterial Endocarditis. II: Survival of Bacteria in Endocardial Vegetations. *Br. J. exp. Path.*, **53**, 50.

DURACK, D. T., BEESON, P. B. & PETERSDORF, R. G. (1973) Experimental Endocarditis. III: Production and Progress of the Disease in Rabbits. *Br. J. exp. Path.*, **54**, 142.

GARRISON, P. K. & FREEDMAN, L. R. (1970) Experimental Endocarditis. I: Staphylococcal Endocarditis in Rabbits Resulting from Placement of a Polythene Cannula in the Right Side of the Heart. *Yale J. Biol. Med.*, **42**, 394.

HARDIE, J. M. & BOWDEN, G. H. (1974) Cell Wall and Serological Studies on *Streptococcus mutans*. *Caries Res.*, **8**, 301.

KAYE, D. (1976) *Infective Endocarditis*. Baltimore, London and Tokyo: University Park Press.

McGOWAN, D. A. (1977) Scanning Electron Microscopy of Heart-valve Vegetations. *Lancet*, **ii**, 822.

McGOWAN, D. A. (1978) The Initiation of Infective Endocarditis by Oral Micro-organisms in Rabbits Subjected to Endocardial Damage. Ph.D. Thesis. University of London.

NIE, N. H., HADLAI HULL, C., JENKINS, J. G., STEINBRENNER, K. & BENT, D. H. (1974) Statistical Package for the Social Sciences, 2nd edn. New York: McGraw-Hill.

PELLETIER, L. L., JR, DURACK, D. T. & PETERSDORF, R. G. (1975) Chemotherapy of Experimental Streptococcal Endocarditis. IV: Further Observations on Prophylaxis. *J. clin. Invest.*, **56**, 319.

SIEGEL, S. (1956) *Non-parametric Statistics for the Behavioural Sciences*. New York: McGraw-Hill.