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EXPERIMENTAL BACTERIAL ENDOCARDITIS
II. SURVIVAL OF BACTERIA IN ENDOCARDIAL VEGETATIONS

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SUMMARY.—A method has been developed for assessing metabolic activity of bacteria in the vegetations of bacterial endocarditis using a labelled metabolite and autoradiography. Evidence provided by this technique suggests that there are different degrees of activity between superficial and more deeply placed bacterial colonies, and that variations in activity also exist within a single group of organisms. The possible relevance of these findings to the antibiotic therapy of endocarditis is discussed.

CLINICAL experience in the therapy of bacterial endocarditis has shown that even when bactericidal agents such as penicillin are employed against highly susceptible organisms, the therapy must be maintained for weeks in order to eradicate the infection. Considering the diffusibility of penicillin in an artificial thrombus (Weinstein, Daikos and Perrin, 1951) and the high levels of the drug that can be achieved in body fluids, it seems unlikely that inadequate penetration of the vegetation can account for its relative ineffectiveness in this situation. An alternative possibility is that a proportion of the bacteria in the vegetation, though alive and capable of multiplication, are in a " resting " stage and hence not liable to rapid killing when exposed to penicillin (Hobby, Meyer and Chaffee, 1942).

In bacterial endocarditis of man, masses of bacteria can be seen in the vegetations, usually clustered in discrete colonies near the surface (Allen, 1939). In experimental streptococcal endocarditis the majority of the bacteria enter a resting phase within 2 days of first infection (Durack and Beeson, 1972). Examination of vegetations in man and experimental animals, especially when healing has been favoured by penicillin therapy, reveals evidence of bacterial death within the vegetations, in that the morphology and staining are altered and that necrotic tissue and bacteria may undergo calcification (Moore, 1946; McGeown, 1954).

It has not so far been possible to determine what proportion of bacteria seen in sections are potentially viable. We have used an experimental model for bacterial endocarditis to test for evidence of viability of bacteria within the vegetations. The technique employs autoradiography after incubation in the presence of a labelled metabolite.

Tritium labelled L-alanine was selected as the metabolite since this small, freely diffusible molecule not only takes part in intermediary metabolism but is incorporated as D- and L-isomers into the sub-units of the mucopeptide component of cell walls (Strominger and Tipper, 1965). Since penicillin inhibits the final transpeptidase reaction in assembly of these sub-units, it was reasoned that those

cells shown by autoradiography to be active in incorporating alanine might also be susceptible to the effect of penicillin.

METHODS

Experimental streptococcal endocarditis was induced in rabbits by a method previously described (Durack and Beeson, 1972). One animal was killed on the 3rd, 9th, 10th and 14th days after infection and the vegetations excised with aseptic precautions. They were immediately placed in glucose broth to which had been added 5 μCi (4·7 μg) per ml of ^{3}H-labelled L-alanine, and incubated at 37° for 18 h. The isotope was supplied by the Radiochemical Centre, Amersham, England, as a solution containing 90 μCi tritium per μmol L-alanine. The vegetations were then washed and fixed in 10 per cent formol saline for 12 h. The formol saline solution was changed 3 times to allow unincorporated tritium to diffuse out of the specimens.

After routine embedding in paraffin, 5 μm sections of the vegetations were cut, dewaxed, and dipped in Ilford Nuclear Research Emulsion Type K2. They were stored in the dark at 4° for 3–9 days before development for 5·5 min in Kodak D19 developer, fixing for 5 min in Kodafix and staining with haematoxylin and eosin.

RESULTS

Distribution of label in autoradiographs

Preliminary examination of the autoradiographs showed that colonies in a vegetation which had taken up ^{3}H-alanine could be identified easily by the presence of grains overlying them (Fig. 1).

The degree of labelling varied widely between colonies in a single vegetation and 3 groups could be distinguished. Some colonies were heavily labelled, some partially labelled and the rest unlabelled; examples of all 3 groups could sometimes be found within a single field (Fig. 2).

There was a distinct geographical distribution of these groups within a vegetation. In general, deeply situated colonies were unlabelled, intermediate colonies partially labelled, and surface colonies fully labelled. Most of the labelling was confined to the peripheral 10 per cent of the area of a section, and a majority of all colonies was unlabelled. However, isolated foci of labelling were occasionally found even in those parts of a vegetation farthest from the surface.

There was also a geographical distribution of label within single colonies in the partially labelled group. Sometimes grains were confined to a peripheral rim of bacteria (Fig. 3) while in others centrally situated organisms showed labelling (Fig. 4).

Correlation between labelling and staining

Haematoxylin and eosin was used routinely since dark Gram staining of these streptococci tended to obscure the grains. A remarkably precise correlation between labelled organisms and organisms staining deeply with haematoxylin was always observed (Figs. 2–4). Areas of heavy staining were not artefacts due to the presence of grains, since similar areas were seen in adjacent sections stained without autoradiography.

Morphology of colonies

Centrally situated colonies frequently showed paler staining and dissolution of bacteria, leaving amorphous debris and empty spaces (Fig. 5). These changes

most often occurred in the older central parts of colonies. In contrast peripheral colonies usually showed as uniform, dark staining sheets of bacteria.

There was usually a striking absence of leucocytes in the featureless fibrin matrix surrounding the colonies (Fig. 5).

DISCUSSION

This technique appears to identify micro-organisms in a vegetation which are capable of growth under suitable conditions. Further, it localizes active foci within a single colony.

The distribution of activity described is susceptible of 2 explanations. It may be that some bacteria appear inactive because ^{3}H-alanine did not reach them by diffusion. The obvious activity at the surface of vegetations and on the periphery of some colonies would be consistent with defective diffusion of the label into deeper areas. Failure of ^{3}H-alanine to diffuse throughout seems unlikely, however, since well-labelled areas are sometimes found in the depths of large vegetations, and in the centres of large colonies. The alternative explanation is that older colonies contain fewer bacteria capable of metabolic activity. Since the vegetation appears to grow by a process of accretion, the younger colonies tend to be oriented toward the surface, with middle-aged colonies intermediate and old colonies more deeply situated.

Three major factors tend toward sterilization of a localized infection. (1) Phagocytic cells may effect rapid and specific removal of any bacteria, over a period of hours or days. The central importance of this mechanism is evident from the success of bacteriostatic drugs in treating processes like pneumonia, where the antibiotic holds the population steady while phagocytes clear the infection (Wood and Irons, 1946). However, the almost complete absence of phagocytes in large areas of sectioned vegetations has been a notable feature. This is consistent with findings in human endocarditis (Gross and Fried, 1937), and probably explains the failure of bacteriostatic drugs in endocarditis. (2) Ageing of colonies is a slower process resulting in death of organisms over days or weeks. The numbers may or may not be replenished by new growth, according to circumstances such as the presence of antibiotics. Experiments described in the preceding paper indicate that the majority of bacteria in a vegetation enter a resting phase within 2 days of infection. Subsequently the organisms eventually die, and mature vegetations show bacterial dissolution and debris in the older colonies (Fig. 5). (3) Organization of the lesion by fibroblasts and capillaries proceeds slowly over weeks, accompanied by phagocytosis and sometimes calcification of the

EXPLANATION OF PLATES

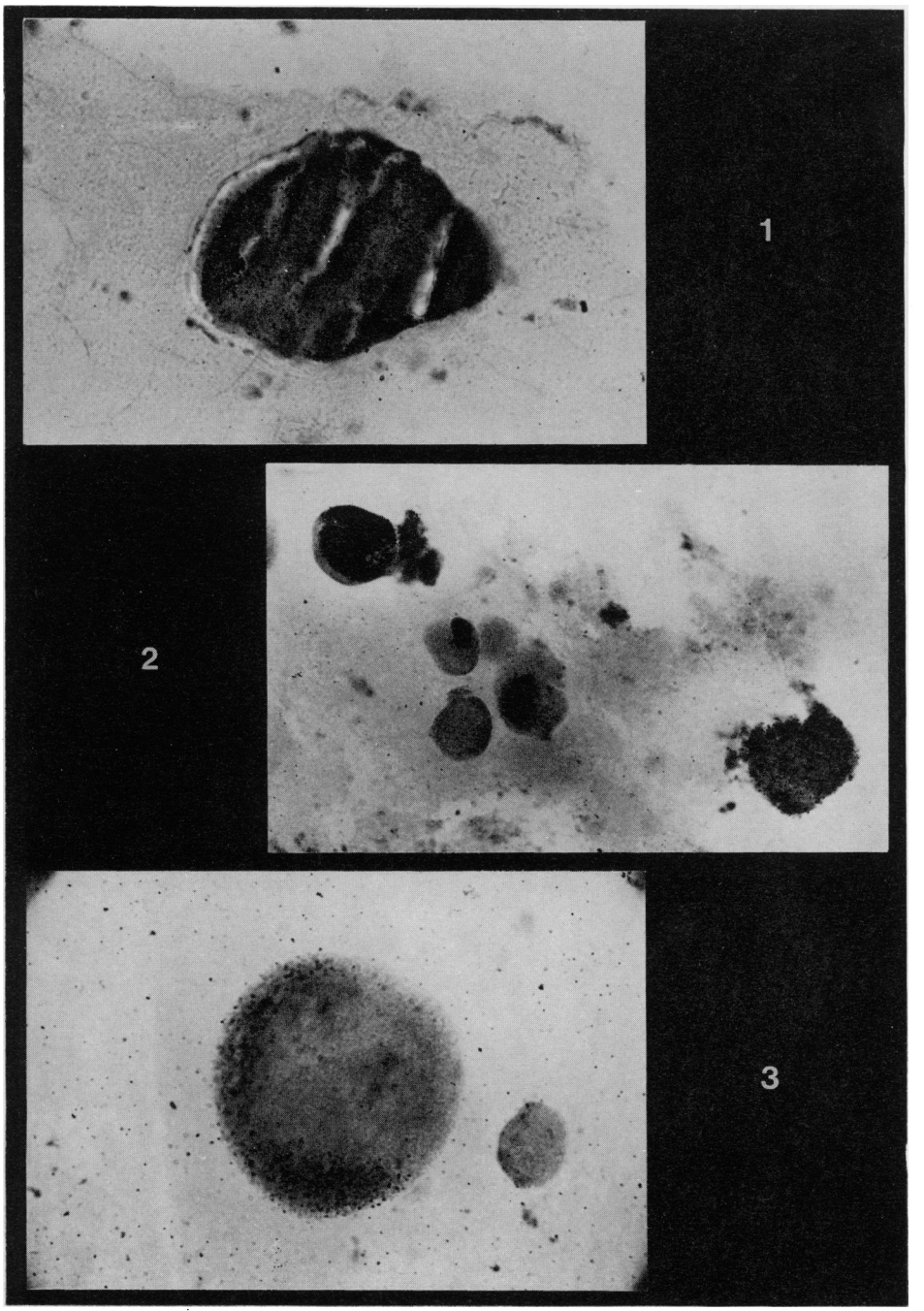
FIG. 1.—Colony of streptococci 3 days after infection showing uniform labelling. Autoradiograph stained with H. and E. × 490.

FIG. 2.—Colonies of streptococci 9 days after infection showing differential uptake of label. Autoradiograph stained with H. and E. × 392.

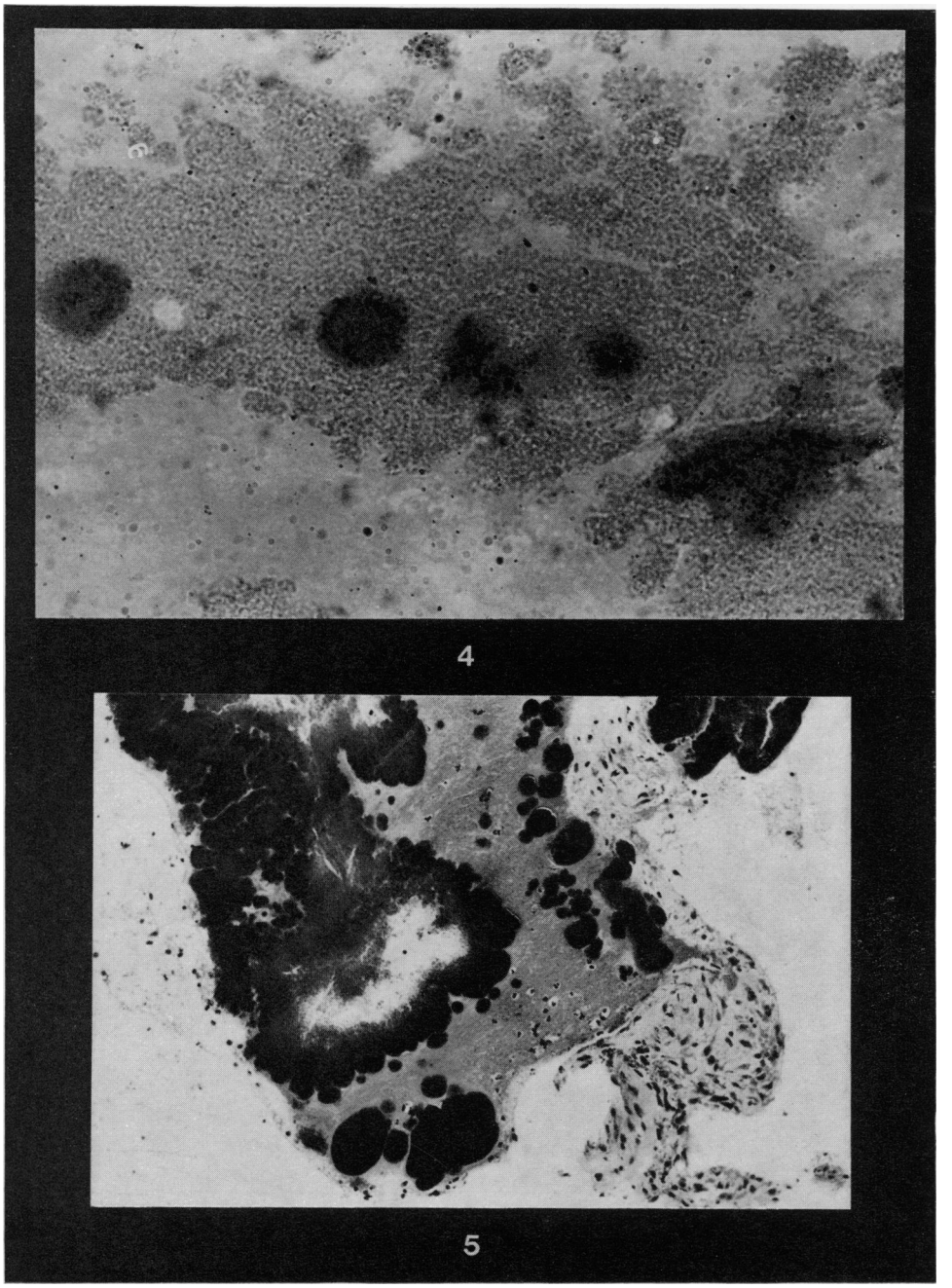
FIG. 3.—Colony 9 days after infection showing peripheral labelling. Autoradiograph stained with H. and E. × 545.

FIG. 4.—Colonies 14 days after infection showing labelling in central areas. Autoradiograph stained with H. and E. × 560.

FIG. 5.—Vegetation 10 days after infection showing dissolution of older central areas in a colony with pale staining, and absence of leucocytes. H. and E. × 120.



Durack and Beeson.



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debris of dead bacteria. This process is favoured by antibiotics and has been well described after penicillin therapy in man (Moore, 1946) and animals (McGeown, 1954) with endocarditis.

Autoradiographs suggest that there is a basic distinction between old, inactive deep colonies and young, active surface colonies. The main action of antibiotics must be confined to the surface, since bacteria more deeply situated are largely quiescent or even already dead. In healing lesions the surface layer of fibrin is invaded by fibroblasts (McGeown, 1954), and bacterial agents may act by reducing the surface population to levels low enough for this to occur. Meanwhile the viable organisms remaining in deeper colonies are slowly sterilized by the processes of bacterial ageing and healing.

Relapse following antibiotic therapy.—These autoradiographs show that old colonies may retain foci of living bacteria which are capable of further growth Such foci are likely to be in a resting phase prior to death resulting from ageing or organization by granulation tissue. If so, they would be unaffected by antibiotics and could cause relapse after therapy. The fact that this healing process requires 2 or more weeks explains why successful antibiotic therapy must be prolonged over the same period to prevent relapse.

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