STUDIES ON THE PROTEOCLAST ('MULLER'S PHENOMENON')

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(With Plates 6 and 7 and 1 Figure in the Text)

In 1927 Léon Muller described a phenomenon (Muller, 1927*a*, *b*) which, although it has attracted the attention of several workers, has remained one of the minor mysteries of bacteriology. He cultivated *Staphylococcus pyogenes* on human blood-agar plates and noted that peculiar areas of clearing appeared near the growth. When the staphylococci were heavily seeded on the surface of the plates which were then incubated for several days, under the film of growth, isolated circular areas of clearing appeared when viewed by transmitted light. The most likely explanation of this phenomenon was that individual colonies varied in their haemolysin production, but this view had to be dismissed since the clear zones also occurred beyond the edge of the growth (Pl. 6, fig. 1*b*). The possibility of contamination was excluded, and transmission of the plaques proved impossible. Muller noted that the effect was a combination of red cell lysis and the destruction of haemoglobin. For this reason he proposed the term 'haemophagy' for it. He further showed that the lytic principle was in the serum and that it was thermolabile. The gelling agent used for the medium in the plates was of no great importance; the phenomenon was elicited using various concentrations of agar and gelatin. In later papers Muller (1928, 1929*a*, *b*) showed that precipitation of serum proteins simultaneously removed the active principle from the supernatant, but it could be eluted from the precipitates. Although there was a correlation between the amount of serum used in the test plates and the number of plaques produced, the relationship was not linear. Most sera showed reduced activity after filtration through Chamberland filters, but some activity was detectable even after filtration through filters of porosity L7 and L9. The development of the plaques was inhibited by an acid reaction of the medium and, perhaps for this reason, by the presence of a fermentable carbohydrate. Eighty out of eighty-six human sera examined showed the effect. Finally, Muller concluded that "most human sera contain a principle or principles, causing haemophagy. The elements responsible are filtrable, but not homogeneously dispersed in the blood. They are present in the form of numerous particles, but they alone cannot produce haemophagy. Activated by specific secretions of certain staphylococci, however, they lead to the disintegration of haemoglobin. The particles are thermolabile and sensitive to a variety of chemical substances." Muller thought it possible that the particles were of a living nature; he pointed out that the nutritive requirements and the need for incubation were consistent with this idea, and stressed the close resemblance of the effect to satellitism (Pl. 6, fig. 2*a*, *b*).

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Relatively little has since been added to Muller's original observations. The effect can only be produced with pyogenic staphylococci, other organisms tested giving consistently negative results (Burnet, 1928; Segre, 1929; Packalén, 1938; Rhodes, 1938). Staphylococcal filtrates were also ineffective. The sera of various animals could be used with success, rabbit and guinea-pig sera being especially suitable (Burnet, 1928; Cucco, 1929; Muller, 1929*a*). The factor in the sera could be absorbed by wood-charcoal, infusorial earth and other absorbants (Dormal, 1927), and in fact some of the plaques appear to be centred round little particles of dirt, suggesting that adsorption plays a role (Burnet, 1928; Grégoire, 1930; Packalén, 1938).

Some improvements in the techniques used for investigating the phenomenon have been made. Human red cells washed free from serum then laked with water and Seitz-filtered provide an excellent indicator to which the sera of various animal species may be added. There can be no doubt that this crude haemoglobin solution acts only as a chromoprotein indicator of the effect; serum protein precipitated by heat also shows lysis under appropriate conditions (Packalén, 1941; Fisk & Mordvin, 1943; Christie, Graydon & Woods, 1945). Attempts to obtain the staphylococcal factor separate from the organisms have generally been unsuccessful (Cucco, 1929; Segre, 1929; Rhodes, 1938), but Packalén (1941) succeeded on a single occasion in obtaining a filtrate of low activity, which was thermostable and produced plaques when a drop of it was placed on each of seven successive days on to the surface of a human blood-agar plate.

In spite of the attention given to the phenomenon no agreement as to its nature has been reached. Muller (1928), Grégoire (1930) and Packalén (1938) were inclined to believe that a living agent was involved, but this idea was challenged by Burnet (1928) and Cucco (1929). To quote Burnet: "It seems most reasonable to regard the discrete areas of haemolysis as resulting from the activation of what may be called a haemolysinogen diffusing from agar growths of all or most pyogenic staphylococci. This activation may occasionally be general, and result in a uniform zone of haemolysis, but usually occurs only when the haemolysinogen comes into contact with a complex of a labile serum constituent (associated with the globulins) and some entity present in meat extract. Each such complex then acts as a source from which an active haemolysin can diffuse."

As long as living and multiplying staphylococci have to be used to demonstrate the effect it is extremely difficult to assess whether any alteration in environmental conditions affects the staphylococci, or their production of the diffusible factor, or the Muller phenomenon itself. Accordingly, in order to dissociate the phenomenon from the staphylococcal growth, after confirming the findings of the previous workers, attempts have been made to produce an active filtrate of the staphylococcal factor. For the sake of brevity the phenomenon will be referred to as 'proteoclast' without any implication as to its nature. This term seems preferable to 'haemophage' since the lytic effect is not limited to haemoglobin.

METHODS AND MATERIALS

Method 1. *For use with live culture of staphylococci*

Materials

(*a*) *Crude haemoglobin solution.* Red cells from citrated human blood were washed three times with sterile isotonic saline, and then an equal volume of sterile distilled water was added to the packed cells.

(*b*) *Serum.* Sterile human serum, or various animal sera were used as specified. The human serum employed was generally filtered through a Seitz sterilizing pad.

(*c*) *Medium.* Peptone (Oxoid), 10 g.; sodium chloride, 5 g.; Lab Lemco, 5 g.; agar (Davis, N.Z.), 20 g.; tap water, 1000 ml. The ingredients were dissolved and the medium made slightly alkaline to litmus paper; it was boiled for 3 min. and filtered through paper pulp. Its reaction was adjusted to pH 7·4 with normal hydrochloric acid. Sterilization was by autoclaving at 15 lb./sq.in. for 15 min.

Method

The basal medium was poured in 10 ml. quantities into Petri dishes of 9 cm. diameter, each plate receiving in addition 0·5 ml. of crude haemoglobin solution and 1 ml. of serum. After drying, the plates were inoculated with streaks of the staphylococci to be tested. The lids were sealed to the plates with plasticine; incubation was at 37° C. for 24–48 hr.

Method 2. *For use with staphylococcal extract*

Materials

(*a*) *Crude haemoglobin solution* prepared as for Method 1 was used for some of the experiments.

(*b*) *Filtered haemoglobin solution.* For other experiments it seemed desirable to use a filtered product prepared as follows. Human red cells were washed with isotonic saline three times and after washing one volume of ether was added to one volume of packed cells. An aluminium hydroxide gel was prepared by adding 10 % ammonium hydroxide to a saturated solution of ammonium alum until the mixture was just faintly alkaline. One volume of the gel was added to one volume of the ether-cell mixture and, after mixing, the coarse sediment produced was removed by filtration through filter-paper. The residual ether in the filtrate was driven off on the 37° C. water-bath. The haemoglobin solution so obtained was sterilized by Seitz filtration and used in 1 ml. quantities per plate.

(*c*) *Serum.* Either Seitz filtered or filtered through Gradocol membranes as required.

(*d*) *Basal medium.* As for Method 1.

(*e*) *Staphylococcal extract.* Petri dishes of 14 cm. diameter were poured with 2·5 ml. of fresh human serum and 25 ml. of the basal medium which differed, however, in having an agar concentration of only 1 %. A strain of staphylococcus giving a profuse proteoclast effect was selected and inoculated in the form of parallel streaks about 4 cm. apart. The plates were sealed with plasticine and incubated for 4 days at 37° C. After incubation they were rapidly frozen and then allowed to thaw out quickly. The fluid exuded was pipetted off and filtered through

a Seitz pad or Gradocol membrane. In this way a potent extract was prepared which usually proved active in quantities of 1 ml. per plate. The minimum quantity to be used had to be determined experimentally.

Method

Plates were poured as described for Method 1 with the addition of a sufficient quantity of staphylococcal extract. This was usually 1 ml. The plates were not sealed. The proteoclast effect appeared after 24–48 hr. incubation.

RESULTS

The role of Staphylococcus pyogenes

The finding that only *Staph. pyogenes* (i.e. coagulase-positive staphylococci) are capable of producing the proteoclast effect was confirmed. Fifteen coagulase-positive strains obtained from human lesions gave the effect, while none of several coagulase-negative strains nor any other organisms did so. In fact, strains of coagulase-negative staphylococci and also some other occasional contaminants apparently inhibited the development of the plaques (Pl. 6, fig. 3). It was not certain, however, whether the inhibition was due to bacterial action on the haemoglobin, rendering it resistant to the proteoclast action, or to some other mechanism. Some coagulase-negative staphylococci are capable of digesting haemoglobin (see B 39, Pl. 6, fig. 3), but this effect is quite distinct from that produced by the proteoclast which occurs in plaques.

The dissociation of the effect from actively growing staphylococci

The preparation of active filtrates presented considerable difficulty. Filtrates of broth cultures prepared to yield high titres of α-haemolysin, lipase or fibrinolysin were inactive. In order to approximate the conditions under which staphylococci produce plaques, plates were prepared containing the basal medium and serum, but no haemoglobin. Another set of plates contained the basal medium only. Both sets were inoculated in parallel streaks about 1 cm. apart with a strain of staphylococcus previously tested for activity. The plates were incubated in 30 % CO_2 in air for 4 days, then frozen and allowed to thaw. The liquid which exuded was filtered through a Gradocol filter with an average pore diameter of 700 mμ, and tested in 1 ml. quantities. The addition of this amount of filtrate to 1 ml. of fresh serum, 0·5 ml. of haemoglobin and 10 ml. of basal medium led to the development of numerous plaques after about 2 days' incubation. Active filtrates were obtained whether serum was present or not in the plates used for making the extract, showing that the staphylococcal factor does not require serum for its production, although the yield was greater in the presence of serum. Similar experiments showed that meat extract was a desirable but not essential constituent of the medium. Results, however, were somewhat erratic, and at times filtrates of low activity were obtained which produced plaques when added in 5 ml. volumes to the indicator system, but not in lesser amounts; at other times no activity of any sort was obtained. Carbon dioxide did not enhance the yield, and its use was therefore abandoned. It was found that the best yield was obtained

when plates were sealed with plasticine or paraffin wax. This effect was not due solely to maintenance of moisture since incubation in a saturated atmosphere was ineffective.

The yield of the staphylococcal factor was related to the time of harvesting. A set of cultures was inoculated diffusely with staphylococci and incubated for periods varying from 1 to 6 days. Samples were taken daily, frozen, and then thawed and the filtrates tested in 5 ml. volumes. Filtrates taken after 1 day's incubation gave no plaques in the test plates, after 2 days a few, and with filtrates taken after 3 days' incubation innumerable plaques were produced. After 4 days the number of plaques was greatly decreased and filtrates after 5 or 6 days' incubation were inactive. These results show that the staphylococcal factor produced during the first few days disappeared later possibly because it was utilized by subsequent staphylococcal growth or, more likely, because another substance is produced by the staphylococci which is capable of destroying it. This sequence of events would pass unobserved in the ordinary plate cultures using living staphylococci, since once the plaques were formed, they would remain unaffected by the destruction of the staphylococcal factor. It also explains the observation that for a while there is an extension of the corona of plaques around a streak of growth, but later this ceases. The effect on the serum of prolonged incubation must, however, also be taken into account, as will be shown later.

That plaques appear around a streak of staphylococcal growth on plate cultures may be due to the fact that for a time at least the staphylococcal factor diffuses out ahead of the substance destroying it. If the concept that there is differential diffusion of the staphylococcal factor and the substance destroying it is correct, it has a very important application to the problem of making an active filtrate, for if harvesting is carried out too early the filtrate will be deficient owing to insufficient active material, while later the staphylococcal factor will have diffused out further from the streak of inoculum than the agent destroying it, and at this stage the filtrate obtained will be active. At a still later date the destructive agent will catch up with the staphylococcal factor required by the proteoclast and will inactivate it; a filtrate obtained in such circumstances will be devoid of activity. Furthermore, the distance between streaks of inoculum must be considered in the light of this concept. If parallel streaks are made too close together, conditions will favour the destruction of the active substance, if they are too far apart, the concentration in the extract will be too low: there is, therefore, an optimum distance between the streaks. The optimum distance between streaks was about 4 cm. for the strain used; this might not be optimal for other strains.

Consistent yields of low activity could be obtained by using basal medium alone, placing the streaks of inoculum 4 cm. apart, sealing the plates, and incubating them for 4 days. Considerably higher yields were obtained when 10 % heated serum was added to the basal medium and still higher yields when the same proportion of fresh serum was added. The addition of 5 % crude haemoglobin solution to the nutrient agar base gave a filtrate without appreciable activity. The activity of the filtrates was assessed by determining the minimum amount of filtrate that produced plaques in test plates made by method 2. A filtrate judged in this way

to be of low activity also gave rise to a few plaques only whose development was delayed. The use of fresh serum might appear theoretically objectionable since the extract was to be used for the investigation of the properties of fresh sera. An active filtrate can, however, be obtained in the absence of serum, and, furthermore, 4 days' incubation of fresh serum at 37° C. robs it of its ability to produce the proteoclast effect in test plates. The staphylococcal extracts could be boiled for at least 2 min. without noticeably decreasing their activity, while the fresh serum factors required for proteoclast activity are extremely heat labile (vide infra).

Nature of the staphylococcal factor. The physical and chemical properties of the active material in staphylococcal extracts were briefly investigated. Activity was retained for many months at 0–5° C. and resisted a temperature of 100° for at least 2 min. The effect could not with certainty be correlated with any defined diffusible products of staphylococci nor with the growth factors which give rise to satellitism of *Haemophilus influenzae* and certain diphtheroids on staphylococcal cultures. Nor is coagulase responsible, for although only coagulase-positive staphylococci have been found to produce plaques, some coagulase-positive strains fail to give the effect. Moreover, a highly concentrated sample of staphylocoagulase proved inactive in this respect. The effect could not be identified with lipase production nor with any of the three known staphylococcal haemolysins. Of all the known diffusible products investigated, fibrinolysin showed the nearest relationship with the proteoclast effect. This observation, made earlier by Christie *et al.* (1945) was confirmed in that none of the five fibrinolysin-negative strains of coagulase-positive staphylococci investigated produced the proteoclast effect. These fibrinolysin-negative strains all came from animal sources. This correlation cannot, however, be taken as proof that fibrinolysin is the actual factor concerned in plaque formation for fibrinolysin dissolves heat-precipitated fibrinogen in the absence of fresh serum while the proteoclast requires the latter. Furthermore, filtrates rich in fibrinolysin fail to give the proteoclast effect.

The role of serum

Variation in plaque appearance. The proteoclast effect could not be elicited in the absence of serum; each of more than thirty samples of fresh human serum was found to be effective in this respect. Sera kept unfrozen in the refrigerator for several weeks showed unimpaired activity, which suggests that complement is not the factor responsible. In addition to adult sera, seven sera obtained from umbilical-cord blood showed this activity.

Adult human serum (Pl. 7, fig. 5*a*) generally gave a profuse crop of medium-sized plaques, closely set but not confluent. Cord sera (Pl. 7, figs. 5*b*, *c*) gave numerous small plaques. Other mammalian sera (rabbit, guinea-pig) were also active; however, when tested under comparable conditions (method 1), there were certain differences in the appearances of the plaques. Plates containing rabbit serum produced a mixture of small and larger plaques (Pl. 7, fig. 5*d*). Guinea-pig serum gave confluent lysis which was hardly recognizable as the proteoclast except on close scrutiny of the edges, where a few isolated small plaques appeared (Pl. 7, fig. 5*e*).

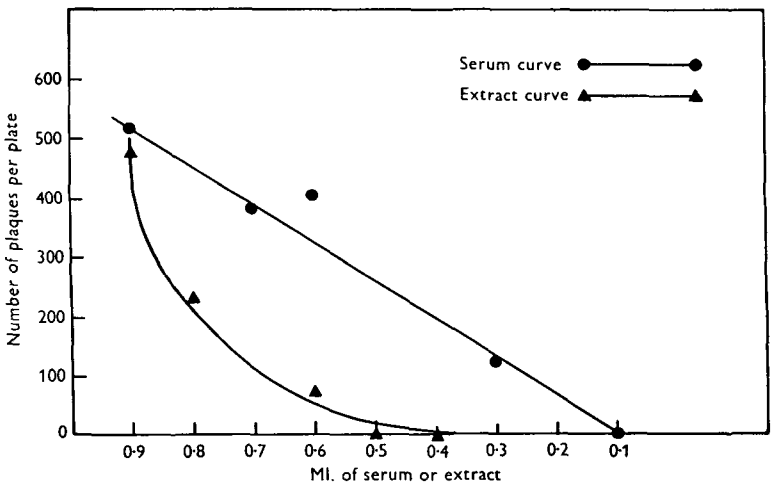
Four main morphological plaque patterns were observed: giant plaques, very small plaques, a mixture of very small and medium-sized plaques, and plaques surrounded by a halo (Pl. 6, fig. 4). The very large plaques were probably due to aggregation of the proteoclast since they were usually observed when the total number of plaques was small. The other three plaque types appeared to be associated with the sample of serum employed, as though the morphology were in some way a characteristic of the serum. A noticeable feature was that the plaques had little tendency to coalesce even when quite close together. Those lying away from the others were larger, as if crowding interfered with development. This behaviour is unlike a chemical effect which should lead to summation where the proteolytic zones approach each other. It is, however, very similar to the appearance of bacterial colonies arranged unevenly on the surface of a culture medium.

The serum constituents required by the proteoclast. The discreteness of the plaques suggests that the effect arises from particles which could be either in the serum or in the staphylococcus. The latter possibility is the less likely as it would have to be assumed that the particulate elements migrate out of the growth for a considerable distance, and then produce an orbit of proteolysis. A third possibility is that the effect is centred on minute crystals or particles of dirt in the agar. Plaques were sometimes seen around minute particles in the medium, but this was exceptional, nearly all the plaques being entirely without any central structure.

If the particulate elements reside in the serum, its progressive dilution in the presence of an excess amount of staphylococcal filtrate should lead to a linear diminution in the number of plaques produced. One ml. or more of an average sample of human serum produced uncountable plaques (Pl. 7, fig. 6), but with smaller volumes fewer plaques appeared. A fair degree of linear correlation between the volume of serum and the number of plaques was found (Text-fig. 1). When the reverse experiment was done, using an excess of serum with diminishing quantities of staphylococcal extract, the decrease in the number of plaques was out of proportion to the dilution of the extract. A critical minimum amount of staphylococcal extract is needed for the development of a certain number of plaques for, in the presence of a slightly smaller amount, only a small proportion of the plaques develop.

The linear correlation between the amount of serum added to a plate and the number of plaques produced does not hold for very small amounts of serum. In Text-fig. 1 this is not obvious as it does not record observations with less than 0·3 ml. of serum. With amounts smaller than this no plaques were obtained. This finding does not militate against the assumption that the particulate phase is in the serum, but indicates that the serum has another property, which is needed for the development of the plaques. That two factors required for the proteoclast effect are present in fresh serum was established by means of the following experiment. Fresh serum was heated to 55° C. for 30 min.; 1 ml. of this heated serum, 1 ml. of staphylococcal extract and 0·5 ml. of crude haemoglobin were incorporated in 10 ml. of basal medium. Plates poured with these ingredients were 'inoculated' on the surface with one drop of fresh serum, which was then spread out over the area of the plate. No plaques developed upon incubating the plates. Control plates

spread on the surface with 1 ml. of fresh serum gave a profuse proteoclast effect with plaques too numerous to count. It is inconceivable, therefore, that the single drop used as an inoculum should not have contained many particles whose failure to produce plaques indicates that the non-particulate factors, which were destroyed in the heated serum, were not in sufficient amount in a single drop of fresh serum to permit the development of plaques.



Text-fig. 1. Graph showing the number of plaques developing in plates prepared by method 2. The 'serum curve' was obtained by incorporating different amounts of fresh human serum, made up to a constant volume of 1 ml. with heat-inactivated serum, in plates containing an excess of staphylococcal extract. The 'extract curve' was obtained by adding different amounts of staphylococcal extract to plates containing an excess of fresh human serum.

The size of the serum particles. Further evidence that the particulate elements are in the serum is provided by the finding that filtration of a sample of human serum through Gradocol membranes removes its activity. Filtration was carried out without using a filter-paper pad under the Gradocol membrane so as to minimize the chances of absorption. As filtration took several days, an aliquot part of the serum was held at room temperature while the remainder was filtered undiluted through a given membrane. The aliquot and a sample of the filtrate were then simultaneously tested with excess staphylococcal extract. The same process was followed for each successive membrane. It was thus possible to be sure that any reduction in the number of plaques produced by the filtrate was due solely to filtration.

Filtration of serum through Gradocol membranes having an average pore diameter of 640, 210 and 100 mμ respectively produced no reduction in the number of plaques, but there was a 99 % reduction after filtration of the serum through a membrane of average pore diameter of 58 mμ. The particles involved must therefore fall into the size range commonly associated with viruses. It is not known whether there are differences in size between particles of different sera.

The effect of physical agencies on the serum constituents. The heat lability of the factors in fresh human serum was confirmed. Their range of sensitivity was found

to be very similar to that of most vegetative bacteria (Table 1). When whole fresh serum is inactivated by physical agencies such as heat, the only direct conclusion that can be drawn is that one at least of the factors required—the particulate or the filtrable—is sensitive. If both are labile, the behaviour of the more sensitive one alone is revealed by the experiment, since destruction of one factor would stop the development of the effect. Thus it is impossible to say to which of the serum constituents the values of heat-sensitivity refer. However, there is evidence that the non-particulate factor is destroyed by 55° C. for half an hour since the use of such heated serum will not allow the development of plaques when the particulate factor is provided in small inocula. There are no data as to the heat-lability of the particulate factor.

The sensitivity of the serum factors to ultra-violet light was tested in the following way. Plates were prepared containing 0·5 ml. crude haemoglobin solution in 10 ml. basal medium. After thoroughly drying, each plate was spread with 0·5 ml. of fresh human serum which was allowed to soak in, and then with a strain of *Staph. pyogenes* so as to ensure confluent growth over the entire surface.

Table 1. *The effect of heating the serum for* 30 *min. at different temperatures*

Temperature (° C.)	Number of plaques
Control	+ + + +
40	+ + +
45	+ +
50	+
55	−
60	−

A circular hole about 2 cm. in diameter was cut in a metal plate and the plate was used to replace the lid of the Petri dishes. The surface of the medium in the plates was irradiated through the hole for varying periods with a mercury vapour lamp. Irradiation for 1 min. under the conditions of the experiment sterilized the irradiated area and also destroyed the proteoclast effect since, upon incubation, no plaques developed in the circular areas free of bacterial growth. In another experiment in which plates were spread with fresh serum, irradiated over their entire surface for 20 min. and subsequently inoculated with a streak of staphylococci, a very occasional plaque appeared after incubation. Sixty minutes' irradiation destroyed the proteoclast effect completely. Unirradiated control plates gave profuse plaques near the streak of inoculum. These findings suggest that the sensitivity of the serum factors to ultra-violet light is equal to, or only very slightly less than, that of bacteria.

Conditions required for the development of the plaques. There are at least four essential requirements: fresh serum, a product of staphylococci, an indicator, and incubation. The conditions of incubation required for the development of plaques are similar to those for the cultivation of bacteria. When plates, prepared according to method 2, were incubated at various temperatures, it was found that the maximal development of plaques occurred at 37° C., although some increase in the number of plaques was observed when plates, previously incubated at 37° C., were

subsequently left at room temperature. No plaques developed at 4° C. or on incubation at 45° C. These results are consistent with the presence of a living lytic agent, but could equally well be explained as being due to the action of a heat-labile enzyme.

There was a correlation between the time required for the first appearance of the plaques and the amount and activity of the staphylococcal extract. The more active the staphylococcal filtrate the earlier did the plaques appear, but the time could not be reduced to less than about 12 hr. This delay provides a further point of similarity with the cultivation of micro-organisms.

Oxygen is not required for the development of the plaques when an active staphylococcal extract is employed. With plates prepared according to method 1, using living staphylococci, plaques do not develop on anaerobic incubation, doubtless due to failure of the bacteria to provide the necessary factor in the absence of oxygen.

The effect of chemical agencies on the development of plaques. The Heatley cup technique was used to test the sensitivity of the proteoclast to various substances. Penicillin (400 units/ml.), aureomycin (2500 μg./ml.), chloramphenicol (2500 μg./ml.), streptomycin (1000 μg./ml.) and urea (2 %, w/v) were without effect on the development of the proteoclast but sulphathiazole (200 mg./ml.) gave a very marked zone of inhibition (Pl. 7, fig. 7). This drug was then tested by incorporating diminishing amounts in the medium, and it was found that 10 mg./ml. gave almost complete inhibition of the proteoclast effect. This concentration was at least a hundred times the amount generally required to inhibit sensitive organisms. Possibly the inhibitory effect was not on the proteoclast but on the haemoglobin.

DISCUSSION

The phenomenon described by Muller consists of a sphere of proteolysis in a gel in which haemoglobin plays the part of an indicator substrate. The experiments described indicate that the zones of proteolysis are associated with, and presumably centred on, particles found in mammalian sera. These particles are probably smaller than 70, but larger than 20 mμ diameter. For the development of the relatively enormous zone of proteolysis two further factors, or groups of factors, must be present; a heat-resistant factor produced by staphylococci and a heat-labile factor present in fresh sera.

What is the underlying cause of the proteolytic effect? Four main possibilities require consideration:

(1) The focal nature of the effect may be an artifact resulting from adsorption of an enzyme in solution on to minute crystalline or other aggregations in the medium.

(2) The particulate element may be a living virus derived from staphylococci.

(3) Serum may contain a living virus which with the aid of a staphylococcal factor becomes capable of proteolysis.

(4) The precursor of a proteolytic enzyme may be present in particulate form in the sera of animals, and when immobilized in a gel may be activated by a substance produced by staphylococci.

(1) The possibility of artifact can be dismissed at once. The number of plaques that develop is closely correlated with the amount of serum and staphylococcal extract employed, while the constitution of the gel is of little consequence. Various concentrations of agar, gelatin and tragacanth are all suitable (Muller, 1927*a*; Dormal, 1927; Packalén, 1941). Inspissated serum combines the properties of gel and substrate, and the proteoclast effect can be demonstrated on it (Packalén, 1941), showing that the physico-chemical properties of the gel cannot explain the focal arrangement of the lytic effect.

(2) The view that the particulate element is a virus originating in staphylococci is equally untenable. It is inconceivable that a virus should resist boiling as the staphylococcal extract does.

(3) The existence of a 'saprophytic' virus in serum is not so startling as it might at first appear. It was suggested many years ago by Theobald Smith that non-pathogenic viruses exist, and this view was also conceded by Doerr & Hallauer (1938) who argued that if parasitic bacteria represent an adaptation of saprophytic forms, then it is reasonable to assume that forms of life of the size of viruses may also exist in a free-living state, the pathogenic strains having been derived from these. There does not appear to be any *a priori* reason why the circulation of a multicellular organism should be free from non-pathogenic viruses and, in fact, the Pasteurian doctrine of the sterility of the blood stream in health has already been breached by the discovery of the icterogenic agent in human blood and the Bittner factor in mice.

Non-pathogenic viruses would certainly fill the intellectual gap in the continuity of living things, but in the absence of a suitable indicator effect the concept must remain speculative. If saprophytic viruses exist, their recognition would depend on some effect which they are capable of producing and which is transmissible; the effect should be associated with particles of the right size having the property of multiplication. There are a few scattered observations worth mentioning in this respect. Barnard (1935) noted that in media containing horse or rabbit serum precipitates occurred at times. The effect could be transmitted, but after four subcultures was lost; it was apparently associated with particles of about 150–170 mμ in diameter. Filtrable organisms in sewage have been described (Laidlaw & Elford, 1936; Seiffert, 1937*a*, *b*) which could be cultivated indefinitely on artificial media. They were, however, relatively large (0·2–0·5μ), approximating the filter-passing forms of bacteria. On the other hand, on more than one occasion effects observed on plates in the absence of bacteria have been traced to artifacts (Twort & Twort, 1921; Laidlaw, 1925).

The proteoclast certainly shows similarity in some respects to micro-organisms. When the effect is brought out by means of a staphylococcal extract, the appearance of plaques and their development is so much like the customary picture of bacterial cultivation, that one has a strong impression that the effect is due to a living agent. The initial lag period and the appearance of pin-point areas of clearing growing rapidly to a certain size and then no further, are all points which have their counterpart in the cultivation of microbes. The striking lack of confluence and the smallness of the plaques in places of crowding are also more difficult

to explain on a chemical basis, but would fit the concept of biological competition.

The findings that the plaques develop slowly at room temperature, but not at all at 45° C. is also interesting. Were the effects due to a proteolytic enzyme, increase of the temperature should accelerate the reaction until the point is reached when the enzyme itself is destroyed and a temperature of 45° C. seems rather low for this. The optimum temperature of the proteoclast is around 37° C.: another analogy with micro-organisms cultivated from mammalian sources.

The resistance of the proteoclast to ultra-violet irradiation is also within the range found with living things; its thermal destruction is complete after 30 min. heating at 50° C., resembling the thermal death point of some of the more sensitive bacteria.

A haemolysin for human red cells has been found in the amniotic fluid of chick embryos infected with mumps virus (Morgan, Enders & Wagley, 1948). This may provide an analogy with the lytic activity of the proteoclast.

Lastly, the size of the particles in serum is of the same order as that of viruses. That the effect has not been transmitted is not a conclusive argument against its virus nature. Admittedly, could self-replication be demonstrated experimentally, all doubts would be resolved and the proteoclast would have to be accepted as a virus. The lack of this demonstration may mean either that in fact it does not multiply or that the available methods do not provide the conditions required for multiplication.

(4) When the alternative theory is considered, that the proteoclast effect is due to an enzyme or its precursor present in mammalian sera it must be admitted that this view would also fit the experimental findings. The lag period is explicable on the basis that minimal amounts of proteolysis are not visible to the naked eye. If the rate of proteolysis then increases plaques will rapidly develop, and if the size of the enzyme aggregations is relatively uniform, the resulting plaques will be of equal size. The edge of the plaques can be taken as representing the sphere at the surface of which the enzyme concentration has fallen below that needed to produce a visible effect. Other things being equal, the size of the sphere of action will depend on the amount of the enzyme precursor present in the aggregate and the available activator. Where the aggregates are crowded together there will be relatively less activator available for each of them which will lead to a diminished zone of action. In this way the finding of smaller plaques in a crowded area and larger ones in a more isolated position could be explained.

The failure of the plaques to develop at 45° C., and over, may be due to the fact that the rate of destruction of the enzyme, or one of its constituents, exceeds the reaction velocity at that temperature. Sensitivity to ultra-violet light is shown not only by living things but also by enzymes and toxins. The wide distribution, almost ubiquity, of the particles in mammalian sera could be taken as another indication that they are the products of mammalian cells. The size of the serum particles is certainly surprising since one does not associate enzyme action with particles of that order of size.

It is well known that the fibrinolytic activity of some bacteria is due to the

activation of a 'prolysin' present in normal plasma. Such activators have so far been found to be produced only by certain strains of haemolytic streptococci and staphylococci (Lewis, Ferguson & Jackson, 1949). No haemolytic streptococcus has been found to give the proteoclast effect (Rhodes, 1938; Packalén, 1938). Nor is there any evidence that 'prolysin' is particulate. However, tissue fragments have been described which are of a similar size to the particulate element in serum (Sigurdsson, 1943), and it is quite conceivable that such cellular breakdown products should find their way into the blood.

Furthermore, it is known that part of the cellular ribonucleic acid is located in cytoplasmic basophilic granules (Claude, 1938, 1946): two kinds of granule have been obtained, the larger with a size of 0·5–2μ, and the smaller, or microsome, having a size of 60–150mμ. A number of enzyme systems have been associated with microsomes, including one, esterase, which is predominantly located in them (Omachi, Barnum & Glick, 1948), showing that they differ in composition from the larger granules. It is interesting to note that these cytoplasmic granules are remarkably similar in composition to certain of the animal viruses. They might be regarded as self replicating units, as are viruses and genes. Indeed, it has been suggested that some cytoplasmic particles ('plasmagenes') might, like the nuclear genes, be the bearers of certain hereditary characteristics (Davidson, 1950).

What is most difficult to explain is that particles so widely distributed in diverse mammalian sera as is the proteoclast, should require for their activation substances produced only by fibrinolytic coagulase-positive staphylococci. This opens up the possibility that the serum particles might be part of the normal blood-clotting mechanism.

There is no evidence at present as to where the proteoclast particles found in the serum originate, except that they are most likely to be derived in some way from the cells of the mammal whose serum contains them. Their particulate form and powerful enzymic activity set them apart from other known constituents of sera. These two properties are consistent with the effect, being due either to an enzyme precursor in a hitherto unrecognized form or to a non-pathogenic virus. When considering elements of the order of size to which these particles belong, it must be borne in mind that the difference between the two concepts may not be so profound as it appears to be at first sight.

SUMMARY

1. When coagulase-positive staphylococci are grown on nutrient agar containing fresh mammalian serum and haemoglobin, discrete areas of clearing appear in the medium near to the growth.

2. These 'plaques' are due to proteolysis, the haemoglobin acting as a chromoprotein indicator substrate. For convenience the effect is referred to as 'proteoclast'.

3. The effect is due to the interaction of a diffusible product of the staphylococci with constituents of normal sera. The staphylococcal factor has been obtained in a bacterium-free filtrate and its interaction with serum studied quantitatively. It resists boiling.

4. The number of plaques produced under defined conditions showed a linear correlation with the amount of serum used, but not with the amount of filtrate.

5. By means of serial filtration through Gradocol membranes it was shown that one factor in normal serum required for the proteoclast effect is particulate, having a size of the order 20–70 mμ diameter.

6. It was found that a third factor present in fresh sera was also required.

7. Serum was rendered inactive by conditions of heating and ultra-violet irradiation similar to those required to sterilize vegetative bacteria and viruses.

8. It is suggested that the proteoclast effect is due either to a non-pathogenic virus, or to a hitherto unrecognized particulate enzyme precursor present in sera and activated by the staphylococcal factor.

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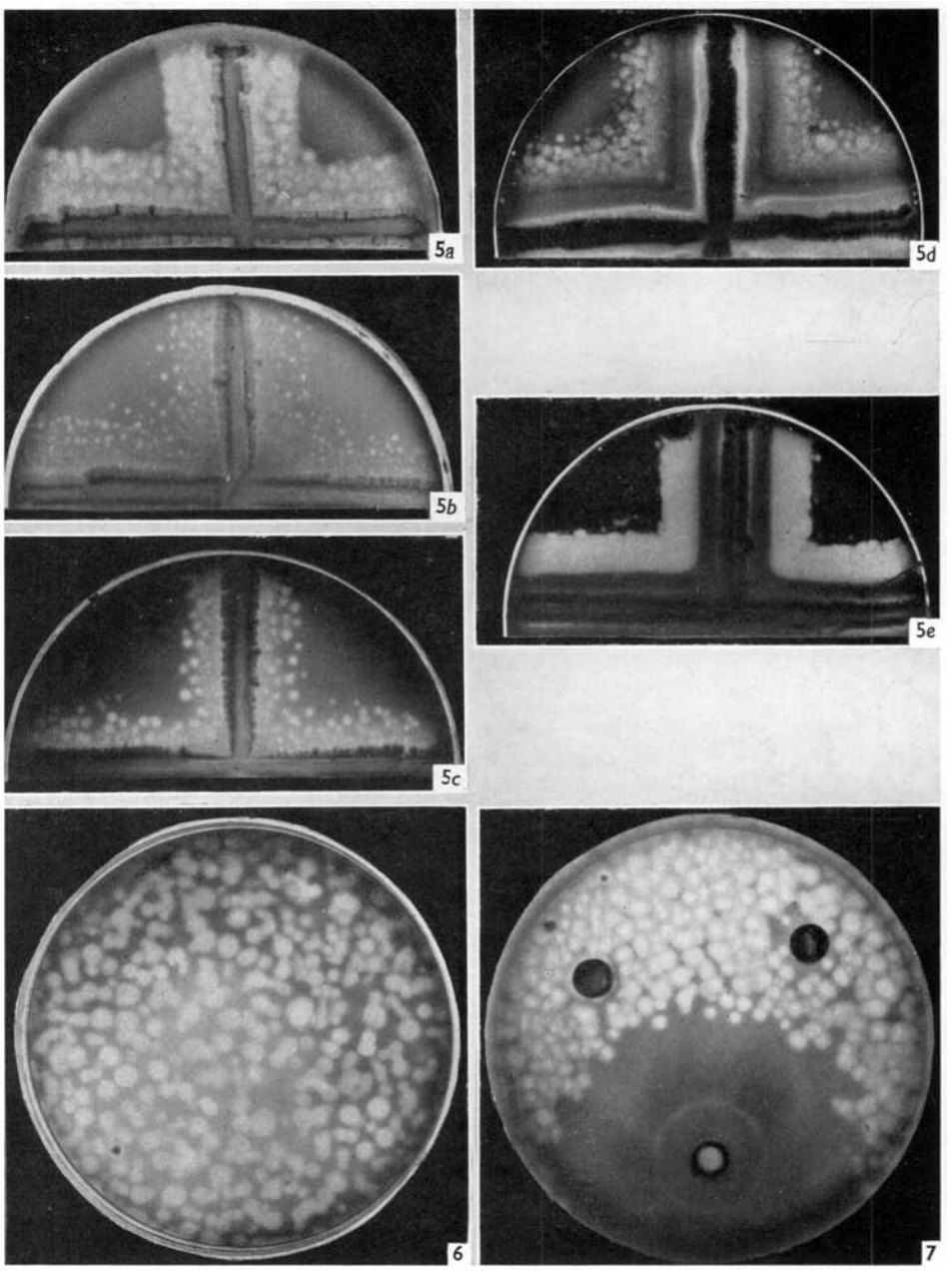

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EXPLANATION OF PLATES 6 AND 7

PLATE 6

ig. 1*a*. A strain of *Staph. pyogenes* on 10 % human blood agar after 24 hr. at 37° C. seen by ansmitted light, showing some haemolytic colonies.
ig. 1*b*. The same plate after 4 days' incubation, showing circular areas of clearing away om the bacterial colonies as well as under confluent growth.
ig. 2*a*. Satellitism of a diphtheroid around a streak of *Staph. pyogenes*.
ig. 2*b*. Proteoclast plaques around a similar streak.
ig. 3. Two strains of coagulase-positive staphylococci ('Muller' and 'Oxford') which have iven rise to plaques, and two coagulase-negative strains ('B 25' and 'B 39'), neither of which as done so. 'B 39' has produced inhibition of plaque development and also shows a band f haemo-digestion. The medium consisted of 10 % human blood agar and the plate was sealed nd incubated for 4 days at 37° C.
ig. 4. A comparison of different plaque morphologies photographed at the same magnifica- on. *a*, large plaques; *b*, small plaques; *c*, mixture of medium sized and small plaques; , plaques surrounded by a halo.

PLATE 7

ig. 5. Plates prepared according to Method 1 poured with different sera. After sealing they ere incubated for 2 days at 37° C. *a*, adult human serum showing the typical morphology; , human cord serum showing 'pin-point' plaques; *c*, another human cord serum showing mixture of larger and 'pin-point' plaques; *d*, rabbit serum showing a mixed morphology; , guinea-pig serum showing a confluent effect with 'pin-point' plaques at the edge.
ig. 6. Bacteriologically sterile plate prepared according to Method 2 showing the typical ppearance.
ig. 7. Plate with three Heatley cups according to Method 2. Marked inhibition of the roteoclast effect around the cup containing sulphathiazole, but none around cups containing treptomycin and urea.

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