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[PLATE 12]

A group of organisms of small size has been discovered which can be cultivated in indefinite series. The organisms form a well-defined group and have both relatively large and small forms. The smaller forms appear to be as small as vaccinia virus and from them the larger forms readily develop.

OCCURRENCE

The organisms have been obtained from all samples of raw sewage so far examined from four London districts during the summer. They have not been detected in London tap-water, or faecal material from man, pig, rabbit, or rat.

ISOLATION

The organisms are readily obtained by successive filtration of raw sewage through " Gradocol " membrane filters (Elford, 1931) of diminishing porosity. Samples of about 15 to 20 cc of sewage are mixed with about 10 cc of nutrient broth and shaken thoroughly to break up clumps of bacteria and protozoa. The mixture is first clarified by centrifugation or filtration through paper pulp to remove any large aggregates, and different samples are filtered through membranes with average pore sizes of 1·0 μ, 0·8 μ, and 0·6 μ. The membrane filtrates are sown in quantities of 0·5 to 1·0 cc into Fildes's broth, or onto Fildes's agar slopes, and incubated at 30° C for some days. The first sign of growth in the broth tubes is an alteration in the colour of the medium from a warm brown tint to a dirty yellow; this is soon followed by a hazy appearance which gradually develops into a definite but not always pronounced turbidity. With the solid medium it is advisable to flood the fluid at the bottom of the agar slope half-way up the exposed surface after two or three days and incubate again for some days longer. Growth is indicated by the development of a ground glass appearance on the lower part of

the slope with an obvious high water mark showing the limit of the flooded area. Subculture is readily effected from solid or liquid cultures by transference to fresh tubes of medium.

On one occasion one of these organisms was recovered on Fildes plates by the plating methods used in routine bacteriology. This indicates that the organism must be present in sewage at times, in fairly large numbers. In filtrates the numbers are naturally reduced, but 0·5 cc samples of 0·8 μ membrane filtrates usually give positive results.

In this study all strains were purified by plating high dilutions of the cultures and picking single colonies under a low power binocular microscope and working with the cultures resulting therefrom.

In many early experiments other types of medium were inoculated and other organisms were recovered from the coarser filtrates such as spirochaetes, and an organism apparently identical with *Spirillum parvum* of von Esmarch (1902). This organism grew freely on highly dilute nutrient broth but very badly, or not at all, on Fildes's or other rich medium. *Spirillum parvum* was studied to some extent as it is of historical interest and is readily filterable through filters of the Berkefeld type. This organism was also recovered from London tap-water. As a rule all obvious bacteria were discarded and attention confined to organisms suspected to be of virus size.

Cultivation

The most generally suitable medium so far discovered for these new organisms has proved to be Hartley's digest broth made from horse meat, set to a reaction of about p_H 8·0 and enriched with a peptic digest of red cells as described by Fildes (1920) for haemophilic bacteria. Media with a neutral reaction give very inferior results, and those with a reaction more acid than p_H 7·0 give no sign of growth. Alkaline serum broth is also suitable for the majority of these organisms but proved useless for one strain. Alkaline broth without serum enrichment gives irregular results. Serum agar slopes, and blood agar slopes, give a delicate growth but are inferior to Fildes's agar. Blood broth gives a good growth but is inconvenient. Growth in liquid medium is accompanied by the development of a fine haze which progresses to a slight turbidity; this reaches a maximum and thereafter declines leaving a faintly hazy medium with a small granular deposit. There is no lysis of red cells in blood broth nor alteration in the colour of the haemoglobin in media containing blood. In serum broth the growth is more granular and there is usually an obvious if small deposit at the bottom of the tube.

This is due to the organisms growing in clumps of hundreds or more Dilution of a good medium with water rapidly converts it into an inferior one, and in view of the natural habitat of the organisms this is rather surprising. On the whole growth is slightly better under aerobic conditions, but anaerobic conditions are quite suitable for all strains.

The optimum temperature for recently isolated organisms appears to be about 30° C, though growth proceeds quite well, if more slowly, at room temperature (22° C), and satisfactory cultures are usually obtainable at 37° C when large inocula are used. At the highest temperature some irregular results were experienced and colony formation was less perfect.

The organisms are readily killed by heat. Heating to 45° C for 15 minutes destroyed the majority of them and none withstood 55° C for 5 minutes.

Growth on solid medium can be very deceptive as it is often fine and delicate. When the whole of the surface is flooded with inoculum the only change to be observed is a faint ground glass appearance which can easily pass undetected. It is always advisable to leave a fair proportion of the surface uninoculated so that a contrast between the normal surface of the medium and the growth-covered area can be obtained. When very dilute inocula are employed on a large solid surface and incubation is prolonged for five or six days giant colonies up to 0·35 mm may be obtained on suitable medium. The colonies when fully developed are umbonate; the centre is rough and usually of a pale brown colour; the periphery is flatter and smoother but may show irregular radial markings. Quite young colonies are usually lenticular, colourless, and quite smooth.

It is possible to count the number of viable organisms in a fluid culture by plating a known volume of an appropriate dilution onto solid medium, incubating for 4 or 5 days, and counting the separate colonies under a low power binocular microscope. The procedure is tedious but gives useful information. Growth curves may be drawn, and it is found that a medium which looks clear may contain 40,000,000 organisms per cc, and the most turbid culture may contain 3 to 10 thousand million organisms per cc. In old cultures the population diminishes and this is accompanied by considerable clearing of the medium. From the counts in developing cultures the generation time during the logarithmic phase of growth was calculated for strain A on the assumption that simple division occurred. This was found to be about 1·6 hours at 30° C, and 1·1 hours at 37° C after the strain had been cultivated under artificial conditions for some months.

Morphology

Dark ground illumination of the living unstained organisms gives the most reliable information regarding their structure. A thin film of a young culture of any of these organisms grown on Fildes's broth, examined by dark ground illumination with a 1/12-inch objective, funnel stop and a 12 X or 15 X ocular shows large numbers of bright rings and minute particles. The largest elements appear to be spherical in some instances and discoid in others. They vary in size from large forms which look like small cocci (about 0·5 μ in diameter) down to small dots which are only imperfectly resolved by the optical equipment specified. The rings are usually single but may be paired. Occasionally the ring appears to show a granule or have a local thickening at the periphery, in other instances a short filament may be attached to the margin of the ring. In older cultures local thickenings giving a signet-ring appearance are more common and large bloated forms may also be seen which may have one or more interior granules.

In cultures grown on medium containing serum the rings are clumped together in aggregates of a hundred or more and there are relatively few free forms. Study of a number of preparations from such cultures shows that the rings are connected together by very fine filaments. At the margins of the aggregates rings may, from time to time, be seen attached to the main mass by a thin filament giving a drum-stick appearance. When the clumps are loose the whole mass may flicker as the spheres and filaments sway about under molecular bombardment.*

Stained preparations are not quite so satisfactory for we are convinced that the organism is readily distorted. Films are best prepared from cultures on solid or liquid media on cover glasses. The wet films are fixed by floating on saturated mercuric chloride to which acetic acid up to 5% has been added. They are then dealt with as wet films and never allowed to dry during staining or mounting. Giemsa's stain, carbol fuchsin, and Heidenhain's iron-haematoxylin have been found to be the most suitable stains. Successful preparations show rather more diversity of form and size than is observable under dark ground illumination. Large coccoid bodies are prominent but many small forms can be seen down to granules which are so small that one is in doubt as to whether they are organisms or particles from the medium (*see* fig. 1, Plate 12). Some of the larger forms stain more deeply at the periphery, which is

* It is hoped that J. E. Barnard will, in the future, give an account of the microscopic appearances of these small organisms as revealed by more refined optical methods.

what is to be expected if the form of the stained body is a biconcave disc; others stain more uniformly, which is the expected picture from a spherical body. The smallest bodies appear uniformly stained, but their proper resolution is impossible. Here and there thin rod-like structures are to be seen; these may be distorted organisms or, as we consider equally possible, discoid bodies viewed on edge. In preparations from older cultures forms with irregular thickenings at the margin can be detected.

It will be observed that these organisms show structures which recall a number of those which are met with at certain stages in cultures of the organism of bovine pleuropneumonia.

We have not been able to determine in what way the organisms multiply. It is certain that large forms may develop from the smaller, but whether the organism multiplies by separating off small particles or not is undecided. We never see septa in the larger ring forms which are so striking in actively growing cultures of staphylococci and we regard it as improbable that multiplication occurs by simple fission of the larger forms. We believe that there is some more complex method of development. Attempts were made to observe directly the division process under the microscope in a hot room at 37° C but these were not successful. It was shown that light rays did not interfere with the multiplication of the organism, but when it was discovered that the generation time was more than one hour assuming a simple method of division, this line of study was abandoned.

Biochemical Activities

It was hoped that it would be a simple matter to determine the nature of the foodstuffs required for the growth of these organisms but the results have been, so far, very disappointing. Whenever growth occurs acid is developed and when the reaction reaches p_H 7·0 or thereabouts growth ceases. The change in reaction has been followed by colour changes with indicators and more accurately with the glass electrode. None of the sugars usually employed in typing bacteria favoured the growth of these organisms and there was no sign of fermentation. This result may be associated with the failure to grow whenever the reaction becomes faintly acid. However that may be, media which gave typical fermentation results with the organism of bovine pleuropneumonia gave negative results with the new organisms, though it was demonstrated that active growth had occurred. Ammonia is not produced from urea. Sodium tellurite is slowly reduced but nitrates are not reduced to nitrites. The haematin of Fildes's medium is partly converted under anaerobic conditions into haemochromogen, and later is altered in some way, being

converted into a yellow pigment the nature of which is unknown. The addition of sodium salts of a number of organic acids to the standard rich culture medium did not produce increase of growth or the development of an alkaline reaction. In brief the metabolic activities of these small organisms, even though the population may be large, are not great enough to be followed by rough and ready methods, and greater refinement will be required.

The precise role of the Fildes enrichment is obscure. The addition of separate X and V factors (haematin and yeast extract) did not give so good results as a peptic digest of red cells. Moreover there is some constituent in the Hartley broth which is essential for copious growth. None of the commercial peptones can be substituted for digest broth if good growth is desired. Casein digests we found to be useless.

Multiplicity of Strains

We have isolated three strains which show small but definite differences in cultural behaviour corresponding to well-defined serological differences, but morphologically the three organisms appear to be identical. Strain A was isolated most frequently and was encountered in sewage from Finchley, Hendon, and Croydon. It grows more freely than the others and produces a more pronounced turbidity in liquid cultures. Digested blood is not indispensable for its development though this addition is certainly advantageous. Serum broth is quite suitable for maintaining indefinite subculture in series and even ordinary nutrient broth made alkaline will permit of fairly satisfactory growth. Strain B, obtained from Hornsey sewage, is very similar in growth requirements to A but shows a great tendency to grow in masses at the bottom of a culture tube (particularly if a little serum is present), and the supernatant fluid is often quite clear. Strain C does not grow at all readily and requires digested blood for its development. It never produces a pronounced turbidity, and gives very small colonies on the surface of solid media. Strains A and B give colonies up to 0·35 mm, and strain C up to 0·17 mm. The colonies of strain C are not umbonated but rough and irregular on the surface. Strain C when originally isolated showed two different sizes of colony and these were picked off and grown separately. For a time the larger colony bred true and gave large colonies and the small always gave small, but on prolonged subculture mixed sizes of colonies developed in both instances. At present we regard the three strains, A, B, and C, as variants of a single group on account of their morphological and cultural similarities.

Serological Reactions

Two rabbits were immunized by a series of intravenous injections of suspensions of organisms of strains A and C respectively. The organisms were grown in Fildes's broth, centrifuged down and suspended in a small volume of saline prior to injection. The injections caused no bodily disturbance in the rabbits. After five injections at four-day intervals, samples of serum were collected and found to agglutinate the corresponding organisms very well. Tests with this serum are shown in Table I. It will be observed that antiserum to strain A agglutinates and precipitates strains from Finchley (i) and (ii), Croydon, and Hendon; has only a slight action on a strain from Hornsey (strain B) and no action at all on strains C (from Finchley sewage) or pleuropneumonia. Conversely antiserum for strain C has no effect on the A and B type organisms, but shows a striking action on strain C. There can be no doubt that strains A and C are quite distinct antigenically, and that strain B is more closely related to A than to C. None of the strains shows any relationship to pleuropneumonia and corresponding with this, antiserum to the latter organism is without action on any of the strains of the new organism.

After six weeks' rest the rabbits were given four further injections and the final serum harvested. The only difference observable in the serum after further immunization was that the antiserum to strain A now produced much more striking agglutination of strain B. Strain C remained unaffected, and strain C antiserum remained without action on both A and B. Normal serum from man, rabbit, sheep, pig, goat, ox, or horse did not agglutinate the organisms.

Ultra-filtrates of old cultures of these organisms also give small precipitates with the corresponding antisera showing that a precipitable substance is formed or liberated in the culture medium.

Filterability of the Organism and Size of the Smallest Units

Preliminary filtration experiments with Fildes's broth cultures suggested that the size of the smallest phase of the organism was probably about 0·2 μ, since membranes of average pore diameter (A.P.D.) 0·42 μ gave positive filtrates while 0·25 μ membranes yielded sterile filtrates. In view of the variable size of the elements seen under the dark ground microscope it was decided to establish the complete curve showing the filterability of the organism through membranes of porosities ranging

TABLE I

	Organism	A antiserum					C antiserum				
		½	⅛	1/32	1/128	1/572	½	⅛	1/32	1/128	1/572
A	Finchley (i)	++	++	++	++	0	0	0	0	0	0
A	Finchley (ii)	++	++	++	0	0	0	0	0	0	0
A	Croydon	++	++	++	0	0	0	0	0	0	0
A	Hendon	++	++	++	+	0	0	0	0	0	0
B	Hornsey	+	0	0	0	0	0	0	0	0	0
C	Small colony	0	0	0	0	0	++	++	++	+	0
C	Large colony	0	0	0	0	0	++	++	++	+	0
Pleuropneumonia		0	0	0	0	0	0	0	0	0	0

Shows the degree of agglutination of the organism specified by the two specific antisera at the final dilutions indicated. Equal volumes of cultures and dilution of antisera mixed and incubated at 39° C for four hours.

++ = complete agglutination with precipitation.
+ = agglutination without precipitation.
0 = no sign of agglutination.

from 1·0 μ down to the filtration end-point. A Fildes serum broth culture of strain A, grown for two days at 30° C, was diluted with an equal volume of Fildes's broth and then filtered through a membrane of A.P.D. 1·4 μ. This provided a filtrate free of any aggregates, and the concentration of the organism, found by making Fildes's broth cultures of serial tenfold dilutions, was 10^9/cc. Measured 7 cc amounts of this filtrate were filtered under 76 cm mercury pressure through selected membranes ranging in porosities from 1·0 μ to 0·22 μ. The results are contained in fig. 2. It will be noticed that upwards of 90% of the organisms may be retained by relatively porous membranes of A.P.D. 0·7–1·0 μ, yet it is not until porosities less than 0·25 μ are reached that complete retention is assured. The least porous membrane to yield a

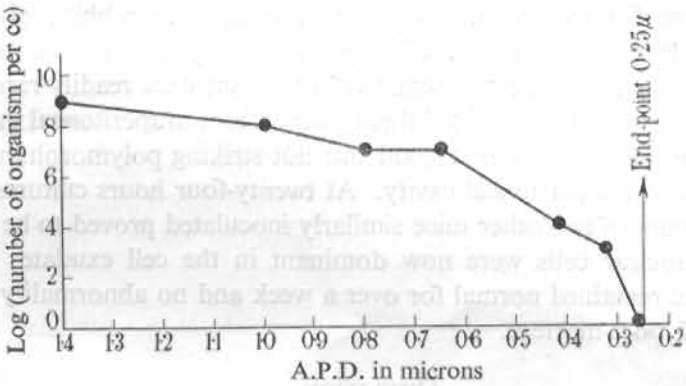

FIG. 2—Filtration end-point for strain A grown in Fildes's serum broth medium at 30° C.

positive filtrate has been of A.P.D. 0·26 μ. The filtration end-point 0·25 μ coincides with that previously found for vaccinia virus (Elford and Andrewes, 1932). The curve of fig. 2 is interpreted as indicating the smallest phase of the organism to be 0·125–0·175 μ in particle diameter, but that there are in addition larger forms ranging up to 0·5 μ in diameter.

In its general filtration behaviour the organism resembles bovine pleuropneumonia and agalactia, and contrasts with viruses and bacteriophages, which, individually, have been found to be relatively uniform in particle size, as evidenced by the fact that no appreciable drop in filtrate concentration, as compared with the original, is detected until the porosity of the membrane used is about twice the true end-point value.

The filterabilities of all the strains studied have paralleled closely that of the particular strain described above in fig. 2. Strain B (Hornsey)

presented difficulties owing to the pronounced tendency to spontaneous aggregation, which made it appear to filter rather less readily.

It is significant that cultures prepared from filtrates yielded by 0·28 μ membranes (this grade completely retains particles greater than 0·2 μ in diameter) behaved in subsequent filtration experiments exactly like the parent culture. They also presented a similar picture when examined under the dark field microscope. It is evident therefore that the smallest phase of the organism can initiate its development and growth process.

Animal Experiments

The organisms appear to be simple saprophytes. Two strains, A and C, were fed to rats, injected intravenously into rabbits, given by inhalation, by subcutaneous and intraperitoneal injection to mice and no ill-effects followed. The two strains of organism were readily recovered from two mice which were killed three hours after intraperitoneal inoculation; at this time there was a good but not striking polymorphonuclear cell exudate in the peritoneal cavity. At twenty-four hours cultures from the peritoneum of two other mice similarly inoculated proved to be sterile and mononuclear cells were now dominant in the cell exudate. Four further mice remained normal for over a week and no abnormality could be detected post-mortem.

Discussion

The chief interest in this group of micro-organisms seems to us to centre round the small forms which are to be found in all cultures. The existence of these suggests that the organisms may form another connecting link between the larger bacteria and the pathogenic viruses; and certain appearances we have described indicate that these organisms may be related in some way to the organism causing bovine pleuropneumonia. Other workers have described small organisms which pass bacterial filters but so far as we are aware no one has described this group.

We do not consider that this group is related in any way to the "filterable forms" of the pathogenic bacteria which have been described by many, *e.g.*, Hadley, Delves, and Klimek (1931), or Kendall (1931 and 1932). The existence of these filterable or "virus" forms is still contested, but in any case they arise under ill-defined conditions or as the result of special treatment (Hadley *et al.*), or on a particular kind of medium (Kendall). The "virus" forms are said to multiply for a time, as such, but may, under appropriate conditions, revert to the larger form

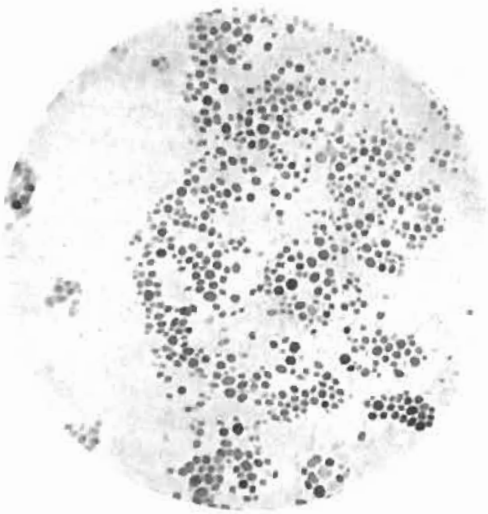

FIG. 1—Impression preparation from Fildes's agar plate culture. Stained Heidenhain's iron haematoxylin. × 1250.

(*Facing p.* 302)

which, in turn, multiplies as such. During eight months' study of several strains of the new organisms we have invariably found the small and the large form side by side and we are of the opinion that the small form will, some day, prove to be merely a regular stage in the development of the large.

Oerskov (1931) described what he regarded as minute organisms growing, on saccharose agar plates, symbiotically with a special bacillus which he grew out of milk. Dienes (1933–34) also has described small colonies around colonies of certain strains of *B. subtilis* on, or in, saccharose agar medium. These he believes to be formed by a small symbiotic organism. Neither of these workers succeeded in separating the small organism and obtaining growth in serial subculture. Klieneberger (1935) has recently described a small organism growing in association with *Streptobacillus moniliformis*, Levaditi, which is regarded by her as showing certain resemblances to the organism causing bovine pleuropneumonia. In an addendum to her paper she states that she has succeeded in growing the small organism separately in pure culture. The relationship of these symbiotic organisms to ours is quite uncertain. From the descriptions there would appear to be great morphological differences and close comparison of the various organisms by one observer would seem to be required.

Barnard (1935) gave an account of the microscopic appearances of certain " saprophytic viruses " which he discovered in culture media. Unfortunately he was not able to secure indefinite serial subculture of the organisms and direct comparison of cultures is now impossible.

We thus do not know how our organisms should be classified but we feel it is highly desirable that the method of multiplication should be established for this would probably determine its classification and moreover might have important bearings on kindred studies with pathogenic viruses. Our study has shown again that organisms at least as small as the vaccinia virus, can lead an independent existence and that small size alone cannot account for our failure to cultivate any of the viruses on artificial media. Moreover it seems desirable that the nutritional requirements of these small organisms should be accurately defined, for it is possible that exact knowledge of this sort might enable us to cultivate some of the pathogenic viruses apart from living cells.

Summary

A group of filterable, saprophytic organisms has been discovered and described. These organisms appear to be closely related to each other

and in the normal course of their development have small forms about the size of vaccinia virus. They may be cultivated on artificial media in indefinite series and may possibly prove to be a link between the larger bacteria and the pathogenic viruses.

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