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SOME OBSERVATIONS ON THE MORPHOLOGY AND BIOLOGY OF *MICROCOCCUS MELITENSIS*.

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[THE following notes summarise observations carried out in the Bacteriological Laboratories of the Medical Schools of Charing Cross Hospital and of Guy's Hospital during the eight years from 1899 to 1906, during which period I have at various times received much valuable assistance from Staff-Surgeon (now Fleet-Surgeon) Stenhouse, Staff-Surgeons Dalton and Duncan, and Captain R. Tilbury Brown, R.A.M.C., and to each of these indefatigable workers I take the present opportunity of expressing my sense of indebtedness.]

I.—MORPHOLOGY.

The *Micrococcus melitensis* (vel *Brucei*) as it occurs in Nature is extremely small, and when observed in hanging drop preparations occurs as a minute spherical coccus not exceeding 0·4 μ in diameter, or of a slightly ovoid cell measuring 0·4 μ by 0·3 μ, arranged singly or in pairs, or more rarely in short chains of four elements. In stained preparations the size is somewhat less (fig. 1), the cocci averaging 0·3 μ in diameter (Bruce, 0·33 μ). In broth cultivations of from one to two weeks' growth it is not uncommon to find longer chains numbering some ten to fourteen individuals. Chains of this length, however, lack cohesion and consequently are never seen in stained preparations. Many writers describe a "bacillary" form

of this micro-organism of which the length is from two to four times as great as the breadth (these forms are said to be most frequently obtained in old gelatine cultivations grown at the room temperature), or refer to it as a "cocco-bacillus," while some contend that it is a true bacillus. These statements appear to be based on the appearances occasionally observed in stained preparations of artificial cultivations, which should, in the writer's opinion, be interpreted in a totally different manner—that is to say, these so-called bacillary forms properly belong to one of two classes :—

(1) Elongated cocci, undergoing binary division, in which fission is as yet incomplete. These are found in stained preparations of young cultures (not in the hanging drop preparations, for the then connecting band of protoplasm remains invisible), and can readily be demonstrated by allowing the cover-slip film of emulsion to dry very slowly, taking say ten to fifteen minutes over the drying process, staining with weak thionin blue or gentian violet, and after washing again allowing the preparation to dry slowly. In such a film every gradation will be seen, from the diplococcus through the bipolar staining cell to the evenly-stained cylindrical form with rounded or oval ends, to which the terms cocco-bacillus or ovoid bacillus have been applied. A control preparation from the same emulsion rapidly dried over the flame and fixed, stained, and, after washing, again rapidly dried, will show nothing but cocci and diplococci, as the more rapid contraction of the cell protoplasm is sufficient to rupture the connecting band between two adjacent cells.

(2) True (but living) involution forms, of irregular shape and size, often approximating closely to the cylindrical form of the typical bacillus, but as often distorted, oval and pear-shaped, only met with in old cultivations, or in cultivations upon or in media of unsuitable reaction, and similar to those forms met with in, for example, cultivations of streptococci under comparable conditions.

Finally, this bacillary form is never met with in film or hanging drop preparations made direct from animal tissues.

Capsule formation is absent, spore formation is never observed.

II.—Motility and Flagella.

In hanging drop preparations the organism exhibits very active and vigorous Brownian, or molecular or vibratory movement, but true locomotion—the translation of individual cocci from one part

of the field to another—is entirely absent. Gordon,[1] staining film preparations from old cultivations by a modified Van Emengen method, claims to have demonstrated flagella, varying in number from one to four, on the majority of the cocci, but other observers, including Durham, Zammit, and the writer, have been totally unable to confirm his observations.

III.—Staining Reactions.

The micrococcus stains well with all the ordinary basic aniline dyes—methylene blue, fuchsin, or neutral red give the best results—it does not, however, retain the stain when treated by Gram's method, but holds only the contrast stain.

IV.—Atmosphere, Temperature, &c.

Atmosphere.—M. melitensis is a facultative anaerobe, it is true, but its rate of growth when deprived of a free supply of oxygen is very considerably diminished. When cultivated in an atmosphere of nitrogen—that is, in atmospheric air from which the oxygen has been abstracted by the action of alkaline pyro, and in which a slight negative pressure exists—many days elapse before the colonies on agar plates are visible to the naked eye, and two or even three weeks' incubation are needed before they attain the same size as those grown on control aerobic plates for seven days only.

Temperature.—The upper and lower limits of temperature at which growth of *M. melitensis* takes place are 45° C. and 6° C. Above the former and below the latter points growth ceases. The "optimum" temperature is 36·8° C. to 37° C. From the "optimum" up to 42° C. growth suffers but little diminution in rate, but from 42° C. up to 45° C. growth is sensibly affected in the directions of slowing and in the increase of involution forms. From 36° C. down to the room temperature (18° to 22° C.) the micrococcus suffers a progressive decrease in rapidity of growth, accompanied by a gradual increase in the number of involution forms present in the cultures.

Rate of Growth.—M. melitensis is, on the whole, a slowly growing organism when cultivated direct from the animal body—or in or upon artificial media of unsuitable reaction—the visibility of the colonies under these circumstances usually being delayed to the third or fourth day. In some instances, if sufficient infective material is planted, a good growth can be obtained direct from the animal tissues in a remarkably short time, twenty-four to thirty-six

[1] *Lancet*, 1899, i. (688).

hours, and when this happens the influence of the reaction of the medium upon the resulting growth is well shown. Growth in broth is also slow. Subcultivations, however, even upon the ordinary laboratory agar (+ 10), can be readily made to yield luxuriant growth within twenty-four hours.

Reaction of Medium.—The extremes of reaction of artificial media in which the micrococcus can be cultivated are, − 7·5 and + 15 (Eyre's scale), at which points growth is extremely scanty and slow. For all ordinary purposes + 10 will be found sufficiently near the optimum reaction, but when rapid and luxuriant growth is desired from material obtained direct from animal tissues the optimum reaction of + 8 must be employed. The following extracts from the laboratory notebook showing date of appearance of colonies on agars of different reactions sufficiently emphasise this point :—

A.—Two mg. of brain substance from guinea-pig 25B were suspended in 10 cc. normal saline solution, and one loopful of the resulting emulsion was employed to inseminate, in series, three plates of each batch of reaction agar. The plates were incubated aerobically at 37° C., until the termination of the observations. In the following tables the sign + indicates growth visible to the naked eye, and the numbers refer to the three plates in the order of planting.

Reaction of medium	Period of Observation				
	24 hours	48 hours	72 hours	96 hours	120 hours
+ 2·5	..	..	..	+ 1	+ 1, 2, 3
+ 5	..	+ 1	+ 1, 2, 3	+ 1, 2, 3	+ 1, 2, 3
+ 8	..	+ 1	+ 1, 2, 3	+ 1, 2, 3	+ 1, 2, 3
+ 10	..	..	+ 1	+ 1, 2	+ 1, 2, 3
+ 12·5	..	..	..	..	+ 1, 2, 3

Reaction of medium	Period of Observation					Total number of colonies on plate
	24 hours	36 to 40 hours	48 hours	72 hours	96 hours	
+ 2·5	..	..	..	..	+	3
+ 5	..	..	+	+	+	30
+ 8	+	+	+	+	+	150
+ 10	..	+	+	+	+	100

B.—A similar experiment using cerebral tissue from guinea-pig 26 as the infective material. Here the number of cocci present per loopful of emulsion was very much smaller, and the first plate

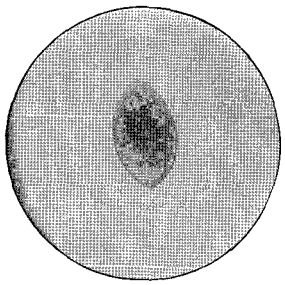

FIG. 1.—*M. Melitensis*, Deep Colony, Glycerine Agar, 3 days at 37° C. × 15.

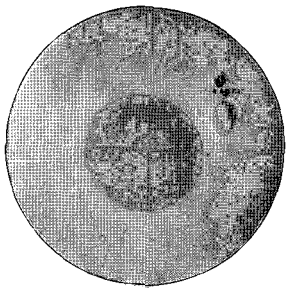

FIG. 2.—*M. Melitensis*, Superficial Colony, Glycerine Agar, 3 days at 37° C. × 15.

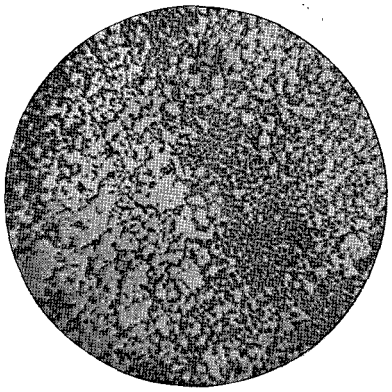

FIG. 3.—*M. Melitensis*, 3 days Glycerine Agar at 37° C., stained Carbolic Fuchsin. × 1,000.

To illustrate article by J. W. H. EYRE, M.D.
"Some Observations on the Morphology and Biology of *Micrococcus Melitensis*."

of each series was countable. Plates 2 and 3 in each series were discarded as they showed no growth up to the end of seven days.

V.—Cultural Characters.

Note.—Unless otherwise stated the appearances are described from cultivations upon ordinary laboratory media (+ 10), after incubation aerobically at 37° C.

Agar Plates.—Growth present in crowded plates at twenty-four to thirty-six hours produces a ground-glass appearance on the surface of the medium, sometimes visible to the naked eye, usually only seen with the $\frac{2}{3}$ inch objective. At forty-eight to seventy-two hours discrete colonies, resembling minute drops of water, are visible. The size of the colonies is inversely proportional to the number present in a plate, and when moderately numerous—say 100 to a plate—attain a diameter of 1·5 mm. at the end of a week. When only two to ten colonies are present in a plate a diameter of 3·5 mm. is not unusual; the measurement of the colonies developing in a series of plate dilutions showing well the influence of abundance of food as compared with proximity and over-crowding on the ultimate size of the individual colonies.

The following experiment shows the point well. Two mg. of cerebral tissue from guinea-pig 25C were emulsified in 10 cc. of normal saline solution, and one loopful of the emulsion employed to inseminate in series nine agar plates (+ 8). These were incubated for twenty-one days under aerobic conditions in an incubator running at 37° C., the atmosphere of which was kept saturated with moisture. The result of the observations was as follows:—

Plate 1	..	Innumerable minute colonies.
,, 2	..	,, ,, ,,
,, 3	..	1,275 colonies—averaging less than 0·5 mm. diameter.
,, 4	..	530 ,, ,, ,, 0·75 ,,
,, 5	..	40 ,, comprising 15 of 1·5 mm., 17 of 2 mm., and 8 of 2·5 mm. diameter.
,, 6	..	20 ,, ,, 18 of 2 mm., and 2 of 2·5 mm. diameter.
,, 7	..	12 ,, ,, 2 of 2·5 mm., 5 of 3 mm., 1 of 3·5 mm., and 4 of 4 mm. diameter.
,, 8	..	1 ,, 5 mm. diameter.
,, 9	..	*Nil.*

The individual colonies are round, nearly circular in shape; surface colonies, convex to pulvinate, occasionally umbonate; deep colonies biconvex, edge entire, structure finely and regularly granular, surface smooth. In the centre of each colony is a darker portion of sharply defined oval shape, better seen in the deep than

in the surface colonies (figs. 2 and 3). No lines, grooves or other structural markings can be seen, although in old colonies the central portion may become more coarsely granular or even grumose.

Colonies when young are translucent. After about four days' growth they become opaque whitish, with a slight opalescence by reflected light, and pale yellow to pale amber by transmitted light. With age the colour passes from rich amber to light brown or even dirty slate brown.

Glycerine Agar.—As above.

Nutrose Litmus Agar.—This medium serves fairly well for the purposes of isolation when prepared from meat essence; if prepared from ox serum[1] and made to a reaction of + 8 it forms the *optimum medium* for the growth of the coccus. The appearance of the colonies is identical with that described above; the reaction of the litmus remains unchanged.

[Agar prepared from a watery extract of either spleen or brain tissue, in place of the ordinary Fleischwasser, is a good medium for the cultivation of the micrococcus, but the growth thereon exhibits no essential differences from those described above. According to Zammit, the organism grows well on an agared solution of normal fæces, but will not develop upon an agar medium prepared from sea water, even when heavily contaminated with human excrement.]

Agar Tube Culture Smear and Streak.—The growth at first consists of discrete colonies limited to the needle track; these rapidly coalesce to form masses and bands of raised, moist, shining growth, at first pale yellow to amber in colour, then passing with age to a distinct brown, like the colour of glue.

Blood Serum (Inspissated).—A white, moist growth, similar to the above.

Blood Serum (Fluid).—In this medium growth usually takes place as a fine flocculent deposit, the bulk of the fluid remaining perfectly clear, and only rarely as a diffused turbidity.

Alkali Albumen Jelly (Lorrain Smith).—A very scanty growth of minute discrete colonies, not appearing under three to four days, and which do not attain any great size, the medium being too alkaline to afford a favourable soil.

Gelatine Plate Cultivation.—At 22° C. the coccus is extremely slow of growth. Practically nothing can be seen with the naked eye until about the end of a week, although the colonies can be seen by the aid of a $\frac{2}{3}$-inch lens by the fourth or fifth day. In

[1] *Transactions of the Pathological Society.*

character and appearance the colonies are identical with those described under agar plate. No liquefaction of the medium takes place even at the end of a month, by which time, if the gelatine has been prevented from drying, the colonies average some 2 mm. in diameter.

Gelatine Streak or Smear Culture.—At 22° C. the growth is restricted to the path of the inoculating needle, and if a fairly large amount of infective material has been sown the growth is visible by the third or fourth day. It resembles the growth upon agar, but is drier. Microscopically, elongated involution forms are often present after about three weeks' growth.

Gelatine Stab at 22° C.—Growth in the form of minute discrete spherical and biconvex colonies, resembling the deep colonies in agar, is visible along the entire course of the stab in about five days. At the end of a fortnight those in the upper part of the needle track have increased in size, are yellowish-brown in colour and coarsely granular, and a few beaded out-growths may be noted in this situation.

Gelatine (*Fluid*).—Growth readily takes place in 10 per cent. gelatine liquefied by heat, and incubated at the body temperature, in the form of a white flocculent deposit, the bulk of the medium remaining clear. Some strains of *M. melitensis*, stricter aerobes than the majority, render the upper layers of the gelatine uniformly turbid, but the growth soon sinks to the bottom of the tube or flask, and multiplication practically ceases after about ten days.

Nutrient Broth.—Growth first appears towards the end of the second day as a diffuse turbidity, and by seventy-two hours the growths in the upper layers of the fluid are denser than those below. Towards the end of a week the rate of growth has slowed and a white deposit has begun to form, whilst after a month or so the bulk of the medium is almost clear, and the deposit, consisting chiefly of short chains easily broken up, has considerably increased. At no period is any pellicle formed.

Peptone Salt Solution (not Standardised).—Scanty growth. Indol absent up to the end of twenty-eight days.

Nitrate Broth (not Standardised).—Scanty growth. No reduction of nitrates to nitrites.

Lead Broth (not Standardised).—Scanty growth. No precipitation of lead sulphide.

Bile Salt Broth (not Standardised).—Growth; no apparent change in the reaction of the medium.

Proskauer and Capaldi's Solution (not Standardised) *No.* 1.—No growth.

Proskauer and Capaldi's Solution (not Standardised) *No.* 2.—Growth; no apparent change in the reaction of the medium.

Neutral Litmus Whey.—Growth. Production of an alkaline reaction in twenty-four to forty-eight hours, which increases until the end of a week; it is then equivalent to from 0·2 to 0·3 per cent. of $\frac{n}{10}H_2SO_4$.

Litmus Milk.—Luxuriant growth takes place in this medium. No change whatever is produced in the consistence of the milk, but the formation of alkali goes on rapidly, and by the end of four or five days has caused the litmus to assume a distinct blue colour. This production of an alkaline reaction is much more marked when using goat's milk as the medium than when cow's milk is employed. Microscopically, the cocci present in milk are rather larger than those observed in other media, except potato.

Potato.—Growth on ordinary potato culture is visible to the naked eye only as a moist area on the surface of the medium—the so-called "invisible" growth—and is never very luxuriant. Upon alkaline potato growth is more vigorous, and appears as a moist film, whitish to pale yellow in colour, by the end of four or five days. Microscopically, the cocci are larger than those grown on agar, tend to chain formation, and are associated with numerous involution forms.

Carbohydrate Media (not Standardised).—Composed of distilled water in which is dissolved 1 per cent. Witte's peptone, and 1 per cent. of the appropriate carbohydrate, and tinted with neutral litmus-solution, previous to tubing and sterilising.

Lævulose media Galactose media Maltose media Saccharose media Rafinose media Mannite media Dulcite media Dextrine media Inulin media	Growth takes place, but is not accompanied by the production of gas or of alteration in reaction.
Dextrose media Lactose media	Growth takes place accompanied by the production of a faint alkaline reaction at the end of a week.

VI.—Resistance.

The experiments referred to under this heading were carried out with each of five different strains of *M. melitensis*, and were repeated many times in order to obtain consistent average results.

(1) *Moist Heat.*—The thermal death-point from the average of five different strains suspended in watery emulsions, under standard conditions, with a time exposure of ten minutes, was found by Dalton and the writer[1] to be 57·5° C.

[1] *Journ. of Hygiene*, iv., 1904 (157).

(2) *Dry Heat.*—The same five strains, similarly emulsified, then dried upon sterile cover-slips and exposed to dry heat with a time exposure of ten minutes, gave a death-point varying from 90° to 95° C. with the different strains.

(3) *Desiccation.*—All these strains of *M. melitensis* resisted drying for long periods (see also VII., Vitality). Working with emulsions subsequently dried in a Müller's desiccator on cover-slips, oncigarette or filter paper, cotton-wool and strips of linen, Duncan and I were successful in obtaining subcultures from these materials up to the twenty-first day. Short lengths of yarn soaked in the emulsions, then dried, yielded growth up to the end of the third month (ninety days), but every attempt to obtain subcultures during the fourteenth or fifteenth weeks failed completely.

(4) *Chemical Reagents.*—In the first instance the resistance of the micrococcus to perchloride of mercury and to carbolic acid was tested by a modification of the drop method; that is to say, a definite quantity of emulsion of the coccus (0·1 cc.) was added to 5 cc. of the test disinfectant, and after short contact periods 0·1 cc. of the mixture was plated on agar; after fifteen, thirty and sixty minutes' exposure, two, three, or even ten such 0·1 cc. plates were poured as seemed desirable. The plates were incubated for seven days at 37° C., and the total number of the colonies present in the plates enumerated, the cocci present per cubic centimetre calculated therefrom and recorded. A tenth of a cubic centimetre of the emulsion, added to 5 cc. of distilled water, served as a control. An average result is tabulated below, and shows that 1 per cent. phenol and 1 in 2,000 hydrarg. perchlor. are both able to destroy the coccus in watery emulsion within fifteen minutes.

Disinfectant	Period of Contact						
	2½ mins.	5 mins.	10 mins.	15 mins.	30 mins.	60 mins.	120 mins.
$HgCl_2$ 1 in 2,000 ..	16	2	2	0	0	0	0
$HgCl_2$ 1 in 1,000 ..	0	0	0	0	0	0	0
Phenol 1 in 200 ..	8,600	3,300	2,900	1,200	624	22	0
Phenol 1 in 100 ..	117	75	7	0	0	0	0
Distilled water ..	64,000	64,000	64,000	64,000	30,000	12,000	2,800

Working on the question of the comparative values of Clayton gas and of sulphur dioxide for the disinfection of ship's holds, Wade and the writer found that Clayton gas supplied to a hold at an average percentage of 1·2 per cent. sulphur dioxide for a period of four and three-quarter hours was sufficient to destroy all the samples

of *M. melitensis* (dried on cigarette paper and on cotton-wool) which had been distributed throughout the cargo in the experimental hold; 0·7 per cent. liquid sulphur dioxide supplied to the hold for a period of five hours was equally efficient; higher percentages were found to be equally efficient in shorter periods, *e.g.*, 2·5 per cent. SO_2, in the shape of Clayton gas for three hours, or 3·2 per cent. liquid SO_2 for two and a half hours.[1]

VII.—Vitality.

M. melitensis retains its vitality for considerable periods in artificial cultivations, even when no particular precautions have been taken to retard evaporation from the medium. Successful subcultivations have been recorded by Shaw from agar cultures two hundred and seventy-six days old, broth cultures one hundred and seventy-three days old, and litmus milk cultures one hundred and forty-four days old. Kennedy successfully established a subcultivation from a litmus milk culture two hundred and eighty-four days old.

A batch of agar cultivations of *M. melitensis*, prepared in Malta under date November 5th, 1902 (and originally sealed with paraffin wax), were received at Guy's Hospital, where they were kept in the cool incubator (22° C.), and subcultivations prepared as required. On September 22nd, 1904, a successful subcultivation was established from the last remaining tube of the batch when the medium was dry and shrivelled (by Duncan), and on February 12th, 1905 (several unsuccessful attempts having been made between these two dates), nutrient broth was poured into the tube, and after incubation at 37° C. for three days, plates were prepared from the now turbid broth. A scanty growth of *M. melitensis*, was obtained a few days later (Eyre). These two periods of six hundred and eighty-seven and eight hundred and thirty days sufficiently indicate the longevity of *M. melitensis* when undisturbed, even when not placed under the most favourable conditions.

When cultivated in large two-litre flasks of sea water (previously sterilised by filtration through porcelain filters, not by boiling or autoclaving) the coccus was found by Stenhouse and the writer to retain its vitality up to the end of seven weeks (forty-two days), and during the first seventeen to twenty days to show evidence of slight multiplication in that medium.

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[1] Report to the Local Government Board on "Further Experiments on Sulphur Dioxide, as Applied in the Destruction of Rats and in Disinfection on Shipboard," by Dr. Wade, No. 232, May 7, 1906.