SOME MORPHOLOGICAL AND BIOLOGICAL CHARACTERS OF THE SPIRILLA (VIBRIO FETUS, N. SP.) ASSOCIATED WITH DISEASE OF THE FETAL MEMBRANES IN CATTLE.

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PLATE 16.

(Received for publication, June 3, 1919.)

In an earlier communication[1] a spirillum of definite morphological and cultural characters was described as being associated in a series of cases with what is commonly known as infectious abortion in cattle. In the following pages the statements there made concerning the biology of the spirillum are amplified and supplemented by fresh observations and by studies on the agglutinative affinities of the various strains. In all, 24 fetal strains have been kept under cultivation. Of these one (No. 308) is a slightly modified, aberrant type. All were obtained from one large herd into which cattle from outside are introduced at irregular intervals.

Morphological Characters.

In films and in hanging drop preparations from fetal fluids and cultures therefrom, the spirilla appear as fine wavy or sinuous lines of various lengths. The smallest forms appear as minute curved S-shaped lines; the longest may stretch nearly across the field of the microscope. If we assume that the spirillum is in the form of a spiral or corkscrew, the diameter of the spiral is small. The spiral is drawn out, as it were, becoming in some cultures almost a straight line. In dried and stained films the spiral becomes a shallow sinuous line (Fig. 7). No segmentations are distinguishable in the longer forms.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

As regards size, the width of the spirillum stained in alkaline methylene blue is probably not over 0.2 to 0.3μ, the shortest form about 1.5 to 2μ long. A common size in the fetal fluids consists of about two complete turns and measures 4 to 5μ in length. As stated above, the diameter of the spiral or turn varies somewhat, but it averages about 0.5μ.

The organism stains fairly well in alkaline methylene blue provided the staining is prolonged, preferably over night. It stains much more deeply in diluted aniline water gentian violet (Fig. 6), but the former stain is to be preferred since it requires no decoloration, a process likely to decolorize the spirillum itself.

When Löffler's flagellar stain, as modified by Moore,[2] is applied, the organism is much thicker, as shown in Figs. 1 to 5. A flagellum is found attached to one or both poles of the spirillum. Actual counts of the spirilla of eight different strains in a given number of fields indicated that organisms having unipolar flagella are far more numerous than those with bipolar flagella. The latter may be considered relatively scarce. The unipolar forms may be individuals recently set free by division, the bipolar forms ready to divide. Longer spirilla have been seen with several short lateral flagella in addition to the terminal ones. This appearance may be due to forms which have divided but not yet separated, the flagella of the daughter cells having been formed in the meantime. The character of the flagella is best seen in the figures. They are very fine hair-like processes in large undulations or waves, of uniform width throughout.

In hanging drop preparations the shorter forms, from $\frac{1}{2}$ to 2 windings long, are very active. They shoot with lightning rapidity in straight lines across the field in all directions. Only occasionally does one reduce its velocity so that details of the movements become visible. It may then be seen in a few cases that the organism revolves around its longitudinal axis. Whether this motion is used in very rapid propulsion cannot be determined. The longer forms move sluggishly or are quiescent.

Another element brought out by staining and referred to in a former paper[1] is the granules which appear on or within the spirilla. They

[2] Moore, V. A., Wilder quarter century book, Ithaca, 1893, 339.

occur more commonly in old cultures and are probably degeneration forms. Usually the long spirilla contain many, but short forms are not without them. In the latter they are terminal. In the long forms they are arranged along the filament at fairly regular intervals. They stain much more deeply than the rest of the spirillum and suggest the Neisser granules of diphtheria bacilli. The feeble resistance of this spirillum to heat and drying makes any interpretation of these granules as spores improbable.

Owing to the recent extensive additions to our knowledge of spiral organisms as agents of disease and the introduction of new methods of culture, the nomenclature is not in a satisfactory state. Most writers on classification have accepted the generic designation vibrio for short, so called comma forms, and spirillum for distinctly spiral forms. The former have one polar flagellum, rarely more, the latter a tuft of polar flagella. The spiral form here described appears, as a rule, in both long spiral and short comma forms in the fetal fluids and may therefore be regarded as standing intermediate between the comma forms and true spirals. Nevertheless since this organism assumes the comma form in its early, most active stage of multiplication in cultures and has single polar flagella, the generic term vibrio appears most appropriate at the present time. It is therefore designated *Vibrio fetus, n. sp.* The term spirillum we shall continue to use in the text as a general expression for spiral organisms.

Cultural Characters.

In discussing the culture characteristics of *Vibrio fetus* we must distinguish between the earliest and later cultures. The earliest represent the still unchanged animal type, whereas later cultures may become saprophytized. The change from one to the other growth type goes on gradually and no boundary lines in terms of generations or transfers can be definitely assigned.

The earliest growths have been obtained in agar slants containing a small quantity of condensation water or added bouillon. To this a bit of tissue about $\frac{1}{8}$ gm. or an equivalent amount of stomach or other fluid or intestinal contents of the fetus is added and the tubes are sealed with sealing-wax. In such a medium the growth may be

feeble and is likely to be overlooked entirely at first. After 3 or more days of incubation two very narrow grayish white, opaque lines of growth extend upward from beneath the surface of the condensation water where the agar slant abuts against the glass. These lines, less than 1 mm. broad, are at first the only macroscopic indications of growth. Soon an exceedingly thin veil-like layer extends from each of these two lines into the capillary space between agar and glass, often meeting behind and then forming a complete barely visible layer with upper margins variably high and undulating or jagged, but rarely more than 4 cm. above the bottom of the tube. The condensation water is very feebly clouded but motile spirilla are usually found in it. Surface growth occurs only very exceptionally.

The above features persist in subsequent cultures but there are gradually added others. If defibrinated horse or other mammalian blood is added to the condensation water, a layer of growth appears on the surface of the sedimented corpuscles, which grows heavier with the number of transfers. Surface growths appear after months of cultivation either as grayish white films or as isolated, roundish, smooth, glistening colonies, 1 to 2 mm. in diameter.

With the increasing vigor of growth it becomes possible to obtain multiplication on agar slants without the blood and thereafter the strain may be maintained on plain agar in sealed tubes. In the condensation water a viscid whitish sediment made up of spirilla appears. This may be drawn out into threads. This phenomenon seems to point to a kind of mucoid degeneration which is suggested by the unstained sheaths around spirilla in Fig. 8.

In the earliest growths fluid media failed, as a rule, to induce multiplication. This failure may be due to the continually changing relation to oxygen of the different layers of fluids on account of convection currents. The agar medium provides stable conditions different at different points of the slant. The method of cultivation described differs from the methods of other workers in which deep layers of culture media, either solid, semisolid, or fluid, have been used for bacteria sensitive to oxygen. The use of slanted agar in a sealed tube gives better access to the growth than do the deep layers. It provides various degrees of oxygen tension and hence a greater variety of conditions, one of which may fit the organism to be cultivated.

With some strains a fairly vigorous multiplication has occurred in the first cultures, such as growth on the agar surface and membrane formation on the condensation water, festoons and mucoid shreds hanging down into this fluid. This is due to a specially favorable medium, which is mucus from the stomachs of the fetuses transferred to tubes when original cultures are prepared.

After thirty or more transfers with intervals up to a week multiplication has been observed in unsealed agar slants. The reduced oxygen tension in such tubes is obtained, when necessary to the organism, by growth in the capillary space between the mass of agar and the sides of the tube.

When agar was used in deep layers in sealed tubes the growth phenomena corresponded with those of *Bacillus abortus* under like conditions.

The following description applies to saprophytized strains. Fluid agar, thoroughly mixed with the spirillum, was allowed to set. Growth was gradual, appearing after incubation of 2 to 3 days as a fine white line around the circumference of the surface of the agar, and slowly growing down between the agar and the glass as a fine film. Condensation water gathered, became viscid, and growth spread over the surface of the agar. A dark line forming in the agar below the surface was observed, which at the end of a week's incubation resolved itself into a zone of individual colonies. The colonies were small, opaque, and of a yellow color. In some instances the zone appeared directly under the surface and in others it grew from 2 to 5 mm. below the surface. In unsealed tubes there was no condensation water and no growth at the surface, but the subsurface zone appeared heavier than in the sealed cultures.

In agar stabs growth developed slowly, appearing on the 2nd day along the lines of puncture. The final growth along the stab varied from 5 mm. to 3 cm. in length. If a lateral puncture was made, a fine white film gradually spread between the agar and the glass. At the points of inoculation a surface growth developed, and any slight condensation water present became viscid. In unsealed cultures no growth appeared on the surface.

Coagulated and slanted blood serum (horse) did not prove superior to the slanted agar plus a few drops of blood. No liquefaction was noted in the usually feeble growth. Growth was obtained when

defibrinated blood was incorporated with melted agar and the latter slanted. Multiplication did not take place in nutrient gelatin, either at incubator or room temperature. It is not known, therefore, whether a liquefying enzyme exists or not.

After strains had become adapted to plain slanted agar, attempts were made to induce multiplication in fluid media. Growth in simple beef peptone bouillon in sealed and unsealed tubes becomes possible, but the addition of a few drops of defibrinated blood makes multiplication more vigorous. The medium becomes faintly clouded and a slight viscid sediment is formed. A filmy stringy deposit may settle upon the sides if the tubes are slightly inclined and a viscid ring may appear at the surface, but no pellicle. Growth is not improved when paraffin oil covers the fluid column. In milk no recognizable multiplication occurs.

Biological Characters.

Since *Vibrio fetus* has many biological characters in common with *Bacillus abortus*, it would seem that these two widely separated morphological species are drawn nearer together in their physiological characters through adaptation to the same environment on the fetal membranes, causing the same sensitiveness to oxygen tension, the same fastidiousness towards culture media, and indifference to carbohydrates. Marked differences, however, do occur, such as failure on the part of *Vibrio fetus* to multiply on potato, on which *Bacillus abortus* produces a fairly rich pigmented growth.

The length of time that a sealed original culture at room temperature may contain living organisms differs from tube to tube. One culture gave rise to successful subcultures after 139 days, another failed to do so after 31 days. Subcultures on agar plus blood displayed the same differences—one dead after $2\frac{1}{2}$ weeks, another alive after 9 weeks. It is therefore important in maintaining strains to transfer once a week.

When cultures are stored in refrigerators at a temperature of 5–6°C. above freezing their vitality is reduced considerably as compared with that of cultures kept at room temperature.

The reaction of the bouillon may be carried slightly beyond the neutral point of phenolphthalein without interfering with multiplication. Indole is not formed in bouillon. Fermented bouillon with

1 per cent of dextrose, lactose, and saccharose was used in fermentation tubes to determine gas and acid production. The bulbs became uniformly clouded and a slight deposit developed; the branches remained clear. No gas was produced. After 5 days incubation the bulbs were titrated, and the reaction was found to be neutral or slightly acid. The original reaction of the bouillon had been 0.5 to

TABLE I.

Resistance to Drying.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	°F.		*days*	
192 (61st transfer)	70–75	1 hr.	3	+
192 (61st ")	70–75	2 hrs.	3	+
192 (61st ")	70–75	3 "		−
192 (61st ")	70–75	4 "		−
192 (61st ")	70–75	5 "		−
192 (61st ")	70–75	6 "		−
192 (61st ")	70–75	1 day.		−
149 (79th ")	70–75	1 hr.	4	+
149 (79th ")	70–75	2 hrs.		−

TABLE II.

Resistance to Heat.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	°C.	*min.*	*days*	
192 (70th transfer)	56	5		−
192 (70th ")	56	10		−
192 (71st ")	55	5	3	+
192 (71st ")	55	10		−
149	55	5	3	+
149	55	10		−

0.6 per cent acid to phenolphthalein. The branch showed no acid production.

Vibrio fetus as it occurs in cultures has but little resistance to drying. Bits of linen thread impregnated with fresh active culture material resisted drying 2 hours at room temperature, but not 3 hours, as shown in Table I. Similarly resistance to heat is feeble. It is able to withstand a temperature of 55°C. for 5 minutes (Table II).

Agglutination Reactions.

The scarcity of positive differential characters of the spirilla isolated from fetuses leaves open the possibility that they may not all be alike and that many, if not all, are harmless invaders of the uterochorionic space and the fetal digestive and respiratory tracts. To test the first possibility agglutination with immune serum was resorted to at an early stage of the work. Rabbits were treated repeatedly with doses of living spirilla injected into the abdominal cavity. After 3 to 4 weeks blood was withdrawn and the serum used in the tests shown in Tables III to VI. Before applying the agglutination test it was necessary to await the time when the various strains could be made to multiply on agar slants without blood or bits of tissue so as to eliminate any possible interference on the part of animal tissues.

At this time one of us fortunately isolated a spirillum from the spleen of a young calf which, though culturally like the fetal strains, was nevertheless wholly different in its agglutination affinities. This culture which will be taken up in another publication was used in the tests as a control. It is designated as No. 174. Somewhat later another strain was isolated from the liver of a calf 8 weeks old and the spleen of a guinea pig inoculated with duodenal contents of the same calf. This agrees serologically with *Vibrio fetus* and appears in the tables as No. 321.

The serum used is from rabbits immunized against Strains 67, 174, 256, and 317. The antigens are saline suspensions of the several strains grown on sealed agar slants at 37°C. for 4 days. The salt solution used is 0.85 per cent. The volume of fluid in each final dilution is 2 cc. The culture control contains 1 cc. of the antigen suspension and 1 cc. of salt solution, the serum control 1 cc. of a 1:10 dilution of serum and 1 cc. of normal salt solution. After a period of from 2 to 3 hours at 37°C. readings are taken. The tubes are then placed in the refrigerator over night when the final readings are made.

Tables III to V show that the agglutination reactions are of the usual kind, except for very young strains which yield a more or less paradoxical reaction, in which the clumping is feeble or absent in

TABLE III.

A. Serum from a Rabbit Treated with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Dilutions of serum.											Controls.	
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	Culture.	Normal serum.
67	64	C.*	C.	C.	C.	C.	C.	C.	+++[1]	+++[1]	++[1]	++	+	−	−
149	75	"	"	"	"	"	"	"	C.	+++[1]	+++	++[1]	++	−	−
159	72	"	"	"	"	"	"	"	"	C.	+++[1]	+++	++	Settling and clumps; cloudy.	−
174		−	−	−	−	−	−	−	−					−	−
179	71	C.	C.	C.	C.	C.	C.	C.	C.	C.	+++[1]	++[1]	++	−	−
192	?	"	"	"	"	"	"	+++[1]	+++	+++	++[1]	++	++	−	−
213	56			"	"	"	"	C.	C.	++[1]	++[1]			−	−
246	25			"	"	"	"	+++[1]	+++	++	+			−	−
251	23			+++[1]	"	"	"	+++[1]	+++	+++	++			−	−
256	21			C.	"	"	"	+++[1]	+++[1]	+++	+++			−	−
258	19			"	"	"	"	++	+	+	+			−	−
263	14			"	"	"	"	C.	C.	C.	+++			−	−
267	17			−	−	−	+++	+++[1]	"	++[1]	++			−	−
289	8		C.	+++	+++	+++	+++	++	++	++	−	−		−	−
290	8		"	C.	C.	C.	++	++	+	+	+	−		−	−

* In this and the following tables C. indicates complete agglutination, +++ nearly complete agglutination, ++ marked agglutination, + slight agglutination, ± doubtful, and − no agglutination.

[1] when following a symbol denotes a degree of agglutination between it and the next higher symbol.

TABLE III—*Concluded.*

B. Serum from a Rabbit Treated with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	90	C.	C.	C.	C.	C.	C.	+++[1]	−	−	−
149	97	"	"	"	"	"	"	C.	C.	−	−
318	17	"	"	+++[1]	+++	+++	++	+	+	−	−
159	96	"	"	C.	C.	C.	C.	C.	C.	−	−
179	89	"	"	"	"	"	+++	++	+	−	−
289	25	"	"	"	"	+++[1]	+++	++[1]	++	−	−
317	18	"	"	"	"	C.	C.	C.	+++[1]	−	−
308	21	"	"	"	"	"	"	+++[1]	++	−	−
317	19	"	"	"	"	+++	++	+	±	−	−
267	39	"	"	"	"	C.	C.	+++[1]	+++	−	−
321	17	"	"	+++[1]	+++	++	+	−	−	−	−
321	13	"	"	C.	C.	+++[1]	+++	++	+	−	−
331	4	+++	+++	+++	++	+	−	−	−	−	−
333	3	++	++	++	++	++	++	−	−	−	−
339	3	+	++	++	++	++	+	+	+	−	−

TABLE IV.

Serum of a Rabbit Treated with Cultures of Vibrio 256.

Vibrio No.	Dilutions of serum.								Controls.	
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	C.	C.	C.	C.	C.	+++[1]	+++	+++	−	−
149	"	"	"	"	"	C.	+++[1]	+++[1]	Slight deposit; cloudy.	−
159	"	"	"	"	"	"	+++[1]	+++[1]	" "	−
174	−	−	−	−	−	−	−	−	−	−
179	C.	C.	C.	C.	C.	+++[1]	+++	+++	−	−
192	"	"	"	"	"	C.	+++[1]	+++[1]	−	−
213	"	"	"	"	"	"	+++[1]	+++[1]	−	−
246	+++	"	"	"	"	"	C.	+++	−	−
251	−	−	+++	+++[1]	+++[1]	"	+++	+++	−	−
256	C.	C.	C.	C.	C.	"	C.	+++	−	−
258	"	"	"	"	"	"	+++[1]	+++[1]	−	−
263	−	+++	"	"	+++	++	+[1]	+	−	−
267	−	−	+	++	+++	C.	+++	++	−	−
289	+	++	+++	C.	C.	+++	++	−	−	−
290	−	−	+	+++	"	C.	+++	++	−	−
321a*	+++[1]	+++[1]	++	+	−	−	−	−	−	−
321b†	C.	C.	C.	+++	+++	+	−	−	−	−

* Liver strain.

† Guinea pig passage strain.

TABLE V.

Serum of a Rabbit Treated with Cultures of Vibrio 317.

Vibrio No.	No. of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
317	20	C.	C.	C.	C.	C.	++[1]	+++[1]	+++	Slight sediment.	−
67	92	+++	+++[1]	+++[1]	++	±	−	−	−	−	−
174	95	−	−	−	−	−	−	−	−	−	−
213	71	C.	C.	C.	C.	C.	+++	++	+	−	−
267	42	"	"	"	+++	++	+	±	±	−	−
318	21	"	"	"	C.	+++	++	+	−	−	−
290	25	"	"	"	+++	++	+	±	−	−	−

TABLE VI.

Serum of a Rabbit Treated with Cultures of Vibrio 174.

Vibrio No.	Dilutions of serum.											Controls.	
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	Culture.	Normal serum.
67	—	—	—	—	—	—	—	—					—	—
149	—	—	—	—	—	—	—	—					—	—
159		—	Slight clearing.	Slight clearing.	—	—	—	—					Slight deposit; cloudy.	—
174	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	+++[1]	—	—
179		—	—	—	—	—	—	—	—				—	—
192		—	—	—	—	—	—	—	—				Slight deposit.	—
213		—	—	—	—	—	—	—	—				—	—
246		—	—	—	—	—	—	—	—				—	—
251		—	—	—	—	—	—	—	—				—	—
256		—	—	—	—	—	—	—	—				—	—
258		—	—	—	—	—	—	—	—				—	—
263		—	—	—	—	—	—	—	—				—	—
267		—	—	—	—	—	—	—	—				—	—
289		—	—	—	—	—	—	—					—	—
290		—	—	—	—	—	—	—					—	—
308*		—	—	—	—	—	—	—	—				—	—
321a		—	—	—	—	—	—	—	—				—	—
321b		—	—	—	—	—	—	—	—				—	—
174		C.	C.	C.	C.	C.	C.	C.	C.				—	—

* This and the following tests were made at a later date.

the highest concentration and rises to a maximum to fall again. The older strains present the usual features of complete sedimentation with clarification of the supernatant fluid in the high concentrations, and gradual increase of clouding of the fluid in the lower ones. The tables indicate a close relationship between Strains 67, 256, and 317, if not identity, as the word is used in establishing a definite species of bacteria. Furthermore, the calf strain, No. 174, shows no agglutination affinities whatever with the fetal strains or with the other calf strain, No. 321.

SUMMARY.

Twenty-two fetal and two calf strains of spirilla have been studied chiefly with regard to the problem of identity. Twenty-one fetal strains are probably specifically the same. One fetal strain differs slightly from these, but in its agglutination affinities it belongs to the same group. Of two strains isolated from calves one has definite agglutination relations with the fetal strains, the other none. In the morphological and biological characters so far investigated all the strains agree closely with one another.

EXPLANATION OF PLATE 16.

Magnification, × 1,000.

FIGS. 1 to 3. *Vibrio fetus* (No. 159) stained to show the polar flagella.

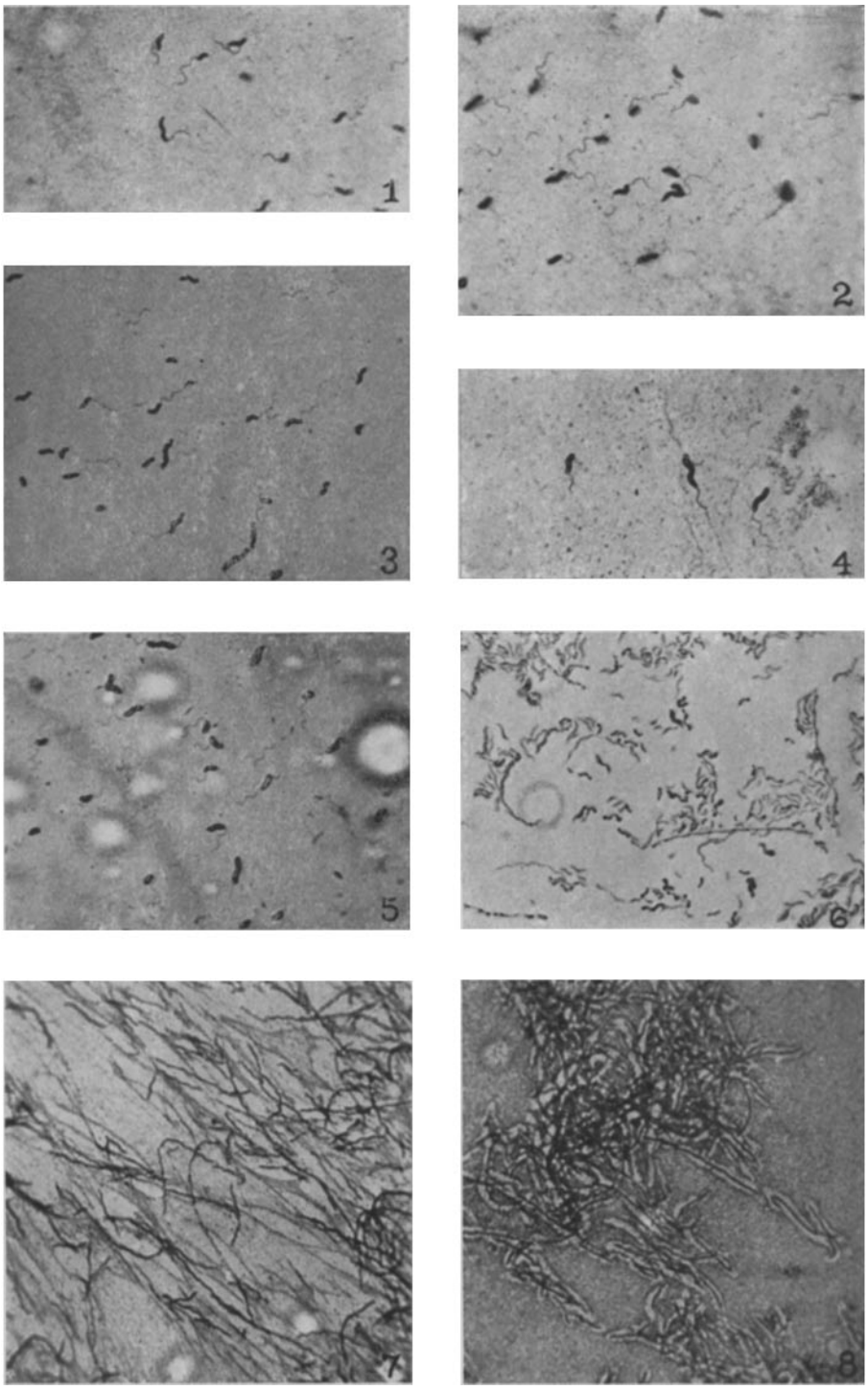
FIG. 4. *Vibrio fetus* (No. 213) stained in the same way.

FIG. 5. Spirillum from a calf (No. 174) stained in the same way.

FIG. 6. Preparation from a culture of *Vibrio fetus* (No. 290), 10th transfer, showing long and short forms, some stained deeply and with granules, others more faintly. Dilute aniline water gentian violet.

FIG. 7. Preparation from a culture of *Vibrio fetus* (No. 333) showing long forms. Stain as in Fig. 6.

FIG. 8. Another field of the same preparation showing some capsular substance, unstained, sheathing the long forms.



(Smith and Taylor: Morphology and biology of *Vibrio fetus*.)