SOME STUDIES ON THE MORPHOLOGY OF B.COLI

by

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For years the majority of bacteriologists have accepted without question the conception of bacterial morphology established by Cohn and Koch. The idea that bacteria reproduce by binary fission only,that each given cell results from the division of another very similar in size and shape,and that they are organisms of more or less fixed morphological types has prevailed in spite of some opposition even early in the development of bacteriology. So the tendency has been to disregard observations of various and bizarre forms,considering them either as "involution forms" and of no possible significance or as purely contaminations. Perhaps a partial explanation for this tendency may be found in the fact that what bacteria do has been of much more interest than what they are,hence this phase of the subject has been somewhat neglected.

Perhaps the earliest opposition to the monomorphic idea of Cohn and Koch was voiced by a contemporary, Nägeli. This worker contended that all fission fungi were capable of transmutation from one morphological state to another and that this was accompanied by various biochemical and perhaps biological changes.

But such a radical idea could not be accepted as a substitute for that of monomorphism and little regard was given it or to the efforts of his followers, notably Hueppe and Kruse. With the work of Massini and of Neisser on B. coli mutabile the terms "mutation" and "mutant" were introduced into bacteriological nomenclature, they having persisted until the present time and having been applied to every form of bacterial variation with utter disregard as to the permanency of the change or to its relation to the original organism.

The idea of cyclical development as applied to bacteria was ushered in probably by Fuhrmann about 1907, but it was not until later that it received any great amount of attention from the standpoint of morphology. Löhnis and Smith (1916) described a rather complex cycle occurring in the life history of Azotobacter. From these and some studies made later on Azotobacter and various other organisms (1923) they have postulated that bacteria pass through a definite life cycle consisting of organized phases and one unorganized phase. The unorganized phase presumably results from the fusion of the cell contents of two or more organisms of a given morphological type, forming thus a mass of living protoplasm from which may develop cells of most any morphological type depending somewhat upon the environment and certain other factors.

Thus the rejuvenescence necessary for the continuation of the species through the mixing and re-arrangement of the chromatin material of the cells is brought about. In this way morphological types which correspond in a general way to the genera Micrococcus, Pseudomonas, Bacillus, and Mycobacterium may be developed from a single strain of Azotobacter. Kellerman and Scales (1917) have reported their studies on seventeen strains of B. coli in which they observed all the stages of a life history as described by Löhnis and Smith except the formation of spores. Jones (1920) attempted to duplicate the results of Löhnis and Smith on Azotobacter and confirmed their conclusions as regards the occurrence of a rather complex life cycle in these organisms, but failed to find any indications of spore formation. He suggested that their observations of conjunction in the Azotobacter were probably examples of incomplete fission.

Smith (1918) found an actinomyces-like organism in the pneumonic lungs of calves which assumed a bacillary and a coccoid phase during growth on different media, these being so entirely different as to suggest different organisms. He assumed that the coccoid phase was perhaps a kind of spore-like stage in the life history of the organism.

Mellon has made an extended study of pleomorphism

and bacterial heredity, assuming throughout most of his work the existence of a rather complex life cycle in bacteria. A brief consideration of each of his discussions will be given. In a study of the diphtheroids (1917), among which were some highly pleomorphic strains, he apparently found a close relationship between these organisms and the streptococci, their morphology in certain phases being strikingly similar to that of the streptococcus. With a fuso-spirillary organism (1919) various phases in a life history were observed including filamentous, coccal, filterable, bacillary, and gonidial phases. These various phases he assumed to be responses to given environmental stimuli but the quality for producing such changes resided within the bacterial cell. From a study of colon-typhoid organisms (1925) he has traced the stages of a process which parallels that of isogamic conjugation and zygospore formation, a process upon which he has laid much emphasis. Two organisms by gradual fusion of their proximal ends give rise in time to a large round body from which the ends of the rods disappear. The resulting zygospore may germinate under proper conditions unless it has undergone too extensive changes, and the direction of the germination depends in a large measure on environmental factors. New characters could be acquired on germination and the process become

a mechanism for the dissociation of the pleomorphic cycle as it occurs naturally in bacteria, in this way giving rise to bacillary or coccus forms of the same organism. Other forms of reproduction were recognized in his study but this form was given greater emphasis. As further evidence of a pleomorphic life cycle of B. coli, Mellon has used his work in the production of B. coli mutabile from a "normal" coli strain (1926 a). In an attempt to show that the Biogenetic Law of Haeckel is applicable to bacteria (1926 b), he concluded that the various morphological changes apparent in the life cycle of an organism were points of potential stabilization and that if realized would constitute its phylogeny. The diplococcoid stage of B. coli which may continue as a permanent form under certain conditions, was taken as an example of such stabilization. That the filterable stages of B. fusiformis might be the virulent forms of this organism and associated organisms was suggested in one of his studies (1926 c). That from the filterable phase may arise organisms of entirely different morphology might lead to the conception of filterable viruses as the virulent filterable phases of certain non-pathogenic organisms was suggested.

Bergstrand (1920) attempted to show that bacteria should be classified along with the Fungi imperfecti

since they grow frequently as buds, either large or small, cocci and rods develop one from the other, and they form structures if cultivated in the right way which might be considered analogous to the chlamydospores of the higher fungi. In a later paper (1923) Bergstrand has described his observations with a strain of Corynebacterium which was obtained from Mellon, and with which he was able to follow the development of reproductive bodies similar to those described by Mellon, but from which he was unable to show any bursting of the body with the release of motile corpuscles. To the reproductive bodies he gave the name "chlamydospores".

Hort (1920) was able to show that binary fission is not the only means of reproduction in bacteria, that gemmation or budding, branching, and endogenous means such as gonidia formation and the formation of chlamydospores might also be found. He assumed that the so-called "involution forms" in old bacterial cultures were in reality the resistant organisms by which the strain is maintained in unfavorable conditions, and that the production of such forms under adverse conditions could not be accomplished from a culture of purely "normal" forms.

Wade and Manalang (1920) using three strains of B. influenzae grown on beef infusion agar containing varying concentrations of sodium chloride were able to

demonstrate filamentous growth and conidia formation. The conidia were developed either as direct transformation of bacillary elements or as terminal buds or as simple lateral buds. They have noted also the formation of structures which they call "cryptoplasmic masses" in these organisms and have suggested the possible relationship of such forms to disease.

Almquist (1922) observed remarkable forms in cultures of B. typhosus, B. diphtheriae, and Sp. cholerae by growing them on a drying substrate at 10-14 C. He observed most of the forms described by Löhnis and Smith including globoid forms and "plasmodia" , forms which were analogous to the amorphous stages described under the name "symplasm" by the latter authors. Filterable forms or "microconidia" were observed also, forms which in the case of B. typhosus were cultivable. Such a form maintained its state as a very small, filter passing organism even after eleven years of cultivation. The name B. antityphosus was given the organism, its close relationship to the original B. tyhposus he claimed as a result of a series of agglutination tests. The suggestion was made that such forms might represent the penetrating stage of the organism in the production of typhoid fever. The growth on a drying substrate seemed to be a means of rejuvenation of all strains studied and

resulted in an increased virulence of the organisms, this being a more effective means of increasing virulence than animal passage. Using the same means of cultivation he grew strains of B. typhosus and B. dysenteriae together with the formation of a hybrid strain to which he gave the name B. diploides (1924). This possibility plus the demonstration of nuclei in bacteria were given as evidences of bacterial sexuality.

Tunnicliff and Jackson (1925) described a small, Gram negative, pleomorphic, anaerobic organism isolated from a tonsillar granule to which they gave the name B. gonidiaformans. During the course of its development bacilli, small and large coccoid forms, filaments both straight and wiry, were observed. It reproduced by simple fission, budding, and by the production of gonidia and gonidiangia, the arrangement of the chromatin material varying in each case.

Enderlein (1925) has been able to demonstrate the presence of nuclei in most of the bacteria which he has studied, considering this a basis for the postulation of sexual processes and cyclical development in these organisms. In cultures of the cholera vibrio he has observed forms similar to those described by Lohnis and Smith for Azotobacter, but in addition found what he claimed was a true sexual process. Cultures of the

vibrio containing gonidia gave rise after a month at room temperature or in a shorter time in the sunlight to small filter passing bodies which he called "gonites". The "gonites" could not be grown on solid media, but if they were transferred to broth they developed and became differentiated into "spermites" and "oites", the former of which was motile and which eventually fertilized the "oite". The fertilized "oite" could then develop into the normal vibrio. The same processes could not be followed with other organisms, a fact taken by Mellon (1925) to indicate that zygospore formation or some other form of rejuvenation obtained in other organisms.

Hadley (1927) has reviewed the literature rather adequately on the whole problem of bacterial dissociation and includes not only the morphological aspects, but the cultural, biochemical, serological, and biological aspects as well. While his own work has been largely from the cultural and serological approach, he seems to favor rather strongly the idea of cyclical development in bacteria. He indicated, however, that the morphological studies alone could progress but little in the solution of the problem and that they needed to be correlated with studies from the other aspects.

Stewart (1928) working from a purely cultural standpoint on the variations in B. coli and on the production

of B. coli mutabile conceived a somewhat different form of cyclical development in bacteria. He postulated that bacteria pass through a two-phase cycle, an asexual and a sexual phase; that the sexual phase occurs on the down-stroke of the wave of asexual fission; that it takes place as a form of autogamic conjugation in which the cells undergo a process of interchange of cell substance immediately before the division is completed. By means of such a mechanism he postulated that the Mendelian Laws were applicable to bacteria.

Recently Evans (1929) has discussed the conception of bacterial life cycles and has shown from a theoretical standpoint the reasons for expecting them to occur. She held that life cycles are a law of nature; that equally wonderful changes take place in the animal and plant world as those claimed for bacteria; that to not expect such complex cycles in bacteria is but to relegate them to a position apart from the rest of the living world. She has used as an experimental basis for her conclusions some of her work on the production of encephalitus in rabbits. From this study she concluded that B. subtilis was probably the resistant resting stage of more pathogenic organisms, and she attributed to its filterable phases the production of encephalitus. It is interest-

ing to note that Tang and Ruz (1929) attempted to confirm her experimental results on encephalitus in rabbits, but were unable to do so, coming to the conclusion that bacteria have no part in the causation of encephalitus in rabbits.

Such is the evidence from the advocates of some form of cyclical development in bacteria. But there is other evidence to be considered, the work from those who have recognized certain morphological variations but who fail to find sufficient grounds for the postulation of sexuality or complex cycles.

Rosenow (1914) has reported some rather remarkable direct transformations of hemolytic streptococci into Strep. viridans, and the transformation of the viridans type into pneumococci by cultural and biological means.

Sherwood (1917) noted some remarkable changes in the morphology of certain strains of pleomorphic streptococci, but was unable to control the factors for their production adequately enough to form very definite conclusions as to their significance. Lowered oxygen tension, and partial dessication of the surface of the medium with the crystallization of salts were offered as contributing factors in their production. He was unable to show any relationship between these organisms and members of the diphtheroid group as was observed by Mellon (1917)

Clark and Ruehl (1919) studied seventy strains of organisms representing thirty-seven different species and found with two exceptions that bacteria normally pass through certain changes in morphology in their growth, especially during the lag and logarithmic phases, returning to "normal" by the end of twenty-four hours usually. "Involution forms" were frequently observed in old cultures. The two exceptions to the general rule were B. mallei and B. diphtheriae, which instead of becoming larger in the early stages of growth were smaller, growing larger as they grew older. No explanation was offered for these exceptions. They suggested that the increased size during the early stages was due to a transfer of metabolic products from the original culture in which they were in sufficient concentration to produce some cell death but which upon dilution in the new medium became cell stimulants instead. They were unable to find any evidences of life cycles in any of the organisms studied.

Reed and Orr (1923) reported the production of various morphological changes in B. influenzae by cultivation upon media either acid or alkaline to the optimum range. The changes which occurred were noted especially in young cultures, and became more marked according to the degree of acidity or alkalinity of the medium. Single

cell cultures of the variants failed to breed true even after several transfers upon the medium used in their production, in every case a reversion to the "normal" form occurring upon transfer to media with the optimum reaction. They concluded that the "involution forms" were simply responses to an adverse condition, the most important factor of which was the H-ion concentration of the medium.

Without reference to any morphological changes that might take place, Sherman and Albus (1923) showed that young cultures of B. coli were apparently less resistant to mild destructive influences than older cultures, thus indicating that some kind of rejuvenescence resulted from simple binary fission resulting in a physiological youth of the organisms.

Gardner (1925) making use of transfers from old cultures to plain agar was able to demonstrate by means of a modification of the Hill hanging block reproduction by branching in at least seven species of bacteria, - B. coli, B. typhosus, B. dysentery Flexner and Shiga, Sp. cholerae, B. paratyphosus B and C, - there being roughly one branched form to a thousand "normal" forms. Multiplication from each of thethree points was observed in each case with subsequent reproduction of "normal" organisms. He suggested that in addition to being another means of multiplication, that branching might

be correlated with some physiological function, as had been suggested for B. radicicola in the utilization of free nitrogen.

Henrici (1925) studied closely the forms occurring during the growth of the cholera vibrio for periods up to ten days, and noted an increase in size for about thirty-six hours after which there was a gradual decrease in size with the appearance of abnormal forms. He assumed that the abnormal forms were injured cells upon which the forces of surface tension and osmotic pressure had manifested themselves in the weaker portions of the cells, rather than as any evidence of life cycles. Later (1926) he studied B. megatherium and found that there was an increased size during the lag and logarithmic phases accompanied by certain other cellular changes, which he considered as indicating a change from the resting cell to the embryonic cell type. From studies on B. coli, Henrici (1926 b) correlated the appearance of abnormal forms occurring in agar media containing sodium chloride or calcium chloride with the autolysis of cells in the death phase. He supposed that "normal" cells maintained their form by a certain degree of rigidity of the cell membrane, but that with death of the cell and beginning disintegration there was a weakening of the cell wall, thus allowing the forces of surface tension

and osmotic pressure to act in giving rise to abnormally shaped organisms. In this work he has made use of a differential staining method (1923) which distinguishes living and dead organisms, and has correlated his results with plate counts.

Orowell (1926) reported the production of various Westbrook types of B. diphtheriae one from the other and claimed that the type had little or no bearing on the virulence of the organism. This may or may not be taken to bear upon the problem of morphological variation.

Beauverie (1928) studying living and stained preparations of Azotobacter chroococcum, observed the formation of resting bodies by the condensation of cellular elements, but was unable to find the sporulating rods or the spore free rods, the gonidia, or the hard walled cells described by Lohnis and Smith. Conjunction appeared to be nothing more than incomplete fission, and "symplasm" appeared to be masses of degenerated cell contents.

Aside from the purely morphological standpoint there has been much work done on the problems of bacterial variation and mutation, but inasmuch as this work is to be restricted to a study of morphology alone, it does not seem necessary to include here reviews of that literature. The whole problem has been excellently reviewed by Hadley in his Monograph (1927).

It seems apparent from a consideration of the literature reviewed that the question of morphological variation is still unsettled, particularly as regards the significance of the forms observed by various workers. The existence of bacterial life cycles and the significance of the so-called "cyclostages" needs further experimental confirmation or some adequate interpretation given to some of the observations that have been made if they are not to be accepted. As luring possibilities which might be become realized providing the existence of sexuality and life cycles could be established beyond question , the following have been offered:

(1). A more adequate classification of bacteria based on the complete knowledge of a species in all its phases or "cyclostages".

(2). Light on certain epidemiological problems, particularly the carrier problem.

(3). Better understanding of group agglutination, double antigens, etc.

(4). Better understanding of the nature of filterable viruses and bacteriophage.

The problem evidently cannot be solved from the standpoint of morphology alone, but some work in this line seems necessary and presents some interesting aspects.

It is the purpose of this paper to record some of

the variations of morphology observed in living and stained preparations of a strain of B. coli, to show the possible relationship of these various forms, to attempt an explanation on the basis of such observations, and to show the extent to which the same forms may be produced in other strains of B. coli.

EXPERIMENTAL METHODS:

Organisms:

For the more detailed study in this work a single strain of B. coli has been used, designated as W23. It is a typical sucrose fermenting colon organism, producing smooth colonies on plain agar plates, and is "normal" in morphology on plain agar as may be seen in Plate I, Fig. 1. Twenty-two other strains of B. coli have been studied in less detail during the course of the work, all except four of which are old stock cultures. In Table I these strains are listed with the source from which they were isolated. The last four strains listed are the freshly isolated strains.

For purification of the strains they were streaked and re-streaked on eosin-methylene blue agar plates, the colonies from the latter then streaked on plain agar plates. The colonies picked from the plain agar plates have been used as the pure line strains in each case except W23.

TABLE I

Strain	Source	Remarks
W23	water	sucrose fermenter
F1	feces	" negative
F2	"	slow lactose and sucrose fermenter
F4	"	" sucrose fermenter
F10	"	sucrose fermenter
F12	"	" negative
F17	"	" "
F18	"	" "
F22	"	" "
F29	"	" "
F50	"	no gas in sucrose
F61	"	sucrose negative
1	"	" "
4	"	" "
5	"	" "
7	"	" "
8	"	" "
F.B.	"	" fermenter
N.5	"	no gas in sucrose
K.1	cow feces	sucrose negative
K.2	" "	slow sucrose fermenter
O.1	oysters	" "
O.6	"	" " "

This organism has been re-streaked several times during the course of the study and it was finally considered necessary to isolate single cells by means of the Barber Pipette Method, one cell of which was cultivated as the pure line strain of the organism.

Media:

Considerable difficulty has been encountered in getting a medium to produce consistent results, so that it has been necessary to constantly modify the medium with which the work was started. A liver infusion agar has

been used as the base medium throughout, however. As first used, this medium contained 1% sodium chloride and 1% Bacto peptone, the reaction adjusted to about pH 5.8-6.0, but this failed to produce adequate results and has been modified several times. Best results have been obtained with the liver infusion agar to which no peptone is added, sodium chloride added to a concentration of three and five percent, and the reaction adjusted to about pH 5.2-5.4 before sterilization. Throughout this work these shall be designated as 3% and 5% salt liver agar, and any deviations from the above will be described. Drying the surface of such a medium enhances its activity with the organism used.

Another medium used in the latter part of the work consists of plain meat extract agar containing 4% dextrose, and this shall be designated simply as dextrose agar. The reaction of the medium was adjusted to pH 5.8-6.0 .

Methods of study:

Attempts have been made to study in detail the strain W23 by means of living and stained preparations . For living preparations Hill's hanging block method has been employed and observations made with a warm stage. Camera lucida drawings have been made in some cases. For a study

of stained preparations the organisms have been grown on slants of the medium made as nearly alike as possible, and at various intervals smears made from them or from suspensions made of the organisms, stains being made with dilute fuchsin. The Henrici Differential Method (1923) has been made use of in some cases. Photomicrographs were attempted but they were not entirely successful.

EXPERIMENTAL RESULTS:

Attention was attracted to this study when upon transfer of the strain of B. coli designated as W23 to the liver infusion agar as described, very large rods similar to those in Plate I, Fig. 2 were found instead of the "normal" small rods. The fact that all the organisms from the preparation were of the same type seemed to indicate that this was not contamination. Transfer to plain agar gave a reversion to "normal" cell types within twenty-four hours, none of the large rods being found in the culture. These tests were repeated several times with similar results, but no permanent changes could be detected. Occasionally peculiar formations were observed but they did not appear consistently enough to be of much significance. Other organisms upon the same medium gave somewhat similar results, though not all of them did so, nor did any of them show quite the changes observed

with the colon organism. The organism was then purified as described.

Stained preparations:

Several duplicate transfers of W23 thus purified were made from a twenty-four hour broth culture to each of the following media: plain meat extract agar, pH 7.2-7.4, with and without 1% lactose, and liver infusion agar, pH 5.8-6.0, with and without 1% lactose. The cultures were incubated at 37 C., tubes removed at the end of 3, 6, 12, 24, and 48 hours, suspensions made in sterile water, and smears made from the suspensions which were stained with dilute fuchsin. In the plain agar cultures a slight increase in size was noted during the early hours of growth with a gradual return to "normal" within twenty-four hours. Somewhat the same changes were found in the lactose plain agar except that the organisms became more plump and maintained that condition throughout, though with the same decrease in size as noted above. A marked increase in size was observed in the cultures on the liver agar, both with and without lactose, these forms remaining large throughout with possibly a very slight decrease in size after the first few hours. Cells from either of the latter cultures averaged roughly 10-20 microns length and 1-2 microns or more in width. Occasional vacuoles appeared in some of the rods

in such locations as to sometimes suggest the appearance of spores, but it has been assumed that they resulted from changes of a degenerative type. In the liver agar cultures considerable cell fragmentation was evident at the end of forty-eight hours.

Upon a second batch of medium made similarly to that described for the liver medium without lactose, several duplicate tubes were inoculated from a twenty-four hour culture of W23 which had been re-streaked after the above tests. At one hour intervals from the third to the eighteenth hours of growth, three tubes were removed, the growth suspended in sterile water, and both dilute fuchsin and the Congo red method of Henrici were used to stain the smears made from these suspensions. The same was done at the end of twenty-four hours and forty-eight hours of growth. Again a decided increase in size was observed in the first few hours of growth, but instead of the organisms remaining large they returned to a size slightly larger than "normal" after twenty-four hours. Considerable fragmentation of the cells was evident at the end of forty-eight houts. As stained by the congo red method slightly swollen forms similar to those described by Henrici (1925 and 1926 b) appeared after about ten hours culture. Occasional branched forms were noted in

both types of preparations but were too few to be considered significant. These results seemed to indicate that the organisms had lost their capacity to react to the medium to a great extent.

It was evident that some modification in the medium was necessary in order to obtain results even as pronounced as those first obtained. Bile salts in varying concentrations, glycerol, phosphates and other substances were added but with no striking results. Equally inconsistent were tests made with the reaction of the medium adjusted to pH 4.4, to pH 5.0-5.2, and to pH 9.0 . Occasional forms were observed, which were striking enough but inconsistent in their appearance. Attempts at varying the concentration of sodium chloride and adding no peptone met with somewhat more consistent results. Best results have been obtained with 3% and 5% salt liver agar as described.

Repeated tests have been made with both of the above media and smear preparations made at varying periods in the growth of the organism. From these preparations generally similar results have been obtained, though several factors have been observed which seem to affect the forms resulting from growth on the media. Among these may be mentioned the drying of the surface of the medium, the size of the inoculum, the age of the culture from where

transfer was made, and lowered oxygen tension.

In practically all cases a marked increase in size has been observed after culture of the organisms on these media, though the forms found and the changes observed varied considerably under different conditions. In fresh 3% salt liver agar before any drying of the surface took place the early increase in size of W23 has been followed after about 12-14 hours by the production of branching forms, most of which were of the three-point type, but some of which have been observed to have several definite branches. At this period as many as 10% to 25% of the organisms in a given preparation have been observed to be branching or beginning branching. Afterward a marked increase in length resulted in most cases, until at the end of twenty-four hours filamentous forms predominated in the cultures. Such forms may persist until autolysis takes place or they have been observed to segment to form shorter rods under certain conditions. If, however, the surface of the medium is allowed to dry considerably, no such regularity of changes takes place. If conditions permit or favor growth after a short lag period, there results the usual increase in size with the later elongation into filaments. At the end of twenty-four hours the filaments and long rods are usually accompanied by small and large round globoid bodies and by considerable

cell debris. There seems to be a progressive autolysis of the organisms after twenty-four hours and it becomes almost complete at the end of a week, as is indicated by the frequent lack of growth upon transfers from such cultures, or if growth takes place it does so in the form of scattered colonies even if the inoculum is heavy.

Similar changes take place in cultures on fresh 5% salt liver agar previous to drying the surface. But after partial drying of the medium the forms may differ from those observed above. Under certain conditions of growth, "mass" forms appear either as the only forms in a culture or along with other more common forms. These "masses" appear as deep staining, irregularly shaped, much enlarged forms similar to those shown rather indistinctly in Plate I, Fig. 3. They apparently develop early in the growth of the organism or in cultures in which little growth may be seen. That they may eventually give rise to rod forms is suggested from Figs. 3 and 4, Plate I, both representing the same culture, Fig. 3 after 24 hours in which very little growth was evident on the slant, and Fig. 4 after 48 hours and considerable growth. If conditions are such that no further growth takes place or if the further growth is inhibited, the "mass" forms persist. Such conditions have been observed in certain anaerobic cultures of the organism, though not in all, and similar conditions have been observed in aerobic

cultures. Occasional forms have been observed similar to those described by Mellon as zygospore formations, the larger globoid bodies being similar to the completed zygospore. No further evidence of the significance of these forms or of their later development could be determined in the cultures. After twenty-four hours the predominant forms are the filaments and in these are frequently found very large swollen areas either terminal or along the filament, more often the latter. Suggestive forms may be seen in Fig. 7, Plate II, and in Fig. 8 it is even more pronounced. The enormous size of the structures in the latter case should be noted. But all these forms revert to the "normal" cell type within twenty-four hours after transfer to the usual laboratory media. Upon transfer of the filaments to plain agar, one of the earliest changes observed is the segmentation of the structure into several shorter rods which subsequently undergo division and decrease in size.

In a series of duplicate tubes of the drying 5% salt liver agar transfers were made from a twenty-four slant culture of W23. This means was used to make transfer to prevent the addition of moisture to the slant surface. It is evident that the size of the inoculum varied considerably, but since the aim was to produce the changes of morphology, it did not seem necessary that they all be inoculated with the same amounts of material.

Dilute fuchsin stains were made at the end of ½, 6, 12, and 24 hours of growth. Stains were made by the Henrici method in certain of the cases. Little growth was evident on the slants until after twelve hours had passed, though the stains indicated that some growth had taken place at this time. The early changes include an increased size with the formation of slightly pointed or fusiform rods which soon gave rise to very large, irregularly shaped forms similar to those shown in Fig. 8. Later the filaments became more slender, though irregular in shape and wiry, and some few round cells of the type described above appear. In all cases a progressive autolysis was apparent from the stains, until after a week there were few staining organisms in any given preparation, and transfer to other media gave only very scant growth even with heavy inoculum. The various forms including the globoid forms were apparently living organisms as shown by the Henrici stain, but no evidences of further development wase observed in the case of the globoid forms.

It has been found that transfer of W23 to the dextrose agar medium results in the formation of small coccus-like forms, as shown in Fig. 5, Plate II. Return to the "normal" morphology takes place after transfer to plain agar. If transfer is made to either of the liver media, the coccus forms increase in size giving rise to yeast like forms and irregular masses which eventually

give rise to the usual filaments and rods.

Results from other strains :

To determine whether the property of variability is common to other strains of B. coli under similar conditions of growth twenty-two other strains have been studied. After purification by plating methods as described and the determination of cultural characters, they were tried first on fresh 3% salt liver agar, transfers being made from plain agar cultures in each case. Later the same strains were transferred first to the dextrose medium and then to fresh 5% salt liver agar. A third series was attempted using 5% salt liver agar after considerable drying of the surface had taken place, and transfers were made from three day old cultures in this case.

In the first case smears made at the end of twelve and twenty four hours and after a week showed that strain No. 1 produced changes similar to thod e described for W 23 on this medium, while the changes occurring in the rest of the strains were considered of no importance. From the second series smears were made from the dexrtose agar cultures at the end of twelve and twenty-four hours an the organisms compared in such preparations with those found in plain agar cultures of the same age. Strains

F2, 0.6, F22, F1, and F29 showed a marked variation in size at both intervals, while strains F10, F12, F17, and 7 became uniformly much larger on the dextrose agar and persisted as such. Strains F4, K2, 4, and 5 became uniformly shorter and somewhat coccus-like similar to the cultures described for W23 on dextrose agar. The other strains failed to show any detectable changes. After transfer to the liver agar as stated, strains F29, F18, 7, and 1 became more or less filamentous and swollen and irregularly shaped forms were observed. A considerable increase in length could be noted in strains N.5, F61, K2, and F2, true branching forms being observed to some extent in each. Strains F12, K1, 4, F.B., 0.6, and F4 were found to be increased in size, while other strains failed to produce any noticeable changes under the conditions observed.

Transfers were made from three day slant cultures in the third series in an attempt to inhibit further the early growth of the organisms thus prolonging the lag phase. "Mass" forms were observed in the cultures of strains F 22, F18, K1, F4, 0.6, F.B., and 8 at the end of twelve hours growth, these cultures apparently showing less growth than most of the other strains at this time and even at twenty-four hours. Large filamentous forms were found with strains F2 and 5. Increased size with

some branching and occasional large "abnormal" forms were observed in most of the other cultures. In cultures of 0.1 and F50 very slight changes, if any, were found. After a week's growth most of the strains had undergone considerable autolysis, though most of them had a few cells which took the stain sufficiently to be called living forms. In such cultures of strain 4 there were found very long, hairlike filaments, some of which were definitely branched, and on some of which were round terminal swellings.

Living Preparations:

By means of living preparations of W23 attempts have been made to watch the development and fate of some of the forms that were observed in test tube cultures. This study was tried with these considerations in view: first, to determine whether the "mass" forms were living organisms and to record their further development if any took place; second, to determine the role of the branched forms in the multiplication of the organism; third, to determine any possible changes that might indicate a complex life cycle for the organism. The objections are raised to warm stage culture in that they are at best abortive conditions for growth and that the organisms are soon lost in the "log jam" after growth has started. Granted

that this is true, it seems to be one safe way of determining the fate of given organisms, a condition which seems of considerable value when correlated with test tube findings.

In Fig. 1 may be seen the growth and division of a form of the organism of the "mass" type which has been found frequently in the early hours of test tube culture. (see camera lucida drawings at end of discussion) Similar forms may be seen in Figs. 3 and 6 (a, b, c). The substrate in the first two was 3% salt liver agar, but the forms in Fig. 6 were observed on plain agar culture after transfer had been made from a few hours' culture on 5% salt liver agar. Each of the figures show rather remarkable forms of division of the "mass" forms with the production of rod forms of the organism. Observation was continued on the preparation represented in Fig. 6 for a considerable time, final observations being made after 14 hours. Practically all organisms found at that time were of the rod type and with only occasional forms similar to those drawn. The position of these with respect to the other forms in the field seemed to indicate that they were dead forms. A somewhat similar condition is represented in Fig. 7 a, but since 3% salt liver agar was the substrate little growth took place during the time of observation. In Fig. 7 b are shown some of the forms

found in this preparation, these forms apparently resulting from the growth of the small coccus-like forms found in dextrose agar, since the transfer to this was made from an eight hour culture on 5% salt liver agar to which previous transfer had been made from the dextrose agar medium. Stained preparations made at the time of transfer showed many yeast like forms which were apparently undergoing division. It is assumed that the forms shown in both Fig. 7 a and 7 b would eventually produce "normal" forms under proper conditions, though observation of further growth was prevented in this case when the preparation was accidentally broken.

Figs. 2, 3, and 4 show the growth and reproduction of true branching forms, so-called three point multiplication, as found on warm stage cultures with 3% salt liver agar as the substrate. Transfer was made from different conditions in each case. The forms represented from Fig. 2 were transferred from a test tube culture on the same medium and was observed for a short time only. Rod types produced from each of the three points are evident. The same is to be seen in Fig. 3, transfer in this case having been made from a sixteen hour culture on 5%salt liver agar (fresh). Fig. 4 illustrates four different branching forms occurring in the same field of the microscope after fourteen hours culture on the warm stage.

In the early stages of growth no branching was observed, the forms appearing only after about fourteen hours when they were found in considerable numbers. Fig. 9 shows the early hours of growth of one organism on this preparation, in which some elongation and increase in thickness may be seen.

Fig.8 shows the beginning growth phases of the organism after a period of three hours had passed with no growth except that commonly seen in the lag phase taking place. The transfer in this case was made from a three day culture on plain agar to the same medium for warm stage culture, and hence may be taken to represent the "normal" development of the organism during such a period of observation. Some increase in size may be noted in the early gowth with a gradual return to "normal" as shown in the latter part of the figure. No "abnormal" types of any kind were observed in this culture, nor were there any present at the end of twenty hours when observation was discontinued. "Abnormal" forms of any kind have not been noted at any time on plain agar cultures of this organism.

Fig. 10 represents some forms present in a warm stage preparation which might be taken as evidence for zygospore formation. The figure shows the presence of globoid or round forms in various positions relative to one or more rods suggesting forms that have been

described as forming zygospores. Attempts were thus made to determine the fate of such forms and the manner of their formation. In all cases the round bodies have been observed to form from the ends of rods by pinching off as terminal knobs either from single rods or from one of two rods that still remain incompletely separated after simple fission. Thus they would seem similar to the gonidia described by many workers in the manner of their formation. Occasionally they may arise as a small granule from the end of a rod and later increase in size. The formation of these structures and their subsequent positions are represented diagrammatically in the Figs. A, B, and C below. Fig. D shows certain other forms observed at various times which are suggestive of very early zygospore formation. The round

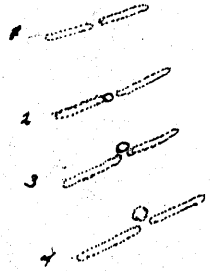

Fig. A

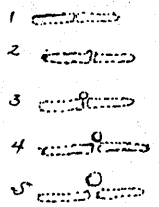

Fig. B

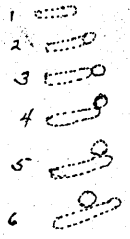

Fig. C

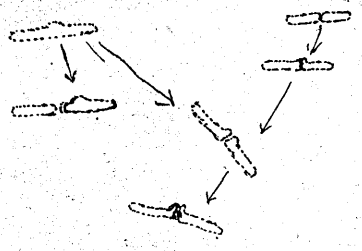

Fig. D

forms arise frequently in conditions under which branching occurs. Further observation of such forms has not revealed completed zygospore formation at any time, nor

has there been any evidence of fusion of the rods observed at any time. While several attempts have been made to determine the further development of the globoid bodies in test tubescultures and on warm stage preparations, yet in no case have they been observed to undergo any change except a slight increase in size and they eventually autolyze. Perhaps under certain conditions of growth these forms might reproduce since they are apparently living forms as determined by the Henrici staining method, but such conditions have not been found in this work.

In most of the conditions under which the organism has been studied except on the dextrose agar and in plain media, fragmentation of the cells has been observed frequently giving rise to small granule forms which might be suggestive of the microconidia described by several workers. Under conditions in which autolysis is not so complete the rods appear as chains of these granules after forty-eight hours or more of culture. All attempts to find evidences of further development of such forms have been unsuccessful, and the fact that very old cultures in which few other forms may be found frequently fail to produce any growth or very scattered colonies seems to indicate that they are dead fragments and in reality degeneration products of the cells.

DISCUSSION:

The occasional observations of rather freakish forms in cultures of W23 very early in this work suggested wide possibilities if followed up. Later studies were considerably disappointing in that no changes could be found except those described by Clark and Ruehl (1919) and by Henrici (1925 and 1926 a and b) as occurring in the usual or "normal" growth of most organisms. However, after considerable effort it has been found possible to produce certain rather peculiar formations in cultures of W23 (B. coli) and certain other colon strains by growth on the media described.

From a consideration of all the observations made three groups of forms seem to stand out, the "mass" forms, filamentous types, and branching and globoid types. A study of the production of "mass" forms has not led to any absolute criterion or method by which they may be produced invariably, but it does seem likely that they result from some inhibitory action in the lag phase of the growth of the organisms giving rise to an abnormal prolongation of this phase. A "normal" increase in size has been observed in the organisms studied by Clark and Ruehl (1919) and by Henrici (1925 and 1926 b) during the lag phase, as well as by other investigators. The explanation for this action has been variously

described. That of Clark and Ruehl has been suggested. Henrici has offered the idea that it represents the transformation of the resting cell to the embryonic cell type. But the mechanism is obscure. In any case it seems probable from this study that the mass forms probably arise through an abnormal magnification of the same mechanism occurring normally in the changes observed in the lag phase. It seems probable that this results from a somewhat selective inhibitory action of the medium on the mechanism of division without an exactly parallel inhibition of the growth mechanism. As evidence for such an idea is the greatly increased size of the "mass" forms and, also, the observations of irregular cleavage of such forms when placed in conditions favorable for their development with the rapid production of rod forms. Whether or not these "mass" forms are similar to those described by Lohnis and Smith (1916), by Almquist (1922), by Wade and Manalang (1920), by Jones (1920), and by other investigators is somewhat questionable. They have described the formation of "symplasm" or plasmodia from the fusion of the contents of several bacterial cells, though it seems probable that in these observations they arise from abnormal growth of single cells. Wade and Manalang do not describe the subsequent development of the forms observed by them with B. influenzae, but

they suggest a possible relationship of such forms to disease. Both Lohnis and Almquist describe the subsequent development of their "mass" forms as a type of organization, in which small reproductive units take form within the mass and are later differentiated into the "normal" forms of the organism or into other forms depending on various conditions. No suggestion is made by them or other investigators which I have been able to find of the cleavage of the entire mass in irregular lines as described in this study.

The second group of forms includes not only the smooth filaments, but the wiry filaments and swollen "monster" forms as well. These usually appear as the dominant types in cultures on the salt liver agar after twenty-four to thirty-six hours, especially if the medium has been allowed to dry somewhat. Their production seems to be peculiar to certain strains, the majority of the strains studied not producing them under the same conditions, though no correlation was possible between this and other peculiarities of the strains. The fact that the filaments and especially the swollen forms usually appear as the last forms to autolyze on old slants might be taken to indicate an adaptive mechanism of the organisms appearing in response to the adverse growth conditions. Such an explanation seems hardly

adequate, however. It seems possible that the same inhibiting factors suggested for the production of the "mass" forms might be effective in these cases also, but the response is different with the more rapidly growing and dividing organisms or in the presence of metabolic products from such growth. One fact seems sure, that both the above types of organisms are not necessarily sterile forms but are capable of further reproduction if placed under favorable conditions.

Perhaps the same mechanism could be applied to the formation of branching organisms and globoid bodies, which appear to be formed under somewhat the same conditions. Because of the regularity of occurrence of the branching forms in cultures usually after 12-14 hours of growth, however, it would seem that some change in metabolism at this time were responsible. Gardner (1925) has suggested that branching might be correlated with the physiology of the organisms he studied. It seems certain that these forms are actively concerned in reproduction since they have been observed to produce rod forms from each of the three points very frequently, in confirmation of Gardner's experimental results.

No further development of the globoid forms has been observed under the conditions of observation,

and hence it seems that they play little part in the reproduction of the organisms. That they may give rise to suggestive zygospore formation described by several workers, notably Mellon, has been shown. It has been assumed that by the fusion of the proximal ends of two rods with subsequent changes zygospores are formed which may germinate under proper conditions. As has been described, the round fonms observed in this study may frequently assume positions with relation to rod forms as to suggest the appearance of such bodies, especially in stained preparations. Other forms similar to the "golf-club" forms described by Mellon have been observed in this work, but instead of resulting from partial fusion of two rods they may arise in either of the ways suggested in Fig. D (page 34). Such structures may or may not be similar to those described by previous workers, but inas much as they present a striking similarity to such forms under certain conditions, some consideration might be given them before discounting their significance entirely in favor of complex life cycles and sexuality in bacteria.

That all strains of the same organism are not equally affected by the same adverse conditions may be assumed from this study. No differential factors in the way of cultural characters have been found by which correlation

might be made with the capacity to produce "abnormal" forms, though agglutination and absorption might reveal such points of difference.

SUMMARY:

Various morphological changes have been observed and recorded with a strain of B. coli. Of twenty-two other strains studied, only one was affected to a similar degree by the same donditions of growth, though it has been possible by modification of the medium used to produce certain changes in most of the strains.

In spite of the fact that long slender rods, coccus-like forms, branching or Y-forms, globoid bodies, large and irregularly shaped "mass" forms, and very long filaments, both smooth and containing swollen and irregular portions, have been observed to be produced by a pure-line strain of B. coli, no evidence for a complex life cycle in this organism can be assumed. That bacterial life cycles do exist is not necessarily discounted by the observations made, yet they may be taken as suggestive and worthy of some consideration before making any too far-reaching conclusions based upon the appearance of bizarre forms found in the cultures of certain organisms.

CONCLUSIONS:

The following conclusions seem warranted from this study:

1. That irregularly shaped "mass" forms may be produced in at least some strains of B. coli, apparently as a result of abnormal prolongation of the lag phase.

2. That such forms under suitable conditions may undergo development into rods.

3. That this development involves a type of cell division not commonly observed in bacterial studies in which cleavage may take place in all directions.

4. That such cleavage suggests a factor which inhibits the mechanism of division with less inhibition of growth.

5. That filamentous forms may be produced under certain conditions of growth, these being either smooth in appearance or present irregular swollen formations.

6. That coccus-like forms may be produced under certain conditions.

7. That branching forms may be produced which give rise to rod forms from each of the points.

8. That these forms are not sterile organisms, but are capable of further development under proper conditions of growth.

9. That globoid forms may be produced either as

terminal knobs or as terminal granules , and may increase in size but do not under the conditions of observation reproduce themselves or other forms.

10. That such forms may become so arranged in relation to rods as to suggest the appearance of "zygospores", but which seems unlikely.

11. That all strains of the same organism are not equally affected by the same adverse conditions of growth, but may under certain circumstances produce certain morphological changes.

12. That the various forms observed cannot be assumed to play a role as stages in a complex life cycle of this organism.

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KEY TO CAMERA LUCIDA DRAWINGS

Magnification... X 1400

Fig. 1. Division of "mass" forms. 3% salt liver agar.

Fig. 2. Division of filamentous and branched form. 3% salt liver agar.

Fig. 3. Reproduction of branching form. 3% salt liver agar.

Fig. 4. (a,b,c,d) Branching forms in single field. 14 hr. warm stage culture. 3% liver agar.

Fig. 5. Division of "mass" forms.

Fig. 6. (a,b,c) Reproduction of "mass" forms on plain agar. Transfer from 5% salt liver agar in which the forms were found.

Fig. 7 a. Growth of "mass" forms. Transfer from 8 hr. culture on 3% salt liver agar, to which previous transfer had been made from dextrose agar culture.

Fig. 7.b. Some of the forms found on the same preparation as 7 a. (apparently resulted from growth of coccus forms.)

Fig. 8. "Normal" development of B. coli on warm stage Plain agar.

Fig. 9. Early growth of organism on 3% salt liver agar.

Fig.10. Forms showing various positions of globoid bodies relative to rods.

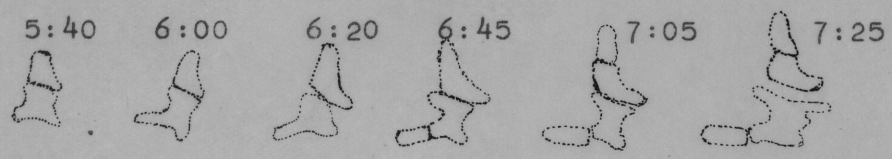


Fig. 1

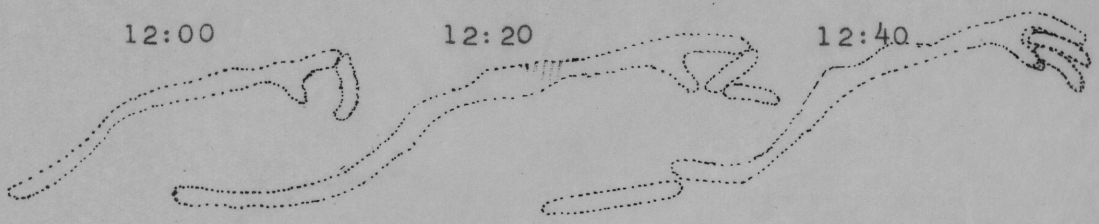


Fig. 2

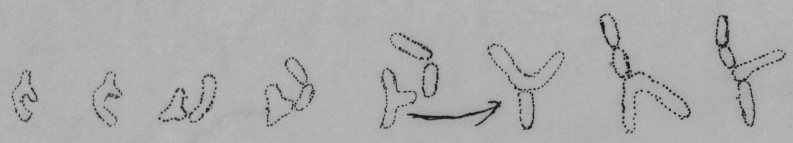


Fig. 3

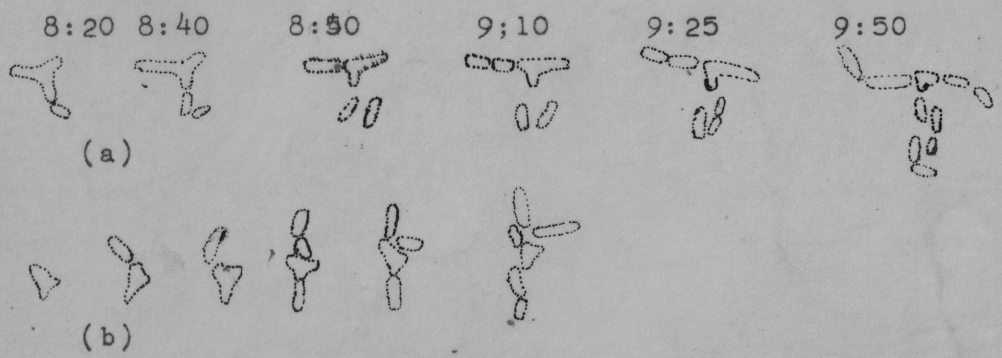


Fig. 4 (a,b,c,d)

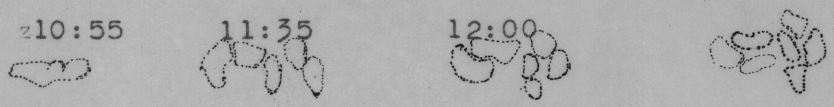
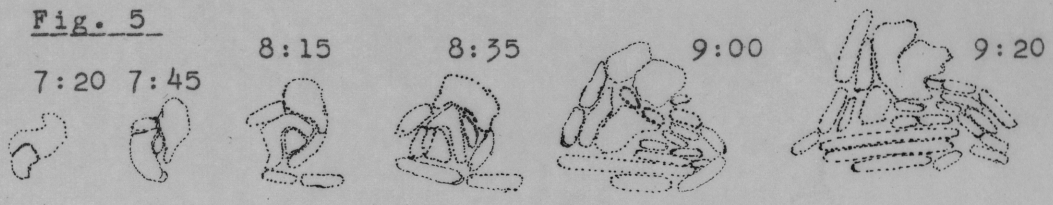


Fig. 5



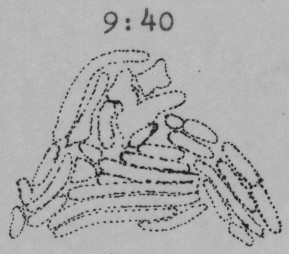


Fig. 6 a

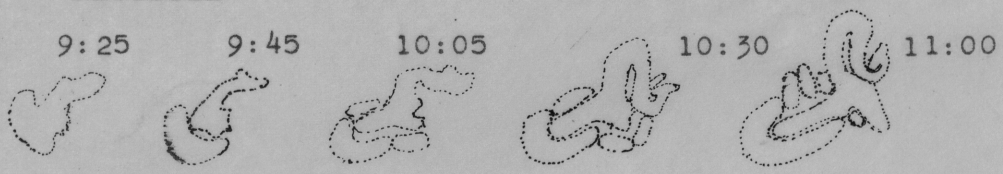


Fig. 6 b

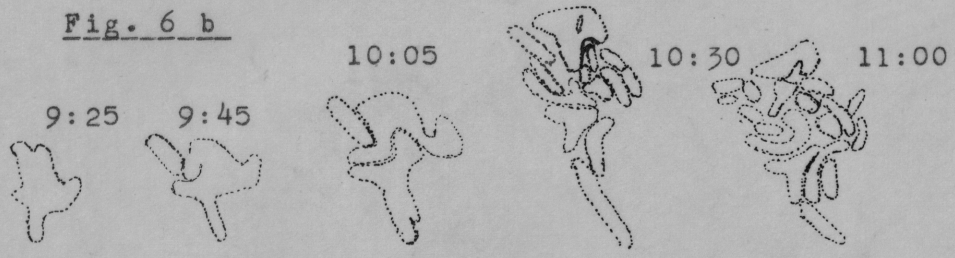
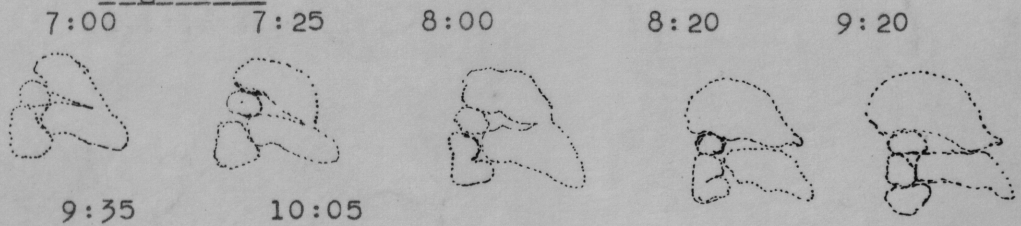


Fig. 6 c



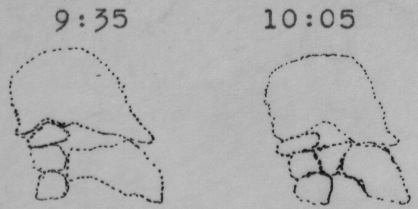


Fig. 7 a

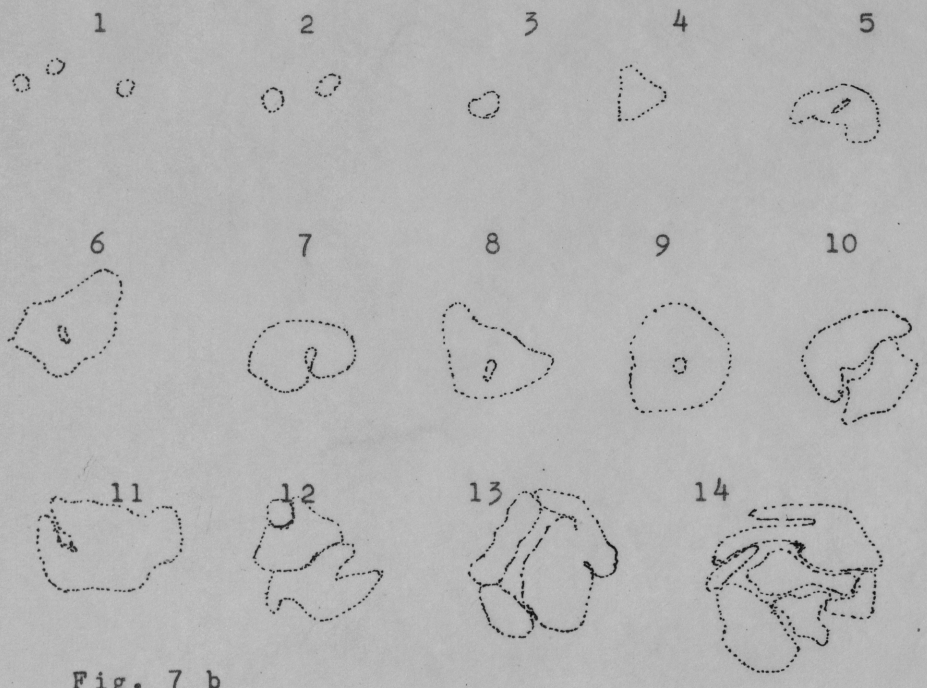

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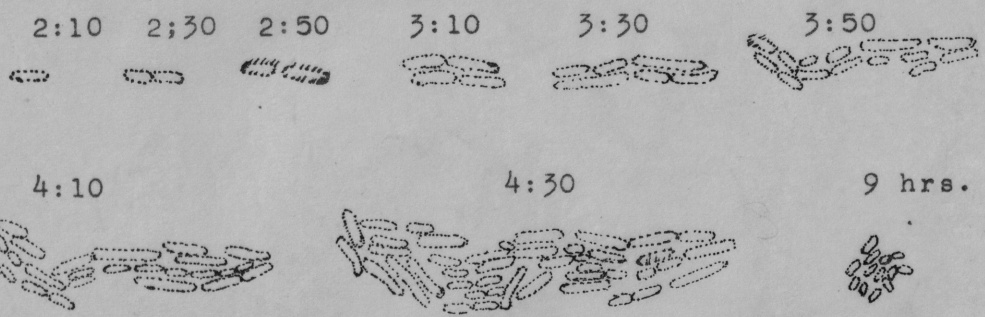
Fig. 7 b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3:50
4:10
4:30
9 hrs. later

Fig. 8

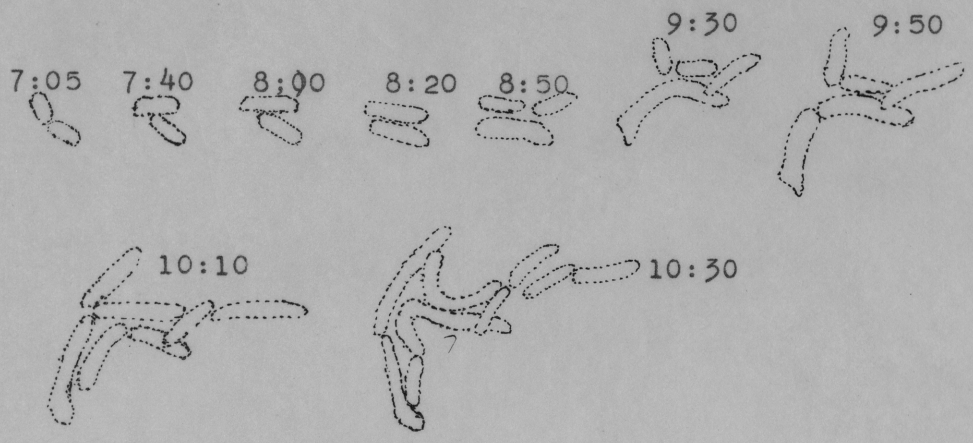

Fig. 9

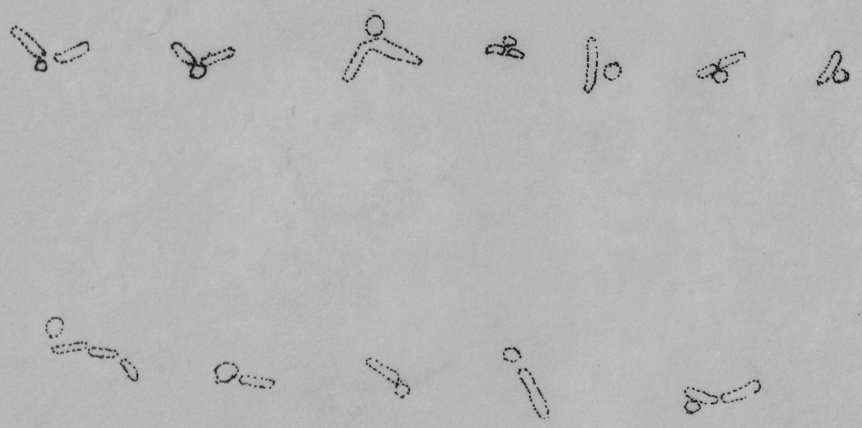

Fig. 10

KEY TO PHOTOMICROGRAPHS

Magnification...X 1000

PLATE I

Fig. 1. The "normal" form of B. coli #23. 24 hr. plain agar culture.

Fig. 2. Large fod forms observed early in study. 24 hr. culture, liver agar.

Fig. 3. "Mass" forms. 24 hr. liver agar (5% salt) culture.

Fig. 4. Same culture at 48 hr.

PLATE II

Fig. 5. Coccus-like forms. 24 hr. culture dextrose agar (4%)

Fig. 6. Branched and some irregular forms. 14 hr. culture, 5% salt liver agar.

Fig. 7. Filamentous forms. 24 hr. culture drying 5% salt liver agar.

Fig. 8. Very large "mass" and irregular forms. 8 hr. culture, 5% salt liver agar.

PLATE I

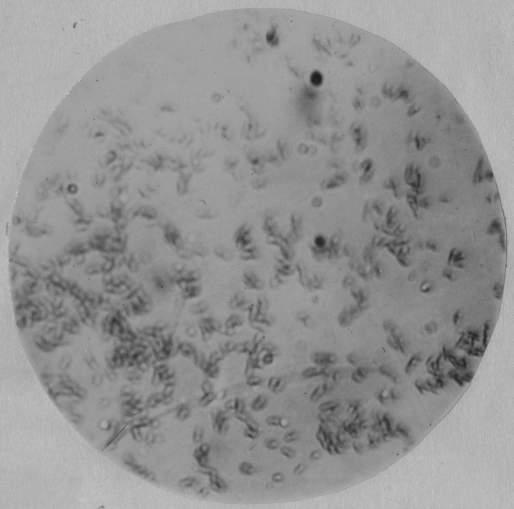

Fig.1 x 1000

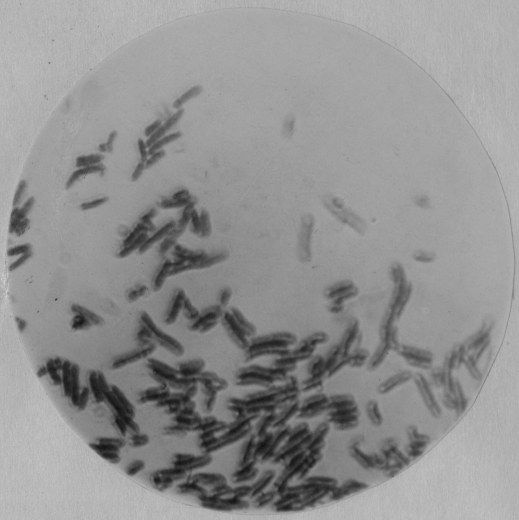

Fig.2 x 1000

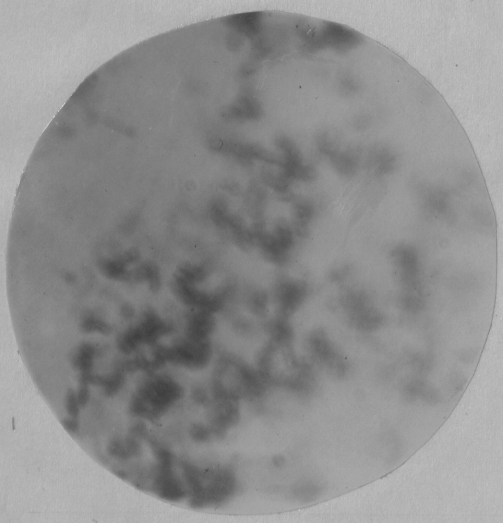

Fig.3 x 1000

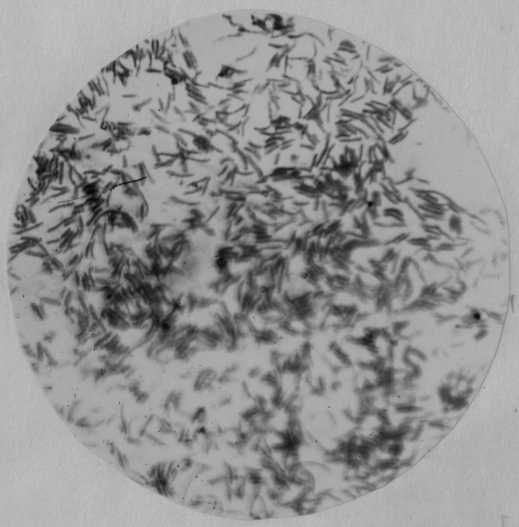

Fig.4 x 1000

PLATE II

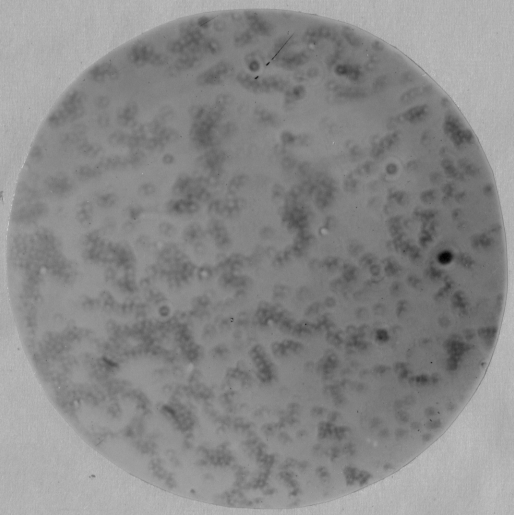

Fig. 5 x 1000

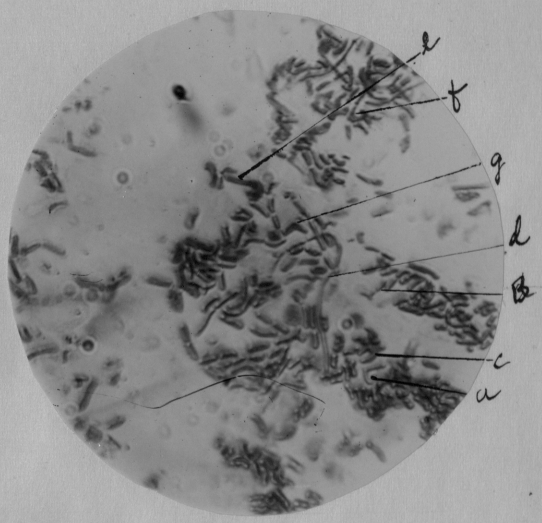

Fig.6 x 1000

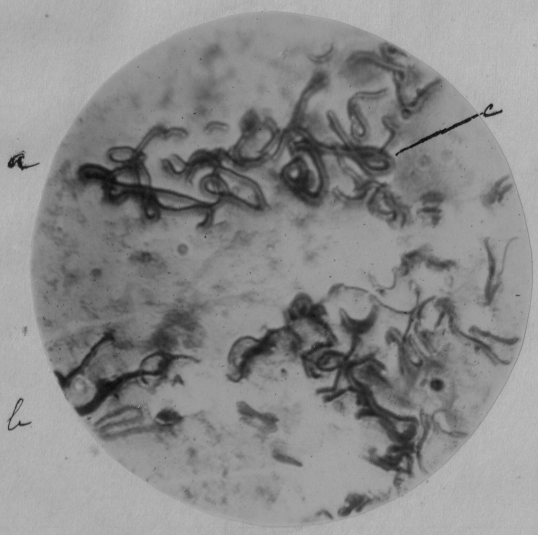

Fig.7 x 1000

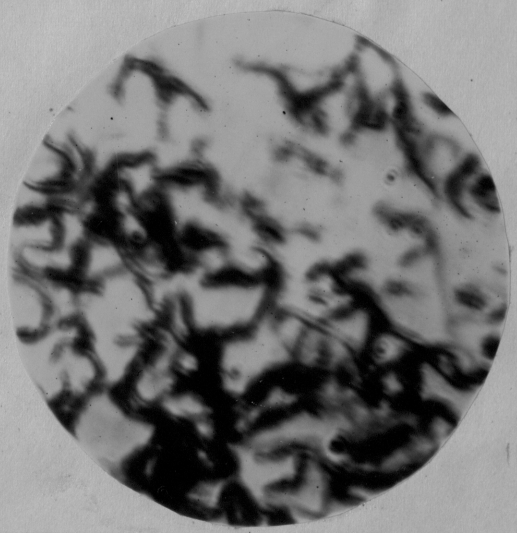

Fig.8 x 1000