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## Observations on certain lactic acid Bacteria of the so-called *Bulgaricus* type.

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With 2 plates, 4 tables and 1 figure in the text.

The attempts to formulate a definite classification of the organisms producing lactic acid fermentations in milk have been rewarded with a more or less incomplete measure of success. There still exists a certain degree of confusion regarding the biological relationships and identities of the more common milk-souring bacteria indigenous to western Europe and America. To the list of organisms already isolated and described there has recently been added those constituting the bacterial flora of the curdled milks of Turkey, Egypt, Bulgaria and other eastern countries. These milks contain bacteria which, at least in so far as their activities in milk are concerned, are unique.

A review of the various published descriptions of Kefir, Yoghurt, Mazun, Leben and the less well known Gioddu draws attention to the fact that in the majority of instances the authors have described the microorganisms isolated and studied in each case as separate and distinct bacterial entities. Kuntze was the first not only to draw comparisons between the different organisms thus described, but also to point out further the possible relationships existing between these particular bacilli and the various species of lactic acid producing organisms already described. During the past two years the bacilli from these milks have received an increased amount of attention from bacteriologists because of their almost universal employment in combating the putrefactive organisms producing intestinal auto-intoxication.

The bacillus isolated from Bulgarian Yoghurt and first described by Grigoroff of Prof. Massol's laboratory -- the so-called *Bacillus bulgaricus* or *Bacillus* of Massol -- has been the organism of this type most discussed, and has generally been considered as constituting a new and distinct species. In this connection however it is of interest

to review the descriptions of other bacilli found in soured milks of similar character. The first published study in this connection is that of Löhnis on the microorganisms present in Russian Kefir. Since his observations were carried out as early as 1881, the nature of the bacteriological methods at that time renders his results of doubtful value for the purpose of comparison. It seems probable, as Löhnis suggests, that the bacillus called *Diospora caucasica* by Kern belonged to the *Subtilis* or *Mesentericus* groups. On the other hand it is quite likely that the *Bacterium caucasica* isolated from Kefir by Beijerinck was the first individual of this particular group to come under observation. A similar bacillus from the same source is described by v. Freudenreich who gives preference to the name *Bacillus caucasicus*. The very thorough investigation of the Egyptian Leben raib by Rist and Houry revealed the existence of two somewhat distinct types of lactic acid bacilli, the *Streptobacillus lebenis* and the *Bacillus lebenis*. The detailed study of their characters establishes their general relationship to organisms of the *Caucasicum* group, and furnishes an accurate basis for comparing allied bacteria. Three years later Grigoroff isolated from Yoghurt two bacilli which correspond in many respects to the two Leben organisms described by Rist and Houry. These he designated as *Bacillus A* and *Streptobacillus C* and it is the former which has been so widely known under the title of *Bacillus bulgaricus*. Although this bacillus, according to the author, produces a higher degree of acidity in milk than does the streptobacillus, and although it shows certain other differences in its characteristics, yet the many striking resemblances observed would seem to denote an intimate relationship between these two types. Unfortunately accurate comparisons between these four organisms are rendered impossible by the lack of corresponding details. In 1906 Cohendy described under the name of *Bacillus bulgaricus* an organism isolated from Bulgarian milk which is probably identical with the *Bacillus A* of Grigoroff. One of the most striking features of this organism was the production in milk of an unusually large amount of lactic acid, 3.23 per cent being found after ten days incubation at 36°. In Armenian Mazun Düggeleli demonstrated the presence of a somewhat similar bacillus, the protoplasm of which showed the presence of more intensely staining granules. It was less active than *Bacillus bulgaricus* in the production of acid, yet it exhibited morphological and cultural features peculiar to the *Caucasicum* group. The existence of a related bacterium is suggested by the observations of Grixonni, who found but failed to isolate a long granule-containing bacillus in the Sardinian sour milk Gioddu. Weigmann, Gruber and Huss described a bacillus repeatedly isolated from Mazun, which is undoubtedly identical with that studied by Düggeleli. Owing to its origin the name *Bacillus Mazun* was applied to it.

Directly comparable to the observations of Grigoroff, Rist and Houry, Düggeleli and Weigmann, Gruber and Huss are the findings of Luerssen and Kuhn who isolated, in addition to a bacillus obtained from several commercial preparations and called by them *Bacillus bulgaricus*, a second organism which they designate as the *Körnchenbacillus*. They found these two bacilli to differ from each other in several important characters, and these differences are sufficient, they consider, to establish their identity as two distinct species. The

latter organism, when stained with alkaline methylene blue or with the Neisser stain, is seen to contain granules which are not observed in the cell body of the *Bacillus bulgaricus*. Although D ü g g e l i mentions these granules these authors are the first to consider this feature as an important ground for differentiation. In addition to this difference, according to Luerssen and Kuhn, the *Körnchenbacillus* is to be distinguished from the *Bacillus bulgaricus* in the following respects;

a) By its longer and more slender form and its greater tendency to the formation of threads and chains.

b) By the appearance of the colonies of the two bacilli.

c) The *Körnchenbacillus* grows at lower temperatures than does the *Bacillus bulgaricus*, good at 37° and 45°, not at all at 22° and only slightly at 50°, while the *Bulgaricus* grows best at 45° to 50°, but develops slowly and feebly at 37°.

d) The former does not grow on potato, while the latter does.

e) Milk is quickly and firmly coagulated by the *Körnchenbacillus* at 37° to 40°, the *Bulgaricus* on the other hand produces only a soft curd in milk and then only after several days incubation.

These characters ascribed to the *Bacillus bulgaricus* by the authors are not in entire agreement with those described by others. This matter will be more fully discussed in another part of this paper<sup>1)</sup>.

When one reviews and compares the original detailed descriptions of the various bacilli mentioned above it is apparent that all these organisms possess in common, to a greater or less degree, certain characteristics which would seem to justify their classification in a group separate and distinct from other more usual organisms producing lactic acid in milk<sup>2)</sup>. It will be found further that many of these morphological features are included in the summary of distinctive characters of the group *Bacterium caucasicum* (Kern) L. et N., as reviewed by Löhnis, who distinguishes six general types, viz. —

- I. Milk coagulating, gas producing, *Bac. casei* Freudenreich.
- II. Milk coagulating, non-gas producing, (*Bac. casei* Leichmann).
- III. Milk non-coagulating, gas producing, (*Bact. caucasicum*).
- IV. Milk non-coagulating, non-gas producing, (*Bac. Delbrücki*).
- V. Slime producing type.
- VI. „Rankenbildener“ type.

<sup>1)</sup> Since the completion of the present investigation Heinemann and Hef-feran report the isolation from various sources of numerous cultures of bacilli which they believe to be identical with the *Bacillus bulgaricus*. From their observations they conclude that “the *Bacillus bulgaricus* is widely distributed in nature, that it occurs normally in human feces, in the feces of cows and horses, also in a variety of sour and aromatic foods, in food for cattle, in normal saliva, in normal gastric juice, and in gastric juice when hydrochloric acid is absent, in various fermented milks, in ordinary market milk, and in soil both manured and not manured.” The further corroboration of these findings would yield much of interest regarding the distribution and occurrence of this organism.

<sup>2)</sup> It is felt that the repetition of the descriptions of the organisms referred to would be superfluous since they have so recently appeared in the literature, and further, since these original articles are published in journals easily accessible.

Among the better known examples of these six types Löhnis mentions the various varieties of the *Bacillus casei* Freudenreich, *Lactobacillus caucasicus* Beijerinck, *Bacillus caucasicus* Freudenreich, *Bacterium casei* I—III Leichmann et Bazarewski, *Bacillus Delbrücki* Leichmann, *Streptobacillus lebenis* and *Bacillus lebenis* of Rist and Houry, and the *Bacillus Mazun* of Düggele. Similar organisms mentioned by Heinemann are *Bacillus panis fermentati* and *Leptothrix buccalis*.

Kuntze has recently suggested the probable relationship existing between some of the bacilli of this group and the *Oppler-Boas bacillus*, *Bacillus acidophilus*, *Bacillus gastrophilus*, and *Bacillus bifidus communis*. Even more recently Rodella asserts that these five last named organisms are identical. The relationships existing between these various lactic acid producing bacilli as might be disclosed by an accurate comparative study of their morphological, cultural, and biochemical characters offers an interesting field for further investigation. Such an investigation would undoubtedly establish the identity of several of these bacteria which at present are considered as distinct varieties, and further would yield a more satisfactory basis for a definite classification. By no means the least service rendered would be the abolishment or change of some of the confusing and inappropriate names. According to precedence the name *Bacterium caucasicum* could be used to designate the group, but this name as well as the name *Bacillus Mazun*, *Bacillus bulgaricus*, *Bacillus lebenis*, and *Streptobacillus lebenis* have scant justification when considered upon the ground of their accuracy or descriptiveness.

It has been the purpose of the present investigation to observe under identical conditions in a comparative way the salient features of a series of lactic acid producing, milk curdling bacilli originally isolated from oriental milks. In order that the results obtained might serve as a basis of comparison in further studies of these and allied organisms the analytical scheme of Lehmann and Neumann, and the plan endorsed by the Society of American Bacteriologists were adopted. Such omissions and variations were made as seemed to be indicated by the conditions met, but these were of slight importance.

#### The Organisms.

The various cultures chosen for study were of following origins;

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|-------|--|
| Bulg. | Received from Dr. Besredka of the Institut Pasteur, March 1908, under the name „ <i>Bacille bulgare Cohendy</i> .“                               |
| B. M. | An original culture marked „ <i>Bacille bulgare</i> “ supplied by the Societe Le Ferment of Paris to their American representative, August 1908. |
| Led.  | From the Lederle Laboratories, New York, August 1908, originally from Prof. Metchnikoff.   |
| B. K. | From Král, October 1908, marked „ <i>Bacillus bulgaricus</i> .“  |
| P. K. | From Král, October 1908, marked „ <i>Bacillus paralacticus</i> “.  |
| 6a—1  | Isolated October 1908 from „ <i>Bacillae</i> “ prepared by the Lactobacilline Co., New York.   |
| 7a—1  | Isolated from Lactobacilline Malt obtained in Paris July 1908.   |
| 7a—2  | Isolated September 1908 from Lactobacilline Malt prepared by the Franco-American Ferment Co., New York.  |
| IX—1  | Isolated from <i>Bacillae</i> in March 1908.   |

- XXIV—1 Isolated March 1908 from sour milk obtained from native Syrians in Brooklyn.  
 W. Isolated by and received from Prof. Kuntze of Leipzig marked „Bact. Yoghurt, Varietät W“.
- 35a—1 Isolated October 1908 from „Zoolak“, a commercial Mazun prepared by Dr. Dadirrian, New York.
- 36a—1 Isolated October 1908 from a commercial Armenian sour milk.
- 41a—1 Isolated October 1908 from Mazun prepared by native Armenians in New York.
- 42a—1 Isolated October 1908 from sour milk prepared by native Armenians in New York.
- K. Isolated by and received from Prof. Kuntze of Leipzig marked „Körnchenbacillus“.

For purposes of convenience the cultures from Bulg. to W. will be referred to as Type A, those from 35a—1 to K. inclusive as Type B.

While it is quite probable that the above list includes strains having a common origin yet these have been subjected to varying conditions of cultural environment. In order to learn the influence of long continued solitary cultivation, of symbiosis with yeasts, bacilli and cocci, and of cultivation in different media the possibly identical strains were therefore included. These sixteen cultures were cultivated and studied simultaneously and always under identically analogous conditions. In addition to the above some twenty different strains of similar bacilli were isolated from various sources, comprising commercial preparations from Germany, France, England, Scotland, Switzerland and the United States, also specimens of sour milk obtained from southern Siberia, Sofia, and from the natives of Armenia, Syria, and Egypt living in New York City. These isolations were accomplished during the course of the investigation, and the organisms thus isolated were compared as to their essential features with the strains already studied. No new types were found and since none of these organisms showed any significant variations from those listed no mention will be made further than to associate them with the types included in the present study.

### Detailed Features.

#### I. Morphology.

1. Whey agar.<sup>1)</sup> The following characteristics are common to all strains; wide variation in length, from 2  $\mu$  to 50  $\mu$ , breadth about 1  $\mu$ . Nearly all individuals are intensely Gram positive and these show regularity of outline. They are straight to slightly curved, with rounded ends and show no vacuoles or granules. All strains show involution forms exhibiting vacuoles, often appearing as empty cell membranes. These forms are Gram negative and vary greatly in both dimensions also in form. All show a tendency to chain formation, some being arranged in chains of 6 to 25 segments. The chain may contain both Gram positive and Gram negative individuals. Gram negative spherical bodies, varying from 0.25  $\mu$  to 1  $\mu$  are seen adhering directly to the sides of some Gram negative individuals in strains of Type B.

2. Whey. In their behavior in whey these organisms exhibit some features of decided interest. The tendency toward degeneration and involution is marked. In the early stages of incubation at 37.5° or 44° the bacilli are uniform in size and intensely Gram positive while in the succeeding stages the irregular, vacuolated, inflated and ruptured forms predominate. These latter forms invariably decolorize. Between the 18th and 24th hour of incubation at 44° the strains of type A develop a morphological peculiarity first noted by Kuntze. This is the formation of oval to kidney-shaped

<sup>1)</sup> The whey, whey agar, and whey gelatin were prepared according to the method of Cohendy.

nodules attached to a small stem extruding from the cell substance. As the incubation is prolonged these nodules increase in size, often measuring 1  $\mu$  to 2  $\mu$  in length, and their increase always takes place at the expense of the cell protoplasm. It is further observed that without exception these bodies as well as the parent bacillus are Gram negative. Their formation may undoubtedly be considered a true plasmoptosis. Two forms may be distinguished. In one the stem apparently projects from the cell body, while in the other the nodule is situated at the junction of two dividing bacilli which form an angle greater than 90°. or this juncture may be considered as a point of marked indentation of a single bacillus. (See plate I). While this feature is observed to a slight extent when the bacilli are cultivated on whey agar, and in a very few instances in milk, it appears to be a marked characteristic of the growth in whey.

Cultures of type B present quite another appearance. No stemmed nodules are present, but instead small spherical bodies are seen which are more or less securely attached to the cell wall. These may become detached and their presence in the medium lead to the suspicion of a coecal contamination. In the case of the stemmed nodules a single bacillus rarely if ever extrudes more than one bud, while in the latter instance the bacterium may have a number of these small spheres adherent to it.

The second feature of interest is the tendency shown by the strains of type A to grow in the form of short bacilli arranged in chains, while the strains of type B develop to a greater length and exist almost exclusively as single isolated forms. Forms have been observed in the cultures of type B which exhibit true branching. These bacilli are Y shaped, the segments of which rarely exceed 2  $\mu$  in length.

Examined in a hanging drop at various stages of development motility was never observed.

3. Milk. Since milk is the natural habitat of the bacteria of this particular group, the morphological features which they manifest when cultivated in this medium may be considered as constituting a criterion. As in whey the age of the culture has a marked influence upon the form of the bacteria. In young cultures the bacilli show regularity of outline although wide variations in their length are observed. In each of the cultures individuals exist whose length scarcely exceeds their breadth, while others show a decided tendency to thread formation. Threads may be seen which exceed 50  $\mu$  in length. It is therefore difficult to make any definite statements in regard to average length. The breadth is more constant, varying between narrow limits. The average breadth is about 1  $\mu$ , although the longer forms are more slender and average about 0.7  $\mu$ . There is less tendency to chain formation than in whey yet chains of 4 to 10 segments have been observed in cultures of type A. Type B shows longer and less straight forms. In older cultures longer individuals predominate in all strains. When grown in symbiosis with yeasts and the more trivial milk organisms the development of the longer forms is restricted, the length averaging 4 to 8  $\mu$ . As might be inferred there is little or no degeneracy and very rarely are nodules observed.

4. Endospores. The presence of spores was observed in no case. In the degenerated forms certain lighter staining areas are seen which have suggested the nature of spores to some observers, although they are usually considered as vacuoles. Piffard suggests the name „lucidoles“. The fact that these bacteria, when young and most active, exhibit a low degree of

vitality and resistance would argue against the possibility of reproduction by spore formation.

5. Capsules. Nothing to suggest the presence or formation of capsules was observed.

6. Staining reactions. All strains are readily stained by the usual anilines.

a) Gram: — Young individuals invariably show intense staining with this method. Older bacilli are more easily decolorized, and both dead and degenerate forms are always negative. In those forms not intensely stained there may be observed the presence of minute, deeply stained granules irregularly distributed throughout the protoplasm. This feature was found to be common to all strains. When chain formation is present not infrequently a segment is seen which is decolorized while the other elements of the chain are deeply stained. Further, single bacilli have been observed which exhibit color gradations from positive at one pole to negative at the other.

b) Loeffler's Methylene blue: — By means of the behavior of the organisms studied to this stain a separation into two types seems possible. The protoplasm of those of Type A is uniformly impregnated while that of Type B shows a distinct differentiation. The cell body is seen to contain a varying number of round to oval bodies or granules having a diameter equal to that of the cell and stained purplish red. These bodies are present whether cultivation has taken place in milk or whey. In the latter case they are more marked in the younger individuals, becoming less distinct and fewer in number as development progresses, while in milk they are to be found in old cultures. These granules have been observed during varying periods of incubation and their presence noted at the end of 18 hours and after 21 days. Generally they occur only in the minority of forms and are less conspicuous in the longer individuals.

c) Neisser's stain: The granules just described are intensely stained by this procedure. In Type A none of the strains has ever exhibited the least affinity for the polar stain.

The granules were noted by Duggeli in the *Bacillus Mazun* and under the name „Körnchen“ have been described by Luerssen and Kuhn. More recently this characteristic has been discussed in detail by Kuntze who holds the opinion that their presence is by no means a constant or permanent character, and that their formation is dependant upon conditions of growth which may be lacking at times. He therefore is disinclined to consider this feature a valid point of differentiation. In the present study, on the other hand, no variation has ever been noted. Fourteen of the sixteen strains have been under observation during a period of about sixteen months, in which time the influence of solitary cultivation as well as that of symbiosis has been noted. None of the organisms thus observed has been found to either gain or lose this character. On the ground of this evidence the presence of granules is tentatively held as establishing a mark of dissimilarity of type. No inquiry into the nature of these bodies has been attempted. (See plates III, IV).

## II. Cultural Features.

The majority of the cultural features described by Löhnis as distinctive of the group *Bacterium caucasicum* are displayed to a conspicuous degree by all of the organisms studied. When freshly isolated from their natural environment — milk — they do not develop on any of the usual nutrient media, even though sugar be present. Isolation can only be effected by the employment of whey agar (Cohendy). Even upon this medium under the optimum conditions as to temperature, etc. the growth is tardy and feeble. This fact, combined with the appearance of the colonies is markedly characteristic. In the structure of the colonies a resemblance is seen to those of *Bacillus anthracis*, and in a less degree to certain strains of *Bacillus subtilis*.

1. Whey agar plates<sup>1)</sup>. The following description is applicable to all the organisms studied with the exceptions as noted:

Growth was first noted at the end of 24 hours at 37,5°. Colonies are then minute: a) Surface; at 48 hours about 0.25 to 1.0 mm. diameter, irregular, filamentous, curled, flat, weakly refractive; with low magnification the structure is found to consist of a mass of curled filaments, often in parallel strands, as in Anthrax colonies, often streaming; are translucent, tinged slightly bluish by reflected light and showing bluish white flocks or islands more or less rectangular in form. The medium is slightly clouded immediately about the point of growth. No tenacity to the surface is shown. b) Subsurface; Minute woolly tufts, delicate not dense, composed of hair-like processes radiating from the center which is yellowish by transmitted light. Exceptions: Strains 6a—1, 7a—1, 7a—2, IX—1 show a distinct, entire, even periphery. At times the growth produces no clouding of the medium. Frequently the colony appears to be nucleated; the center is darker in color and has a slight elevation, being umbonate in character. This feature becomes more apparent as development progresses. The nucleus may be surrounded by a comparatively clear zone and this in turn by a bluish white filamentous border. No chromogenesis is observed. A sour odor is noticeable after 48 hours incubation. (See plates V. VI.).

While the growth on whey agar furnishes no points of type differentiation yet it serves to definitely separate these organisms from all bacteria not included in the group *Bacterium caucasicum*. The resemblance to *Bacillus anthracis*, and in a way to *Bacillus subtilis*, although borne out to a slight degree by the morphology is no more than interesting. Luerssen and Kuhn consider that the growth of the *Bacillus bulgaricus* in the form of round regular colonies having a smooth periphery is diagnostic of this type. In the present instance it was observed only in those strains which had been subjected to cultivation in pure culture for a period of a year or more. Since it was never observed in the other organisms of type A it might seem that this was a casual or acquired character?

2. Whey agar stabs. The growth first appears at the end of 24 hours in the upper part of the stab as a woolly filament. At the end of 48 hours the development progresses downward, delicate hair-like processes extend laterally from the line of growth and the medium becomes slightly clouded. After 3 days incubation the growth is marked along the entire line of inoculation and becomes arborescent with dense clouding of the agar. The surface is scanty with little spreading and is nearly transparent. Further incubation intensifies these characteristics without causing the appearance of further distinguishing features.

None of the strains studied showed any marked variation from the above. This growth would seem to constitute a group characteristic.

3. Whey gelatine plates. (10% gelatine.) — Owing to the comparatively low incubating temperature necessitated, this medium is poorly adapted for the cultivation and differentiation of this class of bacteria. In several experiments the only strains showing growth were Bulg. B. M., 6a—1, 7a—1, 7a—2 and IX—1, and in each case after long incubation at 25° to 27° the colonies were minute, although a microscopic examination showed their structure to be identical with that of the whey-agar colonies. No growth was observed before six days while all freshly isolated strains failed to develop.

4. Whey gelatine stabs. At first no growth whatever was observed in the case of the organisms of type B, although repeated attempts were made to cultivate them in this manner. Later, after continued cultivation in milk a slight tendency toward growth was manifested, which never exceeded a

<sup>1)</sup> In this series as in all others unless special mention is made the medium was inoculated from a 24 hour whey culture planted in turn from a 24 hour milk culture. The medium contained 2 per cent agar.



faint tracing in the gelatine. With the strains of Type A, a distinct but variable growth was obtained, which may be described thus:

At the end of from four to seven days at 25° a faint line is noticed along the track of the stab. This appears as a finely beaded filament which on further incubation extends downward. In the lower half of the growth single colonies appear which are spherical and whitish in color. The bead-like colonies become mossy and from these mycelioid projections extend laterally. When the colonies are closely segregated the growth appears markedly arborescent, while single heads are beautifully echinate. (See Fig. 1.)

The greatest adaptability to this medium was shown by Led. B. K. and P. K.. Surface growth, gas production or liquefaction were never observed. The medium was not clouded.

The filamentous character of these colonies is analogous to their appearance in agar stabs and is distinctive of the group, although not of the type. The variations noted are probably referable to cultural influences and may be developed by long cultivation.

5. Whey. At the end of 24 hours at 44° a slight, diffuse clouding of the medium is observed. At 37.5° the appearance of the cloud may be delayed until 48 hours or even later. Strains of the B type show the heavier growth. Of type A 6a—1, 7a—1, 7a—2, IX—1 and XXIV—1 are the most vigorous. In cultures of this type the cloud is composed of a suspension of macroscopic particles while the appearance of those of the second type resembles that seen for example in bouillon cultures of *B. coli*. The most intense clouding appears during the second 24 hours at 44°. Directly after the height of the growth has been attained the A cultures begin to clear. The suspended particles collect in large flocks adhering to the walls of the tubes and some fall to the bottom forming a grayish white sediment.

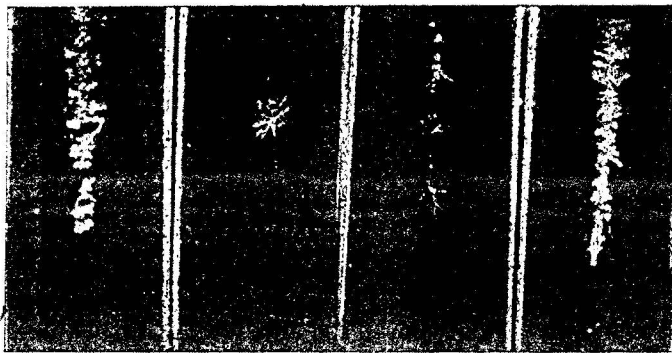


Fig. 1. 21-day whey gelatine stab cultures Led. B. K., P. K., IX—1. Natural size.

Between the sixth and eighth day the medium is entirely clear, complete sedimentation of all suspended matter having taken place. In cultures of type B this turbidity persists for a longer time usually until the twelfth to fourteenth day. No pellicle is formed.

Although these variations may be ascribed to degrees of vigor, they are constant in each case and tend to emphasize the type differentiation indicated by the other described features.

6. Potato. Slants were cut in the usual way. In some instances the potato was merely washed in distilled water, in others immersed in soda solutions of different dilutions for varying lengths of time. Therefore both acid, neutral and alkaline strata were provided. The medium was heavily inoculated with either a 24 hour whey culture or a 24 hour milk culture. In repeated attempts at 25°, 37.5° and 44° no demonstrable growth was obtained with any of the sixteen organisms.

The results in this case although confirming the observations of Grigoroff, Rist and Houry, and Cohendy, are contrary to those obtained by Luerssen and Kuhn, who describe the growth on potato as a constant and distinguishing character of the *Bacillus bulgaricus*. The failure to grow on this medium is in agreement with the behavior of organisms of the *Bacterium caucasicum* group.

7. Milk. When inoculated with young cultures and incubated at 37.5° to 44° milk is rapidly coagulated into a firm solid curd by all the strains studied. The coagulum produced showed slight variations due to the composition of the milk, and to the amount

and nature of the material used for inoculation. The curd was solid, with no shrinkage or cavities, and was accompanied by the expression of a few drops of clear whey. Thickening appears first in the lower part of the tube. The production of gas, or an appreciable peptonisation of the curd were never observed even after several months preservation.

Strains Bulg. and Led. at times produced a viscid curd, often increasing to sliminess. This character was repeatedly lost and regained, and was peculiar solely to these two organisms. When litmus is added to the milk the change from blue to red is abrupt and intense, the curd assuming a deep pink hue. Coagulation takes place in 6 to 12 hours at 44° to 45°, in 8 to 16 hours at 37.5° and rarely before 9 to 12 days at 25°. At the last named temperature coagulation was noted on lyin type A. cultures. Tubes inoculated with the strains comprising type B. were observed for 31 days at room temperature and in no case had any change taken place. On the thirty-second day these tubes were incubated at 44° and at the end of 18 hours all showed typical coagulation. From these cultures further successful inoculations were made, showing that although growth was inhibited at 25° yet the bacteria remained viable during this period. In a similar series of tubes embracing the 16 strains inoculated and allowed to remain for 31 days at refrigerator temperature the milk remained unchanged. When incubated at the expiration of this period of exposure all strains produced coagulation within 40 to 48 hours.

Two of the points of type differentiation mentioned by L u e r s s e n and K u h n are contradicted by the above observations. First, in the present instance the organisms of type A. produced coagulation slightly more rapidly than did those of type B. (comparable to the *Körnchenbacillus*) while the nature of the curd produced by both was precisely the same. Second, the strains of the former type, though showing a marked preference for higher temperatures, still multiplied and produced coagulation at 25°, while those of the latter type remained inactive. The viscosity noted in two strains is a phenomenon previously observed by S e w e r i n. This feature as developed by *Bacillus casei* has recently been studied by B u r r i and T h ö n i. This bacillus however, exhibits this peculiarity only when grown in symbiosis.

8. Other media. When freshly isolated from their natural symbiotic environment no growth is obtainable on the ordinary media. After a year's solitary cultivation in milk these bacteria show an increased vitality and adaptation to foreign environment. Recently a feeble growth has been obtained on nutrient agar in the case of strains of type A. The same agar enriched with 2% lactose, strange to say, was less favorable, while the addition of the same amount of dextrose resulted in the development of many large typical colonies by all 16 strains. In bouillon, lactose bouillon, and dextrose bouillon no growth was observed. However, if calcium carbonate be added to the last named medium these organisms grow well<sup>1)</sup>. The synthetic medium of U s c h i n s k y containing 2% dextrose or lactose remained unchanged. An extract of malt prepared according to the formula of C o h e n d y furnishes an excellent medium for the cultivation of bacilli of this group. It may be substituted for whey in the preparation of agar and gelatine. In lactose bile enriched with peptone a feeble though constant growth was observed with all strains. This lack of inhibitory power on the part of the bile is interesting in relation to the persistence and growth of these bacilli in the human intestine following internal administration. The growth in this medium is unaccompanied by gas formation.

Observed in whey, malt and milk and on whey agar and whey gelatine no differences were noted in the growth under aerobic and anaerobic conditions.

<sup>1)</sup> This medium was suggested by Dr. Garside of New York City.

### III. Physical and biochemical Features.

#### 1. Fermentation.

a) Whey in Smith tubes: — Good growth observed in both arms. No production of gas.

b) Other media: — Grigoroff states that the *Bacillus bulgaricus* attacks mannite, saccharose, maltose and lactose, but not rhamnose, dulcitol or sorbitol, but the nature of the medium he employed could not be ascertained. Cohendy observed the active fermentation of lactose, maltose, saccharose, levulose and particularly dextrose. Since the completion of the present work Bertrand and Duchacek report that the carbohydrates fermented by *Bacillus bulgaricus* are dextrose, mannose, galactose, levulose and lactose, while arabinose, xylose, sorbose and saccharose are not attacked. Mannite is not transformed into lactic acid.

In the present instance many attempts were made to discover a suitable medium with which the action of these bacilli upon the various carbohydrates could be studied. Among the different nutrient substances tested were peptone prepared from casein and of other origins, serum waters, the medium of Barsikow and a similar solution of Eucasein. In all cases there was a marked acid production from dextrose and lactose, but the results obtained with the other carbohydrates were too variable to warrant any definite conclusions.

2. Indol. Whey cultures of different ages were tested by several methods. Negative results were constant.

3. Toleration of acids. Since the members of types A and B produce exceptionally large amounts of lactic acid, their toleration seems to be limited only by the maximum acidity produced in each case, beyond which they are unable to utilize further the residuum of unaltered sugar in the medium. This acid toleration may be made use of in isolating these organisms from material containing other bacteria. Such a separation may be accomplished by adding to the medium a quantity of acid sufficient to inhibit the development of foreign species. Leva found that the addition of 0.35 per cent lactic acid to Cohendy agar facilitated the isolation of *Bacillus bulgaricus* from the feces of patients after therapeutic administration. More recently Heinmann and Hefferan report the successful isolation of this organism from various sources by adding 0.5 per cent glacial acetic acid to glucose bouillon.

4. Vitality. In addition to the observations on the behavior of these bacilli in milk it may be further stated that their vitality varies inversely with the period of incubation temperature, but directly with the length of time of special cultivation. Cultures in malt and whey display a greater degree of longevity than do those in milk. In the former case viable cultures have been obtained at the end of three months.

5. Thermal death-points. Methods: a) 48 hour whey cultures were immersed in a water bath for the designated period and transplants were immediately made into milk. b) Tubes of whey were inoculated with a definite amount of an active whey culture and after subjection to the various temperatures were then incubated. A minimum exposure of 15 minutes to 60° was necessary to kill all strains.

6. Dessication. In a dry condition the vitality is greater than in milk. Whey and milk cultures dessicated over sulphuric acid in a vacuum were found to be still viable after a lapse of four months. Considering their short

survival in milk and the absence of a capsule or spores, their resistance in this case would seem to present an anomaly.

7. Enzymes. The addition of calcium carbonate, calcium chloride, and zinc chloride in excess of the amount required to neutralize the acid produced, failed to prevent coagulation of the milk. This might argue for the presence of an enzyme. It is hoped to continue studies in this direction.

8. Acid production.

a) Milk: Methods: Tubes containing 10 cc. each of decreamd milk were sterilized and then incubated for 48 hours at 37.5° and allowed to stand for several days at room temperature to insure sterility. All tubes in each series were filled from the same lot of milk. These tubes were inoculated with 0.05 cc. of a 24 hour milk culture and incubated for the determined periods in a moist atmosphere. At the end of these periods 5 cc. of the milk was diluted with about 45 cc. of water, then brought to the boiling point and titrated with  $\frac{n}{10}$  NaOH, with phenolphthalein as indicator. Each series included two or more control tubes of the same sample of milk incubated and titrated under similar conditions. The titre of the control in each case was subtracted from the titre of the corresponding inoculated tubes. Whey cultures were similarly inoculated, controlled and titrated. The figures in the tables represent the quantity of  $\frac{n}{10}$  NaOH required to neutralize 10 cc. of inoculated milk.

Table I. Acidity produced in milk at 37.5°.

	6 hrs.	12 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.	72 hrs.	5 days	7 days	10 days
Type A.	0.66	5.51	14.29	18.40	22.77	27.27	29.98	33.21	33.77	34.34
Type B.	0.69	3.54	5.53	5.08	6.21	7.54	9.52	11.38	9.74	11.13

Table II. Acidity produced in milk at 44°. Series 1.

	6 hrs.	12 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.	72 hrs.	5 days	7 days	10 days
Type A.	2.62	11.91	17.78	21.79	24.37	25.70	25.01	27.84	28.47	27.84
Type B.	2.82	3.98	5.56	6.44	6.80	9.49	9.46	8.88	13.72	12.04

Table III. Acidity produced in milk at 44°. Series 2.

	6 hrs.	12 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.	72 hrs.	5 days	7 days	10 days
Type A.	0.71	6.96	16.59	19.90	22.42	24.37	23.96	26.09	27.72	—
Type B.	0.36	3.12	10.53	12.60	11.60	12.14	12.45	14.06	15.70	—

Table IV. Acidity produced in whey at 44°.

	6 hrs.	12 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.	72 hrs.	5 days	7 days	10 days
Type A.	0.66	2.17	4.07	4.25	4.58	—	5.71	5.58	6.90	6.05
Type B.	0.65	2.00	3.29	5.19	5.82	—	5.58	6.02	5.89	5.75

The accompanying charts offer a more graphic representation of the acid production in milk and whey.

An examination of these tables and charts would seem to permit the following inferences;

A. In both types the acid production in milk is slower at 37.5° than at 44°.

B. A higher degree of acidity is attained at 37.5°.

C. The acid production is most active in the first 24 hours, then gradually rises, reaching a maximum about the seventh day.

D. The bacilli of type A without exception elaborate an amount of

acid equal to about twice the amount produced by the strains of Type B. This would seem to constitute an important type difference hitherto unnoted.

E. No significant differences between the types or strains are shown in the acidity produced in whey. The small amount of acid formed in this medium is in marked contrast to the quantity found under analogous conditions in milk.

10. Acids produced.

A. Lactic acid: The statements of various authors regarding the nature of the lactic acid produced by this group of organisms show a discordance referable partly to the methods of analysis employed as well as to inherent differences exhibited by separate species. Grigoroff reports that both the *Bacillus A* and the *Streptobacillus C* produce inactive acid, but the method of determination is not given. From a 5-day culture of the *Bacillus bulgaricus* Bertrand and Weisweiler isolated the lactic acid by means of the zinc salts and conclude that the acid produced was a mixture of the laevo and dextro modifications with a predominance of the latter. In quoting from the work of Luerssen and Kuhn certain authors have erred in stating that they found only the dextro-rotatory modification in cultures of the *Bacillus bulgaricus* and *Körnenbacillus*. In the original publication it is specifically mentioned that "the whey turns the plane of polarized light to the right". This can scarcely be interpreted as indicating the presence of dextro-rotatory lactic acid. Bertrand and Duchacek find that the *Bulgaricus* produces exactly equal amounts of both the right and left acids, and that this action is due to an intracellular lactase. The strains studied by Heinemann all produced the inactive type without a trace of active acid.

In the present investigation the following method was adopted;

The whey obtained from 500 cc. of the milk culture was acidified with phosphoric acid 5 volumes of water free ether were added, then thoroughly agitated in a shaking apparatus. This step was repeated five times and, after an exact separation from the whey, the ether extracts were united and evaporated to dryness in vacuo at 45°. The residue was taken up in water, the solution clarified by filtration through animal charcoal, and after the addition of an excess of zinc carbonate it was boiled and filtered. After concentration on a water bath the solution was allowed to stand. The crystals formed were removed by filtration. Further crops of crystals forming in the successive filtrates were preserved and examined separately. The determination of the water of crystallisation was accomplished in the usual manner.

The analyses of the zinc salts obtained from the whey of 24 hour milk cultures are as follows;

Culture Type A.	Per cent water	Calculated for $Zn(C_3H_5O_3)_2 + 3 H_2O$	Culture Type B.	Per cent water	Calculated for $Zn(C_3H_5O_3)_2 + 2 H_2O$
XXIV—1	18.21	18.18	35a—1	12.77	12.90
Bulg	18.21		36a—1	12.91	
B. M.	18.11		41a—1	13.04	
Lcd	18.60		42a—1	13.05	
B. K.	18.40		K	12.88	
P. K.	17.93				
6a—1	18.04				
7a—1	18.20				
7a—2	18.12				
IX—1	18.02				
W	18.10				

Concentrated solutions of these salts were prepared and their rotatory power determined.

In the case of all the strains of type A the solutions exhibited rotations too slight to be of any significance. The solutions from type B, on the other hand, showed a marked rotation to the right, therefore demonstrating the production of the laevo-rotatory acid.

The crystals separated from subsequent fractions from the 24 hour cultures of strains of both types had the same composition and rotatory power as the initial crystals.

A series of observations was made on cultures incubated 7 days, 14 days, 21 days and 30 days respectively at 44°. An unvarying production of the inactive acid alone was noted in all the strains of type A. The type B organisms, 24 hour cultures, showed a production of the active acid. In 7, 14, 21, and 30 day cultures of strains of this type, the crystals obtained represented approximately equal amounts of the inactive and active acids.

The variations in this feature of the activity of the bacilli of the two types are tentatively considered as constituting a further point of type differentiation.

B. Other acids: The acids already demonstrated in cultures of the *Bacillus bulgaricus* are acetic, formic and succinic acids (Bertrand and Weisweiler, Bertrand and Duchacek). Heinemann finds that the volatile acids constitute 5.8 to 6.1 per cent of the total acidity.

All the strains observed in the present instance produced a small amount of volatile acid. The exact amount and the nature of the acids were not determined.

Although the bacilli of these types have little or no action on the casein and fat in milk, yet they produce a bitter, acid taste and odor which may be due to minute quantities of products formed in the cleavage of these substances. This peculiarity was lacking in cultures of type B organisms.

11. Other products. Grigoroff reports the formation of a trace of alcohol by the *Bacillus A* and the *Streptobacillus C*. This substance was also found by Gourbet, while Luerssen and Kuhn note that 24 hour whey cultures of the *Bulgaricus* and *Körnchenbacillus* gave positive iodoform reactions.

A series of experiments included the distillation of the whey (after faint alkalization with sodium carbonate) from 10 day milk cultures of the strains of types A and B. These distillates all contained small but appreciable amounts of alcohol but no acetone or aldehyde.

12. Pathogenicity. These organisms are non-pathogenic to man and the usual laboratory animals. No untoward effects in man have been observed following the ingestion of large amounts of whey and milk cultures.

#### Summary.

The principal characters common to all the strains studied as compared to the characters distinctive of the group *Bacterium caucasicum* as noted by Löhnis are as follows:

Strains of types A and B.	Group <i>Bacterium caucasicum</i> L. et N.
Great variability of cell form.	Great variability of cell form.

Length = 2 $\mu$ to 50 $\mu$ or more.	Length = 2 $\mu$ to 50 $\mu$ or more.
Breadth = generally about 1 $\mu$ .	Breadth = 0.3 $\mu$ to 1.20 $\mu$ .
Chain formation marked in some strains.	Chain formation not unusual.
Longer and slenderer forms seen in older cultures in milk.	Favorable conditions result in the appearance of more slender forms.
Non-motile, non-sporulating.	The absence of motility and spore formation is apparently constant.
Viable bacilli are Gram positive, dead and involution forms Gram negative.	With the exception of the dead and involution forms all bacilli of the group are Gram positive.
Difficult to cultivate, growth in most media feeble.	The intensity of growth is mostly slight and is dependant on the nature of the medium.
Freshly isolated, growth is obtainable only on media containing whey or malt, and in milk.	On the usual meat pepton media, as well as on potato, particularly in the case of freshly isolated strains no growth takes place.
Grow equally well under aerobic and anaerobic conditions.	The disinclination to grow in the presence of free oxygen is marked.
Optimum temperature for growth is 44°—45°. Fair growth at 30°, slight at 25°, none at 20°.	For many forms the optimum lies between 40° and 50°, the minimum at 25°, while other members of the group are able to grow tolerably well at 20° and under.
Colonies on whey agar are round to irregular, grayish white, 0.5—1.5 mm., curled, filamentous structure, periphery mostly filamentous, often streaming, in a few cases smooth and even.	Colonies are white or yellowish white, mostly smaller than head of a pin.
Gelatine not liquefied.	Periphery is either circular, even or sinuate, lobed at times with root-like ramifications.
No surface growth on gelatine stab cultures. Along the stab the	So far liquefaction of gelatine has never been observed.
	The gelatine stab cultures show typically no surface-growth.

- growth is filiform, beaded, later with horizontally projecting ramifications. Medium clear.
- In agar stabs the growth is the same as above, but heavier. The medium is clouded.
- The growth in whey produces clouding which disappears in 5 to 14 days forming a grayish white sediment.
- No growth on potato.
- Milk coagulated in 8—18 hours at 44°, and is the most favorable medium for growth. Most rapid coagulation takes place at 44°.
- The lactic acid formed is either inactive or laevorotatory.
- A small quantity of volatile acid is produced.
- No appreciable peptonization of the curd.
- Non-pathogenic.
- Along the stab the growth is cuneiform, threadlike or beaded, rarely showing horizontal projections.
- Agar stab, as a rule, weakly developed. Narrow ribbon or dotted growth along stab. The presence of sugar in the agar or gelatine causes clouding.
- In bouillon, when growth takes place, only a whitish sediment is observed in the clear fluid, while the addition of dextrose or lactose produces a weak to strong clouding as well as an increase in the sediment.
- On potato as a rule there is no development, at best it is slight.
- Milk in general is coagulated more slowly than by streptococci. Some individuals grow only slightly in this medium. The optimum for coagulation lies mostly between 40° and 50°, for some 30°.
- The lactic acid formed is mostly laevo-lactic acid, more rarely the inactive or dextro modification.
- Only a trace of volatile acids are produced, if at all.
- Several organisms of the group peptonize casein. Pathogenicity has never been observed.

It will be seen that, with a few exceptions, there is a striking resemblance between the various organisms studied and those of the *Bacterium caucasicum* group.



Variations in the characteristics of the various strains pointing to type differentiation are —

Type A.	Type B.
With Loeffler's methylene blue or with Neisser's stain the protoplasm is homogeneously stained.	With these stains the presence of intensely staining granules may be demonstrated in the protoplasm.
Produce 2.7 to 3.7 per cent lactic acid in milk.	Produce 1.2 to 1.6 per cent lactic acid in milk.
The lactic acid formed is the inactive modification.	The lactic acid formed is always the laevo-rotatory modification.

### Resume.

1. A review of the morphological, cultural and biochemical features of the lactic acid producing bacilli from Yoghurt, Mazun, and Leben appears to justify their classification as a single group.

2. This group would seem to be identical with the group *Bacterium caucasicum* (Kern) — Lehmann and Neumann.

3. The significant variations exhibited by these bacilli in regard to the presence or absence of granules demonstrable by differential stains, the degree of lactic acid production, and the nature of the lactic acid formed suggests a further differentiation into two distinct types. These types might be designated as the true type (Type A) and the para type (Type B).

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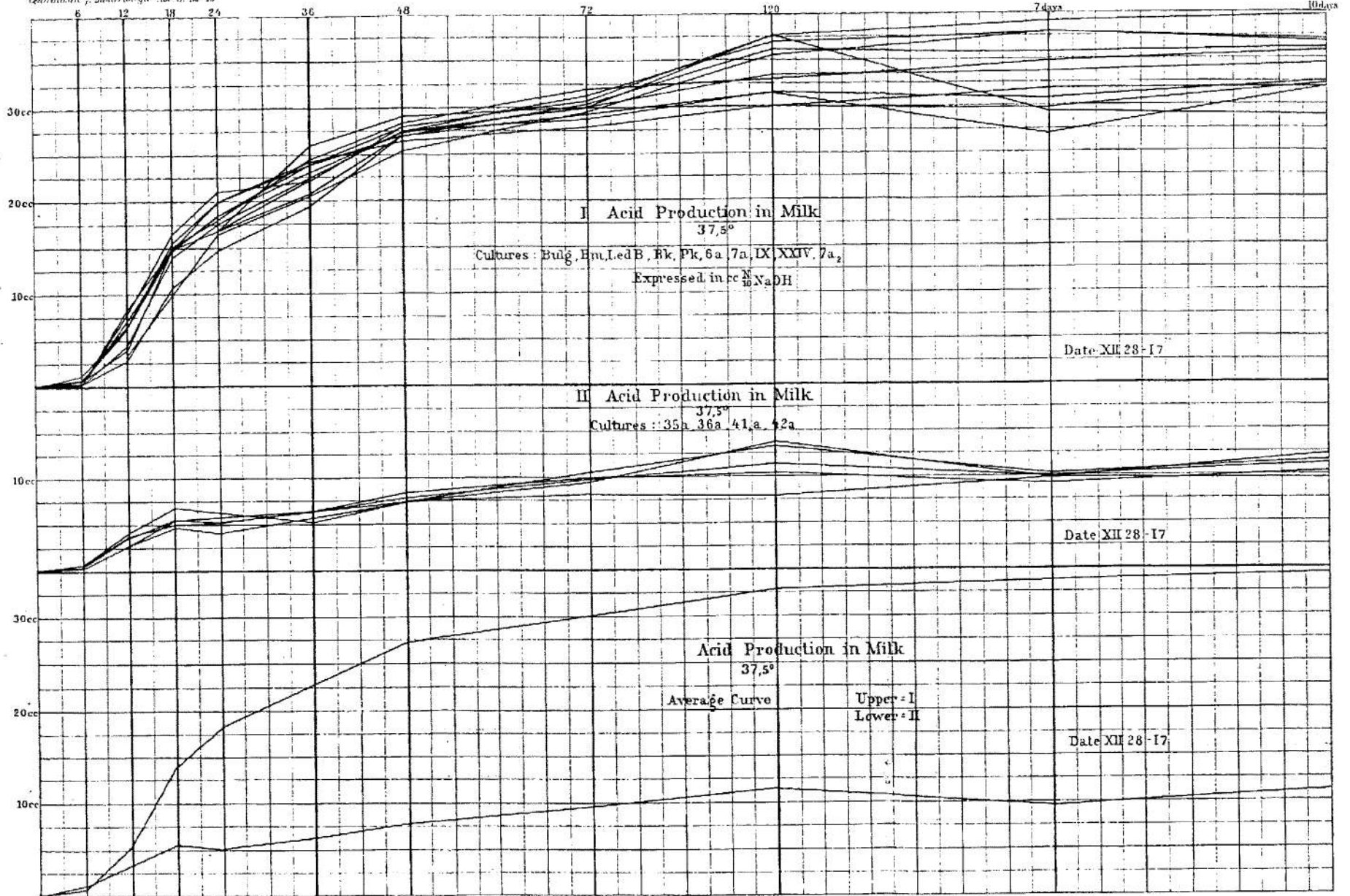
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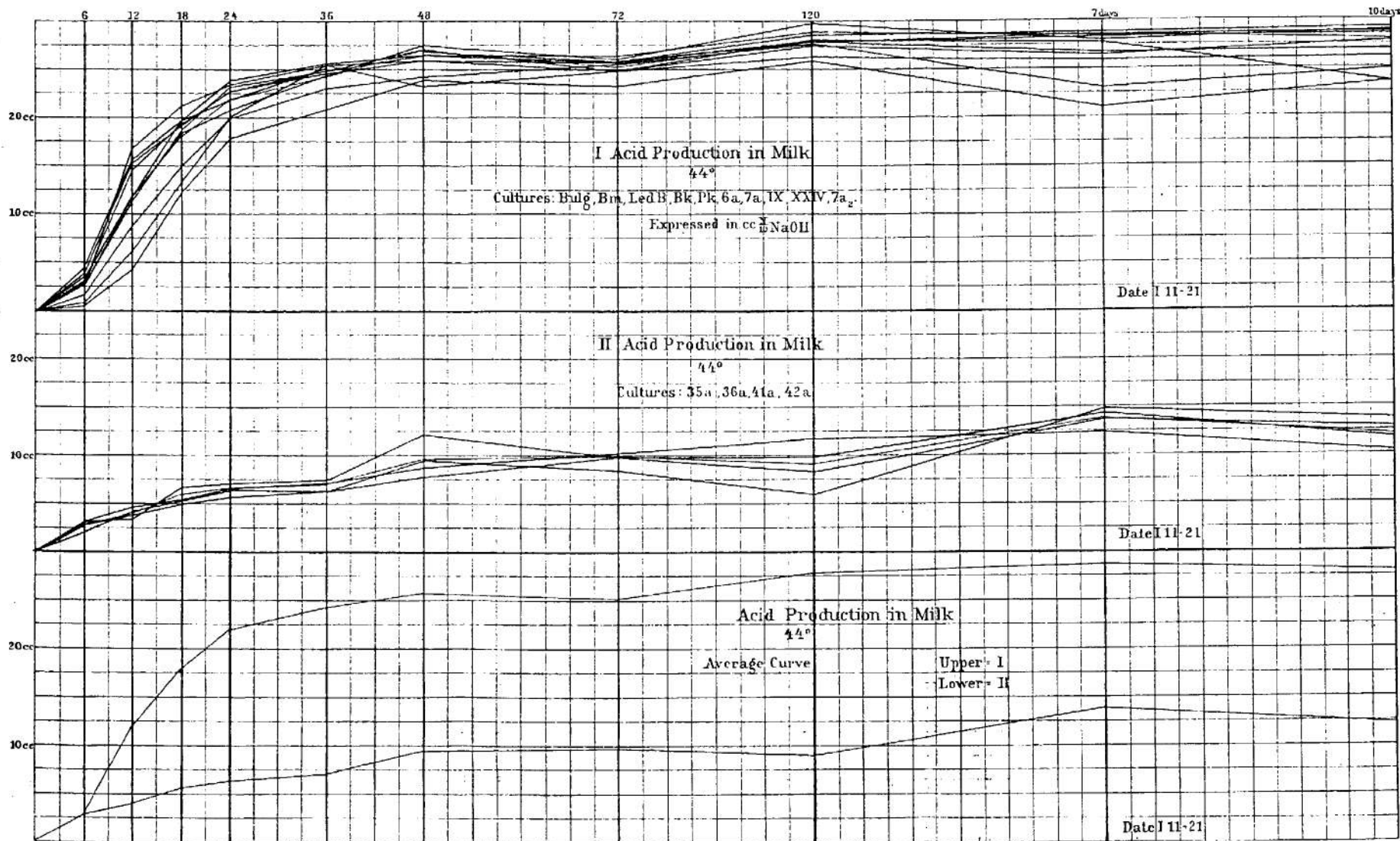
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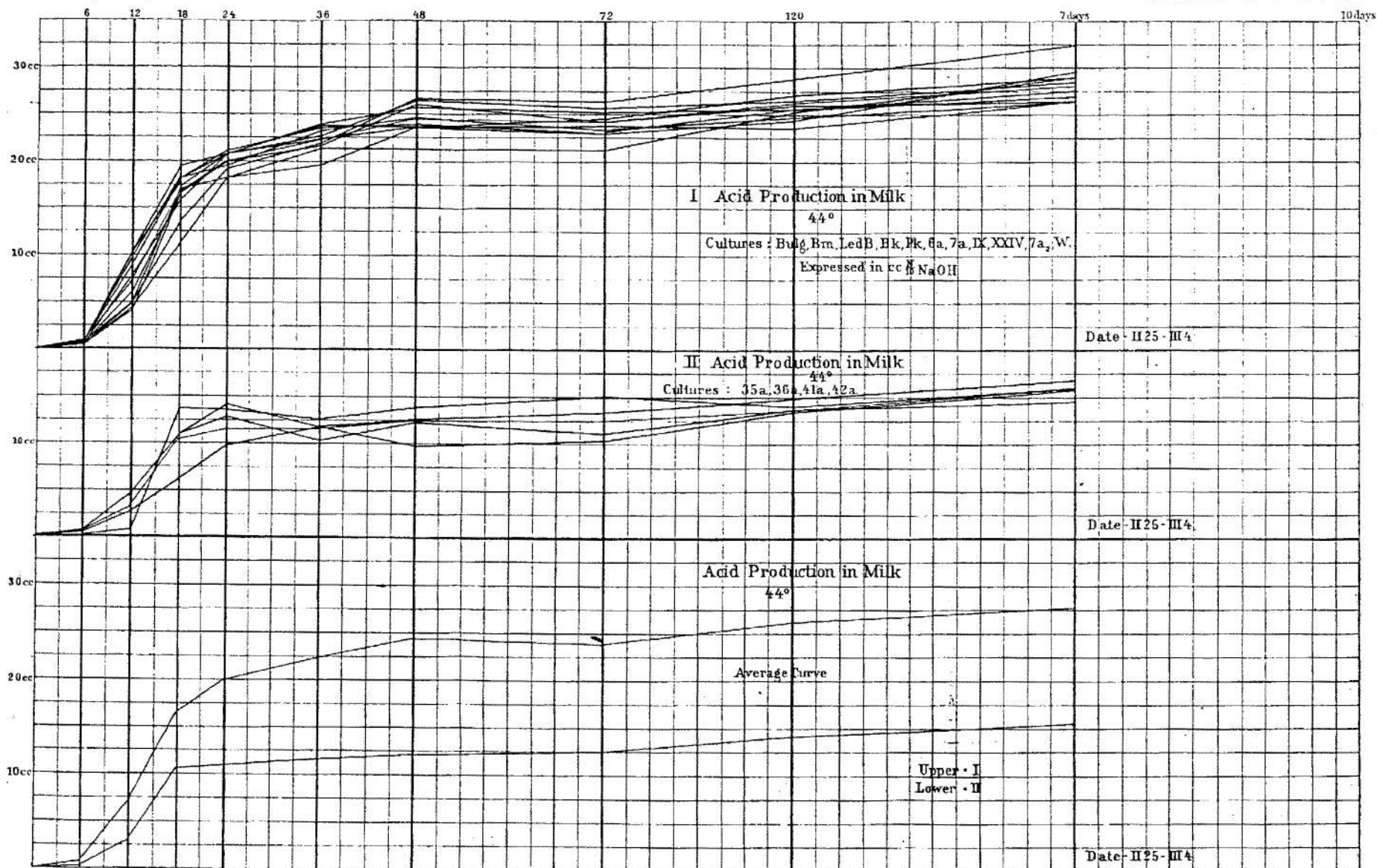
- Fig. 1. 48 hour (44°) whey culture, IX—1, Gram stain, showing nodules. 1 : 1500.
- Fig. 2. 24 hour (44°) whey culture, XXIV—1, Gram stain, showing chain formation. 1 : 1500.
- Fig. 3. 48 hour (44°) milk culture, 35a—1, Loeffler methylene blue, showing metachromatic granules. 1 : 1500.
- Fig. 4. 48 hour (44°) milk culture, 36a—1, Neisser stain, showing metachromatic granules. 1 : 1500.

##### T a f e l II.

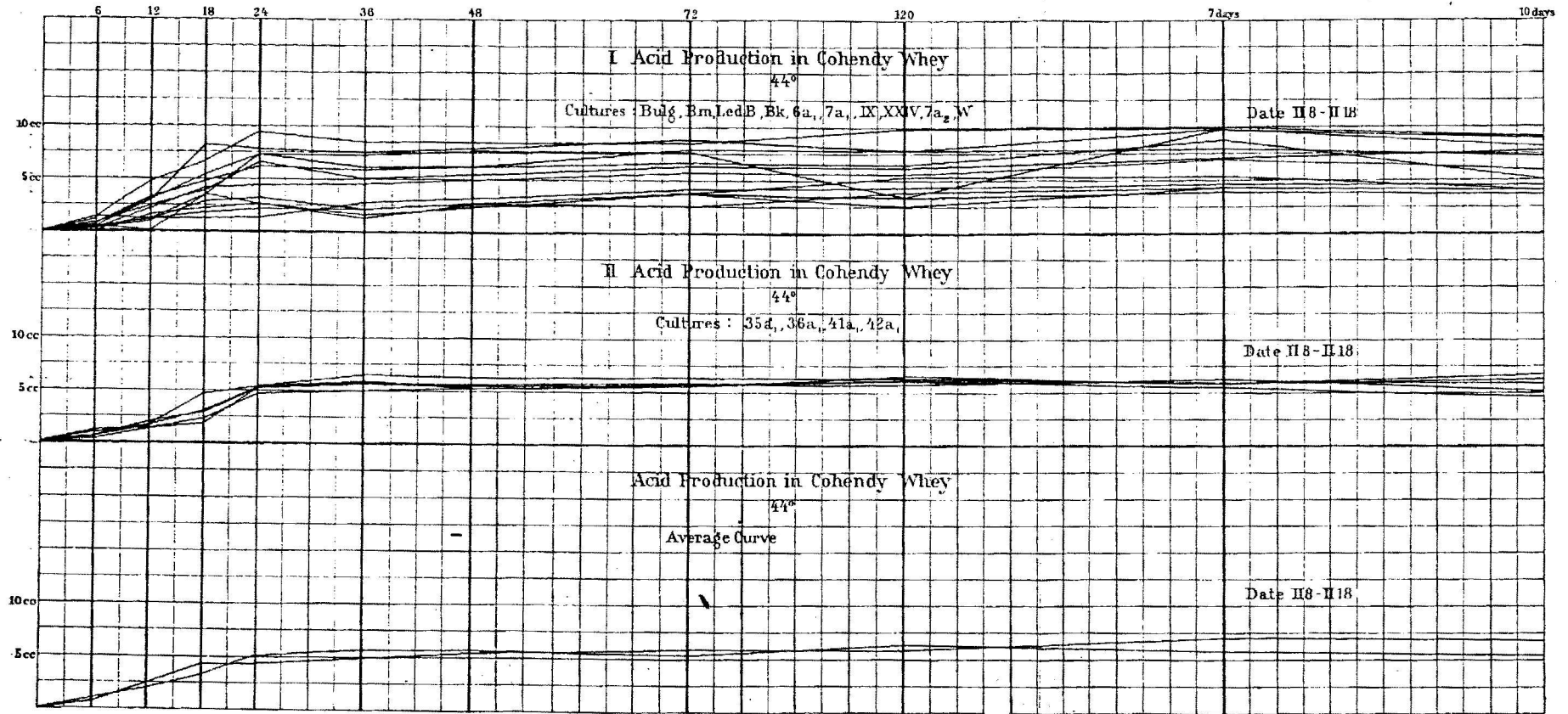
- Fig. 5. Colony of XXIV—1, whey agar plate, 7 days (44°). 1 : 70.
- Fig. 6. Same showing margin of colony. 1 : 350.











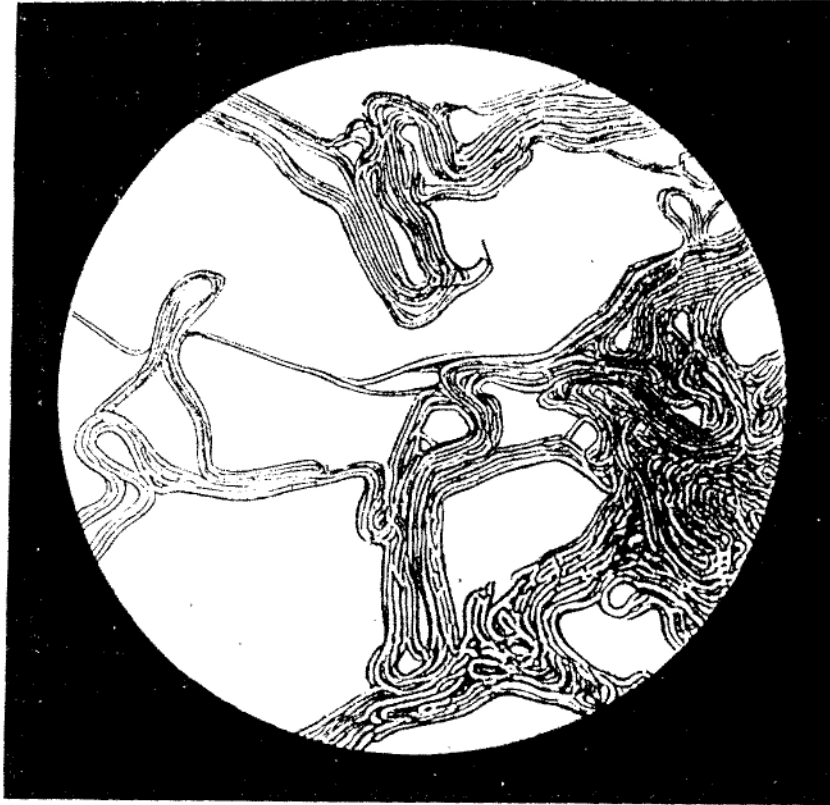


Fig. 6. Margin of colony shown in Fig. 5.  $\times 350$ .

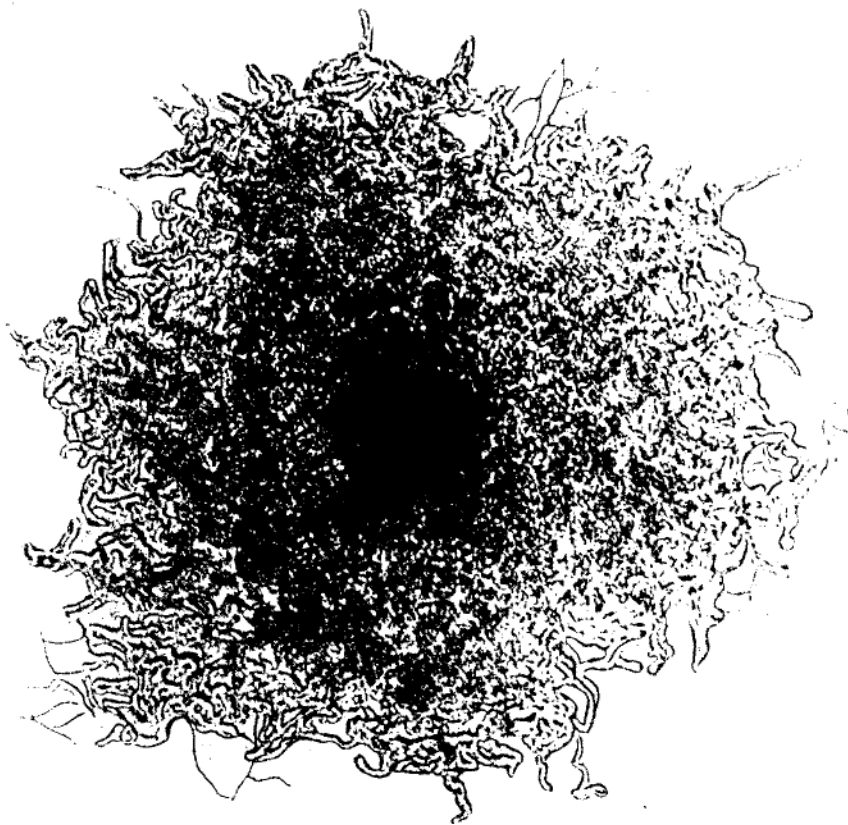


Fig. 5. 7 day (44) colony XXIV-1, whey agar plate.  $\times 300$ .