yond that obtained with either methylene blue or the substrate alone.

It is reasonable to suppose that the increased acetoin formation in the presence of added substrate represented an increased amount of pyruvate available for condensation with the excess acetaldehyde. The ineffectiveness of other members of the citric acid cycle, as compared with α-ketoglutarate, the effect of glucose in brain as compared with muscle, and the comparison of glucose with fructose or glucose-l-phosphate are noteworthy considerations in any interpretation of these results.

Further studies on the disappearance of acetaldehyde in liver brei were made with 0.3 cc of 0.15% acetaldehyde in the side arm. Rat liver removed about 200 γ acetaldehyde during the incubation; added glucose had no effect. Small increases (10%) in the amount removed resulted from the addition of pyruvate, lactate or alanine. 100 μM of citrate or 0.5 mg methylene blue increased the acetaldehyde disappearance by 25% and 40%. Dog liver gave similar results except that methylene blue had no effect. The methylene blue effect in rat liver as contrasted with dog liver is possibly related to the presence of xanthine oxidase in rat liver only; methylene blue stimulates aerobic oxidation by xanthine oxidase.

Summary. In agreement with previous *in vivo* studies, liver homogenate metabolized acetaldehyde more rapidly than other tissues and without the formation of much acetoin. Methylene blue stimulated the aerobic metabolism of acetaldehyde in rat liver but not dog liver.

Skeletal muscle formed more acetoin from acetaldehyde than did brain or kidney. Pyruvate, α-ketoglutarate and phosphoglycerate increased the acetoin formation from acetaldehyde by muscle; pyruvate, α-ketoglutarate and glucose were effective stimulators of acetoin formation in brain.

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17069. Isolation of an L Type Culture from a Gram Positive Spore-Bearing Bacillus.*

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During the last 10 years L type cultures have been isolated from many Gram negative bacilli. The author tried repeatedly to apply to Gram positive bacilli the methods which proved successful in Gram negative species. Success was obtained first in the summer of 1948 with a Gram positive bacillus isolated from a contaminated blood culture. The broth cultures of this bacillus were pleomorphic. After 24 - 48 hours of incubation almost all bacilli swelled to round bodies as in the cultures of some Bacteroides strains. This pleomorphic culture transferred to media of various compositions produced only bacterial growth, but when a penicillin cup was placed on soft horse-serum agar plates and the plates were incubated anaerobically, a few microscopical L type colonies developed near the edge of the zone of inhibition. Growth of these tiny colonies in pure culture was obtained by cutting out a block of agar containing the colonies and transferring it to horse serum agar containing 400 units of penicillin per cc. The colonies grew without difficulty in subsequent subcultures.

Following this observation several samples of dust and earth were planted on similar plates containing varying concentrations of

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penicillin. L type cultures were not observed in any of these plates. However, from one earth sample a large Gram positive bacillus was isolated which, when transplanted on penicillin plates, produced pleomorphic forms and tiny L type colonies. Neither the first nor the second Gram positive bacillus produced spores.

These Gram positive bacilli probably originated from the soil in which pleuropneumonia-like organisms are often present. Thus a contamination of the cultures with such organisms must be considered. Usually it is difficult to exclude this possibility and to prove that the L form grow from the bacilli. The needed proof was obtained with a third Gram positive bacillus. This bacillus produced spores and the L form grew in the same manner from the heated spores as from the original culture. This observation, while it excludes an accidental contamination of the cultures, does not prove in itself that the L cultures represent a changed growth form of the bacillus. They could be parasites of the bacilli and Klieneberger,[1] for example, even at present prefers this hypothesis. In the opinion of the author the evidence in the case of Gram negative bacilli[2-4] supports the conception that the L forms and the bacilli are growth forms of the same organism.

The third Gram positive rod was obtained from a thioglycollate broth inoculated from a wound. Growth in the first subculture developed only anaerobically, and on further subcultures also anaerobic growth was more abundant than aerobic. The colonies on blood agar plate incubated aerobically were flat and slightly irregular, and after a few days the culture spread as a thin film on the surface of the medium. Large elongated spores, usually at the ends of the rods, developed abundantly. A distinguishing property of the bacillus was its tendency to swell into large pleomorphic forms. Some of the bacterial threads spreading on blood agar plate autolyzed and disintegrated and the fragments swelled into small and large round forms. In thioglycollate broth some of the rods grew to large size and became markedly swollen around the developing spores. This tendency to form round bodies is rarely observed in Gram positive rods. Exact identification of saprophytic strains is difficult and has not been attempted. However, the development of spreading growth on the surface of the agar under aerobic conditions and the formation of acid without gas in various sugars makes it probable that it belongs to the genus *Bacillus*.

A thioglycollate broth culture of this strain was planted on soft agar plates containing 10% horse serum and 400 units of penicillin per cc. The plates were made anaerobic using Fortner's technic.[5] Use of a similar medium permitted isolation of L type colonies from various Gram negative bacilli.[3,4] The development of the third strain on this medium was similar to that of Gram negative bacilli which produced L type colonies. Many bacilli swelled within a few hours into large flat round bodies. From these tiny colonies developed which grew best in transplants on soft nutrient agar plates without serum. The tiny colonies on the original penicillin plates and in the subcultures are illustrated in the photographs. Their appearance and morphology were similar to the L_1 and to the other L type colonies obtained from Gram negative bacilli. The colonies grew into the medium and consisted of soft fragile cells which were often very small and had the tendency to swell into large bodies.

In order to determine whether L cultures can be obtained from heated spores, well-sporulated broth cultures were sealed in thin glass tubes and immersed in water at 85°C for 10 minutes. (Previous experiments had shown that the spores were killed in boiling water). The heated spores when transferred to penicillin plates produced no growth. Broth cultures of bacilli obtained from the heated spores produced L type colonies on penicillin plates as the original cultures.

[1] Klieneberger-Nobel, E., *J. Hygiene*, 1948, **45**, 407.

[2] Dienes, L., *Cold Spring Harbor Symposia Quant. Biol.*, 1947, **11**, 51.

[3] Dienes, L., *J. Bacteriology*, 1948, **56**, 445.

[4] Dienes, L., *J. Bacteriology*, 1949, in press.

[5] Dienes, L., and Smith, W. E., *J. Bacteriology*, 1944, **48**, 125.

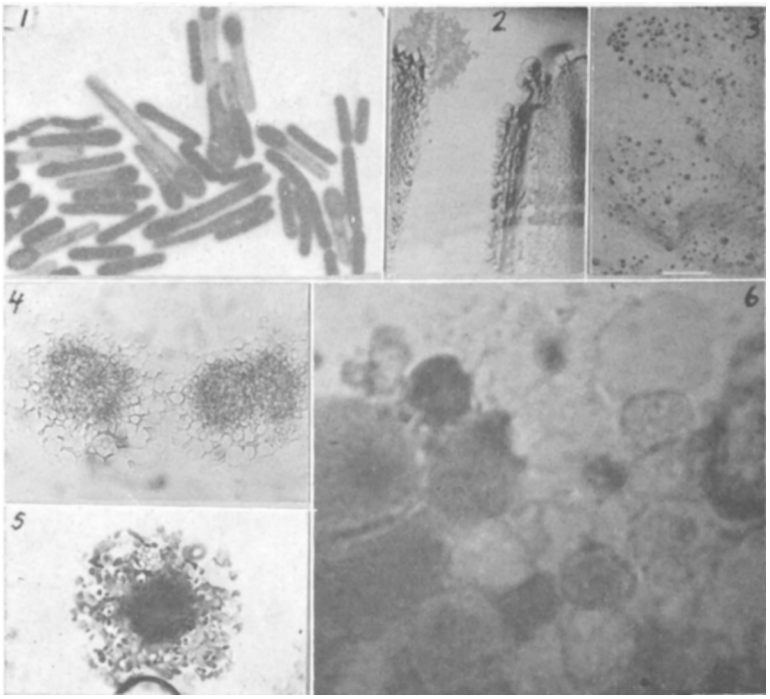

FIG. 1.

1. Bacilli from a broth culture. Slightly stained with crystal violet. (× 2000.)
2. Culture with spreading growth on aerobic blood agar plate. (× 2.)
3. Tiny colonies on penicillin plate after 5 days' incubation. (× 2.)
4. Tiny colonies from a subculture on a soft nutrient agar plate. Unstained. (× 100.)
5. A colony similar to those in Photograph 4 stained with methylene blue on the surface of the agar. (× 100.)
6. Large bodies in the periphery of a tiny colony stained with methylene blue. (× 2000.)

It was interesting to observe the development of the cultures when a soft agar plate was inoculated with heated spores and 100-500 units of penicillin was deposited in a small trough made in the agar. The plates were incubated anaerobically. No growth developed in a wide area around the penicillin trough. The penicillin diffused in this area before the spores germinated. Many tiny colonies developed in the adjoining zone. Here the spores germinated and the young vegetative cells were exposed to the diffusing penicillin. The tiny colonies consisted of large round forms produced by swelling of the vegetative cells and many L colonies began to grow from these large forms. Further away from the penicillin trough the usual bacillary colonies developed. The observations reported indicate that L forms were not cultivated directly from the heated cultures. They apparently survive with the bacteria and develop under appropriate conditions from the vegetative cells. The L strains isolated from the second and third bacilli were tested for heat resistance. Heating of the cultures for 15 minutes at 60°C destroyed their viability.

It is of interest also that descendants of single bacillary colonies varied considerably in their tendency to produce L type colonies and in the viability of these colonies. In one experiment 4 colonies were picked from a culture of bacillus No. 3 on a blood agar plate. Marked differences persisting in subculture were present among the descendants of these colonies. The descendant of one produced more L type colonies than the others and the

L colonies grew easier in subculture. Among the descendants of the colony which was least susceptible to the influence of penicillin, only a few bacilli swelled to large form when their growth was inhibited by penicillin and only a few L type colonies were produced. A similar individual behavior of different strains belonging to the same species has been noted in all species which produce L colonies, but the differences are usually apparent only between strains of different isolation.

The L type cultures obtained from Gram positive bacilli differ from each other and from those obtained from Gram negative bacilli in many respects, although their fundamental morphology and structure of the colonies conform to those of the pleuropneumonia group. The colonies obtained from the two bacilli mentioned first remained very small (10-20 μ) on the original plates and in the early transfers stained poorly with methylene blue. After several transfers they grew more abundantly. They grew equally well with or without horse serum in the medium. The hardness of the agar played an important role in their growth. In the absence of horse serum growth developed only on hard agar plates containing 1.5%-2% of agar. If the agar was softened by the addition of equal amounts of broth, it did not support their growth. Soluble starch markedly improved growth, while glucose, maltose, sucrose and penicillin exerted no influence. The colonies continued to increase in size for a period of two weeks. No growth could be obtained in nutrient or thioglycollate broth, although the colonies transferred with a block of agar into the broth continued to increase in size on the agar. In morphology these cultures were quite similar to the filterable strains isolated from sewage by Laidlow and Elford.[6] The cultures consist of very small (0.3-0.5 μ), fragile coccoid and bacillary forms and they swelled only to moderate size on the surface of the colonies. The strains of Laidlow and Elford grow abundantly in broth and aerobically on nutrient agar. In this respect they differ considerably from ours.

Young colonies of the L strain isolated from the third spore-bearing bacillus were more similar in appearance to the L cultures isolated from *Streptobacillus moniliformis* and Bacteroides and consisted of similar organisms. If the colonies were well spaced, they increased in size for several weeks extending as an opaque mass below the surface of the agar reaching the size of 1-2 mm. They grew best on nutrient agar in the absence of horse serum. In contrast to the other two strains growth developed only on soft agar plates and was inhibited by the usual concentration of agar. Sucrose seemed to favor their growth but its effect was not marked; starch exerted no influence. No growth was obtained in nutrient or thioglycollate broth. The original bacillary forms have not been recovered thus far from any of the three L cultures isolated from Gram positive bacilli.

Summary. Colonies similar in appearance and properties to the L type colonies of Gram negative bacilli were isolated from cultures of several large Gram positive bacilli. The L type colonies obtained from a spore-producing strain grew equally well from cultures obtained from heated spores as from the original culture, indicating that the L type colonies grew from the bacilli and were not an accidental contamination of the culture.

[6] Laidlow, P. P., and Elford, W. J., *Proc. Roy. Soc. London B.*, 1936, **120**, 292.

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