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CYTOGEN[illegible]S OF DIPLOID AND HAPLOID CULTURES DERIVED FROM BACTERIUM COLI, STRAIN K-12

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The isolation of diploid cultures of Bacterium coli, strain K-12, (Lederberg, 1949) has provided an avenue of approach to cytogenetics of bacteria. In this work, "diploid" refers to unstable prototrophs which arose from crosses of auxotrophic mutants, and which are heterozygous for one or more sugar fermentation loci, in which the parents differ. They are identified genetically by plating on a complete nutrient agar medium containing eosin-methylene blue indicator, plus the appropriate sugar. If lactose is added, for instance, this medium is called EMB-Lac. Since the ability to ferment sugar is dominant to its absence, a diploid heterozygous for lactose fermentation will produce dark, Lac + colonies on this medium, but light sectors will appear as the Lac - haploid conponent segregates during the growth of the colony. Such a strain is called Lac v (variegated colonies). The following laboratory abbreviations for media will be used in this paper:

EMB.....Eosin- methylene blue, complete (peptone)
EMS.....Eosin- methylene blue, synthetic, which does not support the growth of auxotrophs.
NSA.....Nutrient saline agar, Difco plus .5% NaCl.

The current investigation deals with a cytological comparison of diploids with their haploid parents, segregants, and wild type K-12. The methods employed are nuclear staining by Robinow's technique (Klieneberger-Nobel, 1950), and more recently, observation of living cells with a dark phase contrast microscope. Diploid cultures have been distinguished from haploid by these methods but it has not been conclusively shown that the difference depends on the number or size of the chromatinic structures which Robinow has called chromosomes (Dubos, 1945).

For nuclear staining, blocks of agar from a plate spread with bacteria are cut out and placed in small Petri dishes for incubation. At desired time intervals, dishes are removed from the incubator and inverted over small wide-mouthed bottles of Osmic acid. The vapor fixes the cells as they grow on the surface of agar; then, an impresion of the growth is printed on a coverslip. The bacteria on the coverslip are post fixed in Schaudinn's reagent, washed in 70% alcohol, water, cold normal HCl, and hydrolysed for

10 minutes in normal HCl at 60°C. They are returned to cold HCl, water, phosphate buffer at pH7, stained with Giemsa for 30 minutes, rinsed in buffer and mounted on a slide in the water soluble resin, Abopon. [1]

Observations of living cells can be made on similar agar sections cut from the same plate, provided a thin plate of colorless medium (NSA) is used. The small section is mounted between slide and Coverslip which have been sterilized by flaming, and the edges are sealed with manometer grease. [2] Then the slide can be incubated between observations or kept at room temperature on the microscope stage. This method for phase microscopy is based on that described by Stempen(1950), working with B.coli and Proteus vulgaris. He identified light bands in living cells with the chromatinic structures that stain with Feulgen and Giemsa. These light bands are visable in the series of pictures of K-12 (1--6). In this experiment, the lag phase was 3hours, and division time thereafter, about 45 minutes. No differentiation at all is visable in the earliest picture made before division began. At this stage, cells ~~of living~~ on stained slides are also small and poorly differentiated. (see pictures 7--9).

The same Bausch & Lomb research microscope with phase contrast aedessories is used for phase and for bright field photography. For the former, illumination is provided by a carbon arc lamp; for the latter, critical illumination from a ribbon filament lamp is used, with a Wratten B (green) filter. The camera is a Bausch & Lomb L type.

In pictures 21, 22, 23, living cells under phase contrast are compared with stained preparations from the same culture, photographed with the same 97X phase objective, and in bright field, with a 90X apochromatic objective. The phase pictures show that osmic acid fixation has not shrunken the cells appreciably. All these pictures are contact prints from 5 X7 Panatomic-X or Super- Panchro-press Eastmen Kodak film. Differences

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1. Staining reagents:
   Schaudinn's reagent: $HgCl$, 10gms.; H_2O, 200cc; Ethyl alcohol, 100 cc.
   Giemsa stain: National Aniline Div. Allied hem. and Dye Corp.
   Dilute, about 5 drops in 10 cc H_2O.
   Abopon: Glyco Products Co. Dilute with heating, 2 prts to 1prt. H_2O.
2. Equal parts lanolin and Vasolin.

in magnification were obtained by using different microscope lenses, or varying the extension of the camera bellows.

Different stained preparations of the same strain show a rather wide range of variability in size of cells and appearance of chromatinic structures. Often the source of variation is unknown and a particular aspect is not always reproducable. In general, the appearance of haploid K-12, during the logarithmig growth phase agrees with the earlier similar study of B. coli by Robinow. (Dubose, 1945). See pictures 7--17 and 24--29, for culture cycle series of K-12 on NBA at 23° and 37°C. Note the symetry of adjacent chromatinic structures that obviously came from a recent division perpendicular to the long axis of the cell. In picture 27, for example, a number of cells contain two sets of double structures that look like anaphase figures. Each is symetrical with respect to the other set and with respect to its two halves. Few cells are seen with less than two distinct rods, but this is probably a matter of inability to resolve the very young cells (pictures 7, 24,25). This interpretation is the same as Robinow's. If each rod is a chromosome, as he believes, the symetry of the paired structures indicates that they are chromatids, rather than two separate chromosomes, and the nuclear unit is probably one chromosome which may divide several times prior to cell division. This agrees with the genetic evidence for one linkage group in K-12 (Lederberg, 1947).

As cells increase in size, permitting better resolution, of the chromatinic structures, they tend to change from condensed rods to thinner, more numerous bodies. (compare picture 27 with 28 and with 30) The same tendency is sometimes noted in K-12 grown at room temperature as compared with 37°C. (Compare 10 with 27). Sometimes haploid cultures in this stage, approach the appearance of diploid cultures where the occurrence of larger cells with relatively disperse chromatinic structures is much more regular.

Since diploids are continually segregating, impressions from plates of complete media give mixtures on the slides of various proportions of diploid and haploid cells. Diploidy of the inoculum must be verified by heterozygosity tests for the fermentation of some sugar. Of course, there is no method of characterizing single cells from a fixed preparation. It is not proven that the large cells with relatively disperse chromatin are diploid, but whatever is rosponsible for their occurrence is more effective in diploid than in haploid cultures.

To test an observer's ability to differentiate between haploids and diploids under identical conditions, some experiments were designed as follows: A Lactose heterozygote was plated on EMS- Lac. Instead of variegated colonies, here, a Lac v cell will usually produce a pure Lac + colony because the medium lacks the amino acids necessary for the growth of most of the segregants. A few prototrophic segregants will occur on the same plate, and those which are Lac + cannot be distinguished from the diploid colonies except by restreaking on EMB-Lac. Using suspensions of single Lac + colonies as inocula, it has been possible to predict which will be diploid from the cytological appearance of the bacteria on the EMB plate after 3 - 5 hours of growth at 37°C, and before the genetic evidence became available the next day.

Because of reports from other laboratories on staining bacteria infected with bacteriophage, (Luria, 1950), it was thought that the presence in K-12 of the lysogenic phage , Lambda, might be affecting the appearance of chromatin in both diploids and haploids. One experiment shows that this is probably not so. Dr. Esther Lederberg provided the stocks from which both lysogenic and λ-free diploids could be synthesized for purposes of comparison. (see pictures 30 -- 35) One parent, W-588 is like K-12 in that it is lysogenic, resistant to the phage it carries. The other, W-1248 is non-lysogenic and resistant to λ. It was derived from a sensitive strain, W-518 by selection with phage. Cytologically, there is no consistent difference between the lysogenic and non-lysogenic haploids. A cross was made by the EMS plating technique, and Lac v colonies were selected. (Lederberg, 1949) In cross streak tests with the sensitive strain, one diploid caused no lysis. It is λ-free diploid, H-232, is cytologically indistinguishable from those previously examined and from a λ + diploid isolated from the same cross. Apparently, λ cannot be detected by this staining method.

The interpretation that granular chromatinic structures, characteristic of diploid cultures, represent two homologous chromosomes, compared to the one in haploids, is an inference from genetics, which this cytological evidence neither supports nor disproves. There does not appear to me twich as much chromatin in the large cells as in those from aploid cultures, but Dr. Hans Ris has suggested an analogy to the spermatocytes in insects of the X O sex type, where the unpaired X may be heteropycnotic and appear at meiotic metaphase, just as dense as the other chromosomes which are paired.

Two diploid stocks have proved parti ularly useful for cytological experiments because the characteristic chromatinic structure is very pronounced. They are H-226 (Lac 1/4 v 2/4 v Mal v Mtl vXylv) and H-267 (same as H-226, but also segregating for streptomycin resistance). (pictures 38-41, 42-45) This heterozygosity for so many characters, (including maltose fermentation, which is usually hemizygous) may mean that they actually have more comp ete nuclei, than the "aberrant heterozygotes" previously examined. (Lederberg 1949). They are relatively stable diploids. Maintained in liquid minimal medium , a high percentage of the cells remain diploid and their segregation can be observed when they are plated on EMB or NSA. To make the impression slided used for photographs 46- 49, about 10^5 cells were spread on an EMB p ate and fixed after about 5 hours growth. Two cell sizes are very d stinct. When the micro-colonies seen at low magnification (46) are resolved, (48, 39), some are seen to consist of uniformly short cells with 2 or 4 compact nuclear bodies. Others consist of bigger and much onger cells which often have their chromatin neatly distributed in aggregates of small granules. Some microcolonies contain both types of cell in sectors. The obvious assumption is that the short cells are haploid, the long ones diploid, and the mixed microcolonies arose from diploid cells that segregated.

Again, there is no direct proof of these identities, but the folloging lines of evidence are now being followed with the aim of describing the cytology of diploids and haploids on a cellular, rather than a cultural basis:

1 - Comparison of cultures from genetically known segregants and diploids:

Practically all the segregants from H-226 and H-267 are Lac - because the diploids are heterozygous fro the two closely linked Lac1 and Lac4 loci. All Lac - colonies on an EMB plate are segregants and the two parental Lac - loci are distinguishable as slightly different shades of light colonies. A few cultures were prepared from H-226 segregants identified in this way and were found to consist of uniformly short cells. (Pictures 38-41) It has not been determined whether they are consistently smaller than the parent strains, as the small cells in the mixed clones from diploids seem to be.

2 - Identifying characteristic staining types with specific sizes of living cells, and subsequently characterizing the living cells by observing their habit and rate of growth or by genetic means, or both.

3- Comparison of series of stained preparations of these diploids under conditions known to produce abnormally high proportions of haploid cells. Methods 2 and 3 will be discussed together:

Growth rate experiments comparing haploids and diploids by usual culture plating techniques have not been attempted because of the difficulty of maintaining diploid cultures owever, direct information on growth rates comes from Zelle's (1951) experiments with these strains. He separated single cells with a micro- manipulator, watched them grow into micro-colonies and then picked them up and identified them genetically. Diploid pedigrees show that when segregation occurs, one cell divides to form one which is haploid and one, still diploid. He has observed that the haploid grows faster.

In my own experience, Dark phase contrast observations of living bacteria have been helpful in establishing growth rates and observing inter and intra clonal size variation, but have been little help, so far, in clarifying the nature of the chromatinic structures. In different experiments, the lag phase and division time of the same strain at room temperature, have not always been the same. No effort was made to keep the temperature constant on the microscope stage and other sources of variation between experiments are the concentration of bacteria and the thickness and moisture content of the agar. The series of K-12 (1--6) at 27 °C is probably roughly comparable in hours to the one of H-267 (61 --69) for which the plates were incubated at 37°C for the first 45 minutes, and subsequently grown at room temperature, which was 23°C on that day. It is obvious that division proceeded faster in the K-12 series. In one experiment the growth rate and general living appearance of the parents of H-267, were found to be similar to K-12. (pictures 50 -60).

In the diploid series (61 - 69) there are two distinct cell sizes "very small" and "medium". The growth of four cells can be traced separately for 8½ hours. Cells 5 and 6 were apparently dead when plated. No. 3 produced uniformly small cells from the beginning and divided at a consistently faster rate than the other three. Nos. 1,2, and 4 produced cells of a uniform medium size except for one "smake" in clone 1. It seemsreasonable to assume that no 3 was haploid and the others diploid at time of plating. The slide was left at room temperature over night and the same field observed the next morning (69). There was no obvious change in the proportion of very small to medium cells,

and this field seemed to be representative of the whole slide. After picture 69 was made, the cells were scraped from the agar and streaked on EMB- Lac. The result was mostly Lac- colonies, indicating almost complete segregation. This seems to contradict the assumption that all the medium cells in picture 69 are viable diploids. It is possible that many of the cells were dead. The slide was not watched long enough on the second day to determine whether division was still taking place. Perhaps the high proportion of haploid segregants was caused by differential survival rather than complete segregation. Corresponding stained slides of the 21 hr. plate might have been informative, but they were not made because slides from such crowded plates usually show very little or are difficult to interpret. Picture 29 was made from such a preparation of K-12. If the large number of ghosts represent dead cells, and the only living ones are those containing chromatin, then it is obvious that such a population has presented a wide opportunity for selective action. Possibly this is the way in which acid production by growing bacteria acts to increase the proportion of haploids. This is a known effect of artificially lowering the pH of culture medium (Lederberg, unpublished). If this idea is correct, one might expect stained slides of a preparation such as picture 69 to show patches of short cells containing chromatin, corresponding to the center clone in the picture.

In comparing stained with living cells certain technical differences must be kept in mind. For fixing and staining, it is usually practical to plate a lower dilution of a culture than can be used for observing growth. Even sections from the same plate are subject to different conditions under the microscope. Besides periodic exposure to a source of light and heat, they differ in that they are growing anaerobically in contact with a cover slip. It is possible to cover the sections which will be used for fixation in a similar manner and fix from the coverslip rather than the agar. A very little experimentation with these alternate methods indicated that there is little difference, but the agar method has been used here because it seemed to result in better stains, and so the question of variation still exists.

In spite of these limitations, similarities between stained and living cells of the same age are close enough to cause little hesitation in identifying the medium cells (in picture 65) with the cells cantaining "diploid type nucalei" that make up the majority of the population of pictures 73 -75, and the fewer very small cells (in 73 -75) probably represent

the type in clone 3 of the living series.

This series was run as a control in parallel with another plating from the same culture after Ultra -violet treatment. A 1/10 dilution of the culture was irradiated for 20 minutes at 50cms. Immediate plating from treated and control on EMB - Lac showed that survival (in the dark) was about 40%. The preparation which was observed after 45 minutes, and photographed at intervals thereafter may have been subject to light reactivation (Kelner, 1949) from the arc lamp. The control was observed on a separate block of agar on the same slide, and photographs were taken alternately. (61 -91).

One effect of Ultra-violet treatment of diploids is a greatly increased proportion of segregants. Here, the assay plates showed 60% more Lac - colonies after treatment. However, it is dificult to count these accurately because many colonies that appear negative after 24 hours develop tiny Lac + centers when incubated for another night, indicating the delayed growth of one or a few diploid cells.

In the U.V. series (77 -91) again assuming that the very small cells are haploid, there is evidence for true segregation during the first few houss.[1] In pictures 83-85, there are perhaps some of the medium sized cells in clones 1 and 4, but most of the population consists of the very small and a third "very large" type. One of these was produced at the first division of cellNo.6, while its sister cell produced many of the small type. No.2 never produced anyting but very small cells, like No3 in the control. Nos. 3,and 5 probably consist entirely of the very large , slow growing type, although this is hard to determine because both clones have merged with neighbors. The very large cells also occur in the control, but less frequently (pictures 70 - 72) This "type" is probably a heterogenous result of many different effects, but the tentative suggestion is offered that some of them are cells which are capable of giving rise to diploids at a later time, thus accounting for the delayed appearance of diploid centers in haploid colonies.

[1] The size differences have been accentuated

The three sizes are also clearly distinguishable in stained slides of the treated preparation, (18 - 23) but the typical "diploid appearance" is conspicuously rare. Note (In 21 - 23) that the very large cells containing disperse chromatin appear mostly dark in phase contrast, as do the very large living cells. This is probably due to their thickness rather than their internal structure. The type containing condensed chromatin (black

in 22, or bright spots, in 23) are not so easily related to any of the living cells at a comparable time. They may correspond to the snake with the clear area in the center in picture 91. After 21 hours such clear spots had occured in practically all the very large cells. They showed no further change when observed an hour later. However these light spots and square areas in living cells may represent some phenomenon entirely different from the condensation of chromatin.

In a previous irradiation experiment with H-226 (no photographs) many snakes similar to the one in picture 91 were observed after 5 hours at room temperature. One which was watched for two hours was alive, as evidenced by two unequal divisions giving rise to two small cells from one end. (similar to picture 84, clone3) There were various bright bands and spots in this snake, that persisted for a while, then grew smaller and after the first hour, disappeared completely, leaving the snake homogeneously dark. The first division occured immediately after their disappearance. In another half hour the ~~spe~~ spots reappeared in the same areas, then disappeared, and the second division occured. In the same culture, a number of cells having persistent ~~light~~ bright bands, showed no signs of life.

It is not clear how the light area arose in picture 91. Its position corresponds to a spot in the previous picture that looks like a constriction, but may be the beginning of the light area. Another, though less spectacular example, occurs in the untreated haploid snake in pictures 53 - 55. Apparent constrictions of the right end (Picture53) have become prominant light spots (in 54) and disappeared completely (in 55) No interpretation will be attempted until this phenomenon has been reproduced and ~~and~~ more carefully studied.

Another feature of the H-226 experiment was the lysis of many of the cells, presumably due to U.V. activation and liberation of lysogenic phage. (Lwoff and Delbruck, unpublished) From a field of six cells, under observation, three disappeared during the fouth hour after treatment, leaving only faint ghosts. This was observed only onee in the H# 267 experiment: In pairs 89 - 91 the long cell in the corner of picture 90 (out of focus) was not there the next morning.

SUMMARY:

In summary:

Diploid cultures of B. coli, strain K-12 have been distinguished from haploid cultures by means of nuclear staining. The former have a high proportion of relatively large cells with a distinctive type of chromatinic structure.

The presence or absence of the lysogenic phage , lambda probably does not affedt the ~~appearance of cil her~~ staining reaction of either diploids or haploids.

In certain relatively stable diploid stocks, known to be heterozygous for a large number of factors, two cell sizes are particularly distinct and can be shown to be localized in microcolonies, suggesting clonal growth from large diploid and small, segregant cells.

Preliminary studies of these stocks by observation of living cells with a dark phase cantrast microscope tend to support this hypothesis, and comparison of living and stained cells from the same culture indicated that the type of chromatinic structure characteristic of diploid cultures, occurs predominantly in diploid cells.

Ultra-violet irradiation of diploids causes some haploidization, for which there is parallel cytological and genetic evidence. Irradiation also has a specific effect on the growth habit and the nuclear material of some cells. This may be correlated with a delayed growth of diploid cells.

References:

Dubose, R. J. The Bacterial Cell; Addendum by D. F. Robinow, Harvard, (1945)

Kelner, A. Proc. Nat. Acad. Sci. 35, 73 (1949)

Klieneberger-Nobel, E. Quart. J. Micr. Sci. 91, 340 (1950)

Lederberg,J. Genetics 32, 505 (1947)

Lederberg, J. Proc. Nat. Acad. Sci. 35, 178 (1949)

Luria, S. E. Science111, no.2889,507 (1950)

Stempen, H. J. Bact. 60, 81 (1950)

Zelle, M. R. & Lederberg, J. J. Bact. 61, 351 (1951)