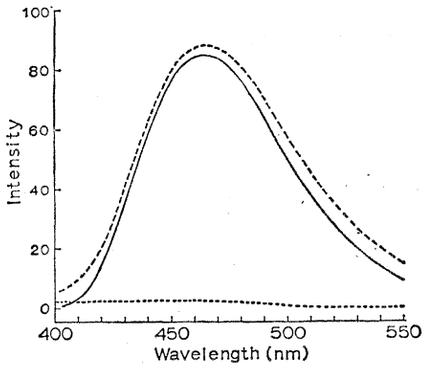

Fig. 2. Luminescence spectrum of luciferin plus luciferase (solid line) and fluoresence spectrum for the equimolar ($3 \times 10^{-6}M$) mixture of oxyluciferin and luciferase on excitation at 360 nm (broken line) in a mixture of $0.05M$ sodium phosphate and $0.1M$ NaCl, *p*H 7.4 at about 0°C within 15 seconds of the mixing. In reference to the latter spectrum, fluorescence without oxyluciferin (dotted line) and without luciferase (not shown) were both negligibly low.

ase with these substances. The data (Fig. 3) indicated that 1 mole of oxyluciferin binds hydrophobically per mole of luciferase, and, assuming that the increase in fluorescence is proportional to the amount of the oxyluciferin–luciferase complex so formed, we calculated the dissociation constant K_D to be $3 \times 10^{-7}M$ at about 0°C. The same result was obtained from titration data in terms of the amount of quenching of protein fluorescence taking place at 345 nm after being excited at 280 nm. Titration of luciferase with etioluciferin showed that the binding is equimolar, and that the calculated dissociation con-

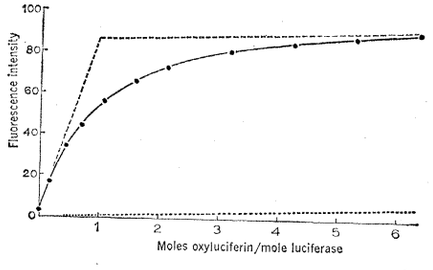

Fig. 3. Fluorometric titration of $2 \times 10^{-6}M$ luciferase with $10^{-4}M$ oxyluciferin, in a mixture of $0.05M$ sodium phosphate and $0.1M$ NaCl, *p*H 7.4. To minimize error due to hydrolysis, various amounts of oxyluciferin were quickly added to 1-ml portions of luciferase solution at 0°C, and the fluorescence measurements were done within 10 seconds of the additions. The broken straight lines are tangent to the titration curve at zero and at large excess concentrations of oxyluciferin, respectively. The dotted line shows the result when luciferase was omitted.

stant, $K_D = 2 \times 10^{-6}M$ at 25°C, was larger than that of oxyluciferin by nearly an order of magnitude. Attempts to obtain a value for the K_D of the luciferin-luciferase complex by methods of equilibrium dialysis or with Sephadex G-25 were unsuccessful because of the extreme instability of luciferin and the very small value to be expected for K_D which should be considerably smaller than the Michaelis constant of $5 \times 10^{-7}M$ at 25°C (*7*).

The foregoing evidence strongly supports our conclusion that the equimolar complex of oxyluciferin with luciferase in an excited state is the light-emitter of *Cypridina* bioluminescence. Moreover, the presence of only one hydrophobic binding center for oxyluciferin on the luciferase molecule suggests that the catalytic center for the light-emitting reaction of luciferin is also only one, and that these centers, if not identical, must be very close together.

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9. This hydrolase activity conceivably could indicate the presence of a contaminating enzyme, undetectable by ordinary methods, in the luciferase preparation. It could also indicate a property of the luciferase itself. While available evidence is insufficient to distinguish positively between the two alternatives, the ratio of luminescence activity to hydrolase activity was constant in the various fractions of the final chromatography, thus favoring the view that the hydrolase activity is indeed attributable to luciferase. In any event, the hydrolase activity can be eliminated as a significant factor in the luminescence reaction merely by using appropriate concentrations of luciferase and luciferin. Thus it has no essential role in the luminescence reaction, and any significant relation of this hydrolase activity to that of firefly luciferase is rather obscure [M. DeLuca and W. D. McElroy, *Biochem. Biophys. Res. Commun.* **18**, 836 (1965)].
10. Aided by NSF grants GB-4086, GB-6836, and GF-143; ONR Project NR-108-860; the Eugene Higgins Fund allocation to Princeton University; and facilities provided by the Whitehall and John A. Hartford Foundations. We thank R. Hyde and B. Bamman for the ultracentrifugal analyses.

17 February 1969, revised 20 March 1969

Centrioles of a Human Cancer: Intercellular Order and Intracellular Disorder

Abstract. *A continuous, symmetric, and periodic pattern of long-range spatial order has been observed among the centrioles of both normal and neoplastic cells. Consecutive ultrathin sections of lethal human esophageal cancers have revealed a defect in the centriolar angle with random angularity and random, long, intercentriolar distances.*

One of the most interesting revelations of electron microscopy has been that ubiquitous constancy with which gross macromolecular aggregates comprise a geometrical pattern of fine structure by which centrioles can be readily identified (*1*). Furthermore, at least among nondividing somatic animal cells, the two centrioles of a single cell are close to each other and are regularly disposed so that their long axes form a right angle, a conspicuous exception to the free-form curvatures that characterize these dimensions of biology (*2*). Heretofore unasked is the question of whether the constant geometry of centriolar ultrastructure and the constant orthogonal relationship of the two centrioles of a single cell is part of a larger intercellular pattern of spatial order among these tiny organelles. This can now be studied, for a procedure has been perfected which consistently yields information from an unlimited number of consecutive sections (700 Å thick) of intact tissues prepared for ultramicrotomy and electron microscopy by conventional methods (*3*).

To minimize the effect of complex time-variable functions upon the spatial orientation of centrioles, tissues were fixed *in situ* or promptly after atraumatic excision, and sections were cut consecutively from the tissue-fixative interface. The area selected for study was centered in a precisely cut block face (0.14 by 0.28 mm) oriented normal to the knife edge. One thousand consecutive serial sections (700 Å thick) were then cut as straight ribbons, none of which exceeded a length of eight sections, each consecutive ribbon being mounted in exact alignment with an unobstructed slot (0.12 by 2.5 mm) in a special specimen grid. Each mounted ribbon was then permanently inserted into a separate electron microscope specimen holder, after which it was checked by electron microscopy

for perfect technical quality and for accuracy of slot alignment. Low-magnification (× 2000) survey electron photomicrography was not begun until an adequate sequence had been verified. Twenty plates (8.3 by 10.2 cm) were required for complete survey of each section, six sections being surveyed per day or one cell thickness per month. Each day's original negative plates were processed overnight and were laid out on a large transilluminated surface in accordance with the pattern in which they were exposed, after which they were analyzed for centrioles with a hand lens (× 4). After higher magnification (× 8000) verification plates of each centriolar section were exposed, a registered overlay tracing was made of every 14th section, and all of the centriolar outlines encountered in this 1-μm thickness of tissue were transferred onto the tracing. When a sufficient number of consecutive sections had been analyzed, all of the interval tracings were registered into a single composite drawing.

Although they measure slightly less than 1 mm in diameter on survey plates, with practice, centrioles are not difficult to identify, because their sections have characteristic shapes and intense electron opacity, and they can be followed from section to section, finally being verified with higher magnification plates. In using this procedure one must be prepared for a dishearteningly scant yield, since most cells require more than 100 slices for complete sectioning and there are only two centrioles in each cell. The centriole is a short cylindrical organelle slightly less than 0.2 μm in diameter and 0.5 μm long; hence, if its long axis is nearly parallel to the cutting plane, it is visible in only two sections; if perpendicular to the cutting plane, at most six sections are seen, and, with obliquity being the usual case, one averages only three consecutive sections. After tracking many thousands of centrioles throughout the large voids of serial sections one becomes most suspect of reports based on random centriolar sections (*4*).

The foregoing procedure has now been used to analyze the spatial distribution of centrioles in a variety of normal tissues in fish, mice, and man; certain normal mouse cells exposed *in situ* to an artificial electromagnetic field; and a variety of spontaneous human tumor cells (*5*). I now report observations made on ten patients with esophageal cancer from whom tissue was obtained by direct incision immediately after exposure of the lesion preparatory to resection. This particular malignancy was selected for study since it is one of the most uniformly lethal human cancers (*6*). All ten primary tumors were identified as squamous cell carcinomas by light microscopy, and although they varied widely in degree of differentiation, their centrioles presented a remarkably consistent pattern. The individual centrioles of these cancer cells were of uniform, normal, short, cylindrical shape and size, with a conventional architecture characterized by nine straight triplet microtubules equally spaced about the

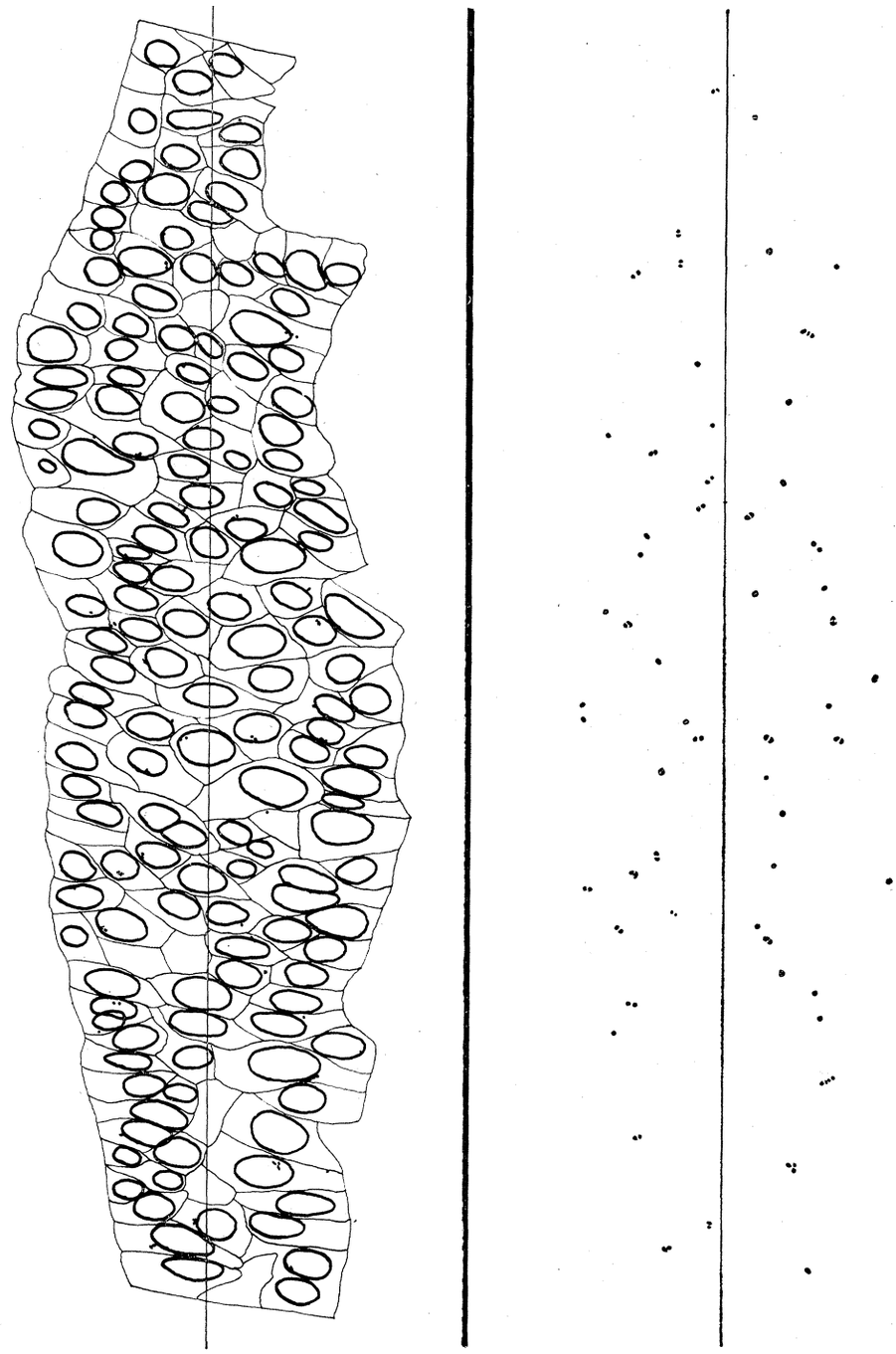

Fig. 1. (Left) Registered tracing of the centrioles, nuclei, and cell walls of 155 cancer cells seen in 1400 survey electron photomicrographs of 71 consecutive sections (700 Å thick). (Right) Registered tracing of the 87 centrioles verified as being all of and the only centrioles present in these 71 consecutive sections. (The vertical black line in the center of each half of the drawing represents the axis of this solid cord of cancer cells and is drawn to an actual length of 0.3 mm.)

circumference of the cylindrical cross section, each triplet with a constant angle to its respective tangent and with no helix evident along the length of the cylinder. Each outside centriole was connected to the cell wall by nine distinct groups of transitional fibers and continued outside the cell as a short cilium, rarely exceeding 1 μm in length (*7*). The normality of these individual organelles was further evidenced by their long-range spatial relation to each other (Fig. 1). The left side of Fig. 1 is a registered tracing of 1400 original, negative, survey electron photomicrographic plates (8.3 by 10.2 cm) ($\times$ 2000) representing 71 consecutive 700-Å sections of 155 cells comprising a single solid cord of the cancer as it invades deep within the esophageal musculature. This tracing has much the appearance of one that could have been made by light microscopy had these 71 ultrathin sections been cut as a single slab 5 μm thick. The large difference is that superimposed fine structures would have thwarted certain identification of more than half of the 87 centrioles which in fact were seen and verified as being all of and the only centrioles present in these 71 sections. In the right half of Fig. 1 is a tracing of these 87 centrioles at their exact locations in the intact tissue. Here is seen a far from random arrangement; indeed, there is a long-range and essentially normal pattern of order in which the centrioles deviate from the axis of the cord in a continuous, symmetric, and periodic manner. Large voids characterize the areas without and within the intersecting curves that could be drawn along the lines of maximum centriolar concentration.

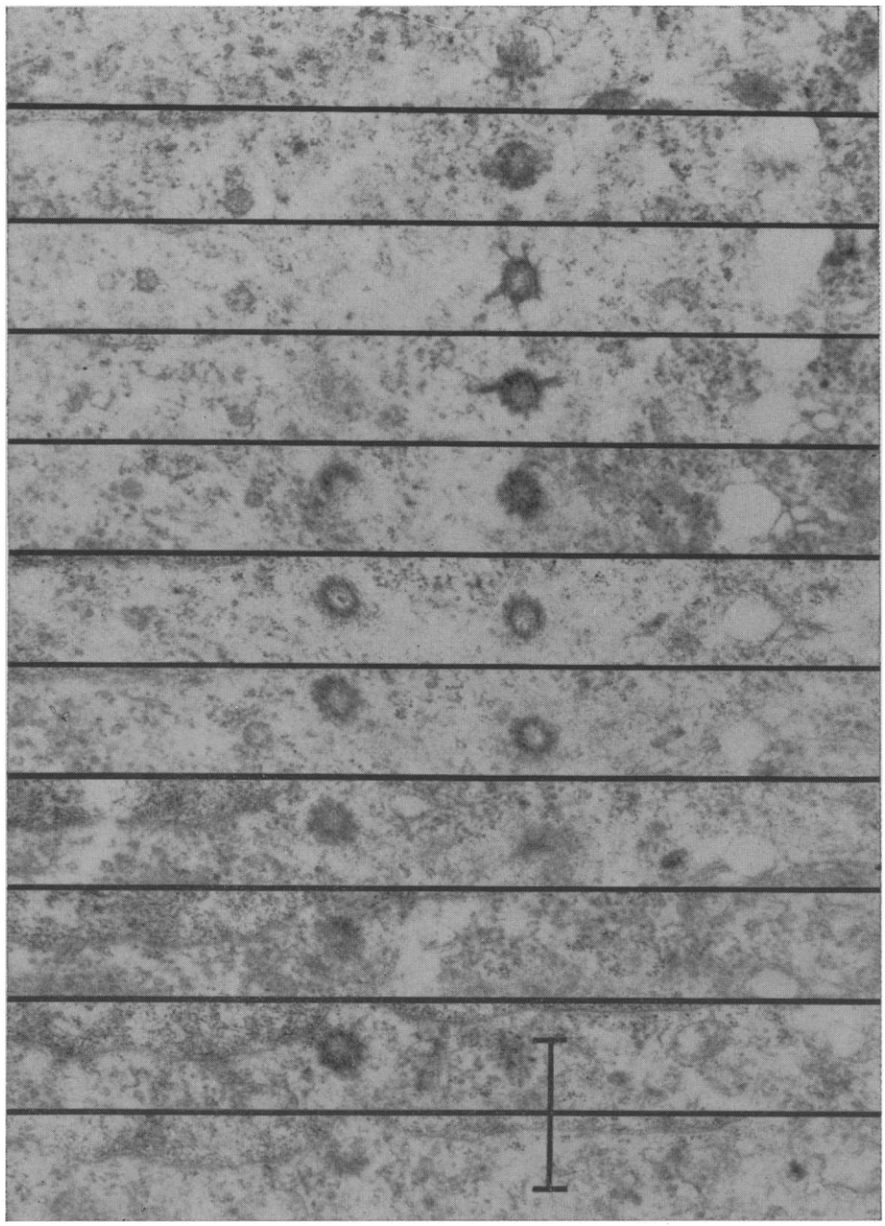

Fig. 2. Eleven consecutive registered serial sections (700 Å thick) through the entire lengths of the two centrioles of cancer cell No. 111 of this series. At left, the inside centriole is cut from its inside to its outside end in the bottom seven sections. The inside end of the outside centriole is first cut at right in the fourth section from the bottom, its outside end is attached to the cell wall by transition fibers and it continues outside the cell as a short cilium in the top two sections. The two centrioles of this cancer cell are parallel to each other. Scale, 1 μm.

However, there the normality ends, as was found on exposure of verification plates at a higher magnification ($\times$ 8000) of each of these centrioles. The two centrioles of single nondividing cancer cells were separated by long random distances and never were found closer together than 0.8 μm. An unusual absence of ultrafine structural elements was observed in the wide interval between them and, equally astonishing, the long axes of these centrioles instead of being mutually perpendicular, were randomly oriented. In one cell the two centrioles were close enough together so that the 11 consecutive sections which cut both centrioles throughout their entire lengths can be seen (Fig. 2). From the bottom up in seven consecutive sections, the inside centriole is cut from its inside to its outside end. Starting with the fourth section from the bottom, the outside centriole is cut from its inside end through the transition fibers which attach the outside end to the cell wall. In the two top sections it can be seen to emerge outside the cell as a short cilium. Although each of these two centrioles is canted slightly oblique to the cutting plane, in these 11 consecutive registered serial sections it is obvious that they are nearly parallel to each other, and there is not even a suggestion of their being connected by ultrafine structural elements in any one of the four sections wherein they approach each other most closely. To whatever extent ordered growth and reproduction of normal cells relates to a regular 90-deg angulation between the long axes of the two centrioles of

each cell and to their close constant physical proximity, to that same extent these relations are absent in this cancer that kills with uniform regularity.

There is increasing awareness that understanding of cell reproduction entails consideration of more than the molecular particulars of chromosomes and their sequelae (*8*), and the centriole more than any other cell organelle must be included in such a consideration (*9*). Compared to the highly ordered reproduction that characterizes normal cells, malignant tumor cells reproduce as if they had been released from a critical regulatory restraint (*10*). The observations reported here may be interpreted in widely varying ways, but inescapable will be the need to explain the pattern of long-range physical order that has now been observed among the centrioles of both normal and malignant cells. If one abjures vitalism, much will be found to commend a hypothesis that centrioles interact electromagnetically; indeed, such a centriolar function may have much to do with that inexorable continuum that has characterized the diverse evolution of cellular life. Human esophageal cancer clearly represents a discontinuity in that continuum, and the data presented here strongly suggest the presence of a defect in integrity of the centriolar angle.

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24 March 1969

Lesch-Nyhan Mutation: Prenatal Detection with Amniotic Fluid Cells

Abstract. *Cells cultured from the amniotic fluid of a 22-week fetus in a heterozygote for the X-linked Lesch-Nyhan mutation, which results in neurological and developmental disorders, lacked sex chromatin and were unable to incorporate hypoxanthine. The diagnosis of a mutant male was confirmed upon birth of enzyme-deficient, hyperuricemic twin boys whose amniotic membrane cells failed to incorporate hypoxanthine.*

We report here the prenatal detection of the inborn error of metabolism that is clinically expressed after birth as the Lesch-Nyhan syndrome (*1*). Amniocentesis ("amnion puncture") was used to sample embryonic cells in the amniotic fluid in order to determine the fetal genotype (*2*). The disease, which involves severe developmental and neurological disorders, as well as overproduction of uric acid, occurs in males having a recessive, X-linked mutant gene determining extreme deficiency in hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8) (*3*). Autoradiography distinguishes between deficient and nondeficient cultured cells; mutants do not incorporate radioactive hypoxanthine from their medium and appear unlabeled (*4*). Heterozygous females, who are unaffected, can also best be identified with autoradiography. Where a woman has had at least one affected son and there is a family history of the disease, skin biopsies have yielded the two phenotypic classes of cells expected if the gene behaves according to the Lyon (*5*) or "single-active-X" (*6*) hypothesis: "mutant" (that is, unlabeled), where the normal allele was on the inactive X, and "normal," where the normal allele was on the active X chromosome (*4, 7, 8*).

Amniotic fluid (about 10 ml) was obtained from a presumptively heterozygous woman [No. 259, in (*8*)] by transabdominal amniocentesis (*9*) during week 22 of pregnancy. Two kinds of observations were attempted with amniotic fluid cells before and after they were cultured (*10*) in vitro: (i) microscopic examination of Feulgen-stained nuclei (*11*) for the presence of Barr, sex-chromatin bodies (*12*) (absence of this indicator of the presence of two X chromosomes per cell suggests that the fetus is male); and (ii) autoradiography (*8*) was performed in order to determine whether the cells could incorporate radioactive hypoxanthine. Together, the two kinds of observations permit distinction between the four probable types of fetuses (*2*).

Most nuclei of uncultivated cells were wrinkled or had clumped chromatin and were unsuitable for scoring sex chromatin. Barr bodies were absent in 100 satisfactory nuclei, thus suggesting that the fetus was male. Too few uncultured cells remained after autoradiography to permit reliable conclusions about their ability to incorporate tritiated hypoxanthine.

The experimental amniotic fluid cells (strain No. A28) failed to grow appreciably for 26 days in Eagle's medium (*13*) supplemented with fetal bovine serum (15 percent) under conditions used for growing such cells from 16 previously sampled fetuses. Cells obtained from a normal, 18-week fetus did grow under these conditions and yielded control strain A26, which was cultured and tested in parallel with strain A28. Our concurrent studies with cultured, nonembryonic, skin fibroblasts indicated that mutant cells grew poorly unless supplied with exogenous adenine or with much higher concentrations of folic acid than are required by normal cells (*14*). While Eagle's medium does supply enough folic acid for mutants, we decided to try medium F10 (*15*): it contains enough folate and, in addition, other factors which promote superior growth of both mutant and normal fibroblasts. Replacement of Eagle's medium with F10 supplemented with 15 percent fetal bovine serum resulted in rapid growth of both cultures and permitted subculture of strain A28 onto cover slips at day 37. Strain A26 cells were fibroblastic (Fig. 1A), while A28 cells were epithelioid (Fig. 1B). The nuclei of both strains lacked Barr bodies (Fig. 1, C and D), and both fetuses were presumed to be male.

The cells of both strains incorporated tritiated adenine (Fig. 2, A and C), an indication that their machinery for synthesizing nucleic acids was intact.