A CYTOLOGICAL AND CYTOCHEMICAL INVESTIGATION OF THE DEVELOPMENT OF THE VIRAL PAPILLOMA OF HUMAN SKIN*, ‡

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PLATES 15 TO 17

Some types of verrucae of human skin have long been known to be caused by a filterable infectious agent (1), and as such, represent the only kind of tumorous growth in man definitely known to be virus-induced. Warts of this kind, characterized cytologically by intranuclear inclusion bodies and cytoplasmic masses (2–4) have been shown to yield crystalline viral particles (5). The cytology and histochemistry of these skin papillomas have been studied, in some aspect, by several investigators (3, 4, 6, 7). Bunting et al. (4) clearly distinguished the cells of this group of papillomas from those of other more common types of wart by the presence of eosinophilic intranuclear inclusion bodies, and failure of infected cells to differentiate normally. Observations with the electron microscope have disclosed viral particles in the cells of the papilloma (8). Further study by Bunting (9), the results of which are detailed in this communication, have revealed a succession of steps in the evolution of the pathological changes in affected cells, which are accompanied by marked tinctorial and histochemical alterations. Since the stages could be readily identified, it seemed desirable to determine the deoxyribonucleic acid (DNA) content of the cells cytochemically at each recognizable step in the progress of the infection. From studies of the relative nucleic acid content per cell it was hoped to gain information on the synthesis of DNA in infected cells in relation to viral development, and on the nature of other cell forms to be described that occur in the lesion.

Materials and Methods

Excised verrucae, usually from plantar or palmar skin, were fixed in 10 per cent formalin, in most cases buffered at neutrality, washed in running water to remove excess formaldehyde,

and processed for paraffin embedding. Blocks were cut at 7 and 10 μ for histological examination, and at about 20 μ, in order to include whole nuclei, in the sections for microphotometric determination. The 7 and 10 μ sections were stained with hematoxylin and eosin, and in some cases with the Giemsa stain for tissue sections, Macchiavello's stain, or by the Feulgen method. Some sections were stained using the alkaline-fast green technique of Alfert and Geschwind (10) for basic protein of the histone type.

The 20 μ sections were first stained with hematoxylin and eosin, and areas of the lesion photographed at relatively low magnification, a montage of adjacent photomicrographs being made when necessary for orientation. The cells of the affected areas were then scrutinized at high magnification to identify the cytopathological stages, and each suitable cell marked on the photomicrograph. In this way a map was constructed which served for relocation of the same cells which had been studied in the hematoxylin-eosin preparation, during subsequent procedures. The same sections were then destained, and submitted to the Feulgen test for deoxyribonucleic acid (DNA), using a hydrolysis time of 15 minutes at 60°C.
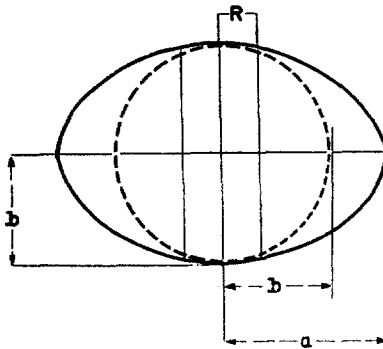
TEXT-FIG. 1. Prolate spheroid circumscribing enclosed isocentric sphere and cylinder, to illustrate computation relating volume of prolate spheroid to that of an enclosed cylinder. Explanation in text.

The relative amounts of Feulgen-colored DNA in the individual cells were determined microspectrophotometrically (11, 12) at a wave length of 575 mμ with an apparatus similar to Moses' (13) and Pollister's (14) modifications of the earlier design of Pollister and Moses (15). The nuclei were treated as complete prolate spheroids (Text-fig. 1), and the following calculation was made. The optical density (extinction) E, through a cylindrical plug of radius R was measured, and the amount of stained DNA in the plug was calculated to be ER^2. The amount of DNA in a hypothetical sphere (dotted in Text-fig. 1) contained within the spheroid, and through the center of which the plug was taken, is obtained by dividing ER^2 by that fraction (F) of the sphere contained within the plug (16). The fraction (F^1) of the spheroid contained in the sphere is equal to b/a (Text-fig. 1). The amount of DNA in the sphere divided by fraction (F^1) gives an approximation of the amount of stained material in the whole prolate spheroid. Thus:

$$\text{Amount DNA} = \frac{ER^2}{F} \cdot \frac{a}{b}$$

Both distributional and geometrical error involved in such measurements could be minimized by adopting a plug radius of 0.6 or less of the short diameter of the spheroid.

Nuclei and inclusion bodies in the later stages of infection were too intensely stained with

the Feulgen reaction to permit valid absorption measurements in one spectral region at or close to peak absorption. Therefore, while relatively less dense nuclei were measured at wave lengths at or near the absorption maximum, the extinctions of denser bodies were determined at wave lengths farther removed from the peak. Two appropriate wave lengths were chosen, and the extinctions of representative numbers of nuclei and/or inclusion bodies were determined at each wave length. The ratio between these provided a factor for interconversion of the optical densities at these wave lengths.

In some of the cytopathologic stages of infection with papilloma virus the distribution of stained material in the nuclei is too heterogenous to be treated in the manner described, without serious errors. To permit reasonably accurate measurement of these stages, and so permit completion of the cytophotometric data on the entire cycle of cellular infection, the two wave length method developed by Patau (17) and by Ornstein (18) was employed. This makes possible the microphotometry of inhomogenous or irregular objects, if they can be delimited in a field containing no other absorbing objects. This requirement, unfortunately, reduced the number of objects that could be measured, since relatively few nuclei in those stages of infection in which the inhomogenity was greatest lay sufficiently isolated from neighboring cells. The calculation used for the two wave length method was:

$$\text{Amount DNA} = L_1 C A$$

in which $L_1 = 1 - T_1$ (T_1 is the transmission of the field containing the object at the wave length of lower absorption, $i.e.$ $T_1 = \frac{I_1}{I_0}$); C is a correction factor which is a function of $\frac{L_2}{L_1}$ and has been tabulated by Patau (17), and A is the area of the field projected upon the photocell. A is proportional to $\frac{I_{2_0}}{I_{CD}}$, where I_{2_0} is the transmission of the blank field at the second wave length chosen, and I_{CD} is the intensity of light flux at constant diaphragm aperture. The latter correction in determining A compensates for error due to discharge of the batteries which are used to supply power to the light source.

Data were plotted as frequency distribution curves on a logarithmic abscissa. The logarithmic scale was chosen because it gives an equal spacing to a distribution of points in all ranges of values, and avoids bunching of data in one sector.

OBSERVATIONS

Cytological Observations[1]

The verrucae that have been shown to yield viral particles (1–5) are typically papillomatous, and are covered by heavy keratinized layers. There is marked acanthosis especially in the rete pegs. In many foci in the thickened spinous layer there are groups of altered cells containing inclusion bodies. Some of the hyperplastic rete pegs, especially near the margins of the verruca appear to contain no inclusion-bearing cells.

Pathological changes in the nuclei of infected cells occur in a definite sequence of stages which can be followed progressively from the lower layers of the stratum spinosum into the stratum corneum.

The first manifestations of intracellular infection is the appearance of small (ca. 1.5 μ diameter) eosinophilic inclusion bodies, in the nuclei of Malpighian cells sit-

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[1] The cytological studies were made primarily by the late Dr. Henry Bunting.

uated in the first or second layer above the basal. Each of these inclusions is surrounded by a clear halo, and may be clearly distinguished from nucleoli or floccules of heterochromatin distributed throughout the same nuclei. The inclusion bodies at this stage are colored blue with the Giemsa stain, and are Feulgen-negative. This earliest stage, in which minute cytoplasmic masses have also begun to form, is designated stage A (Fig. 2). In the second and third rank of the suprabasal Malpighian layer, cells are encountered that contain larger intranuclear inclusion bodies exhibiting the same tinctorial properties and larger cytoplasmic masses than those of the preceding stage, which it otherwise resembles. This stage is called B (Fig. 3). Cells in stage C (Fig. 4) are found at higher levels in the stratum Malpighii. Their nuclei are generally larger, and the inclusion bodies, still eosinophilic and Feulgennegative, are also larger. The Feulgen procedure shows some disorganization and margination of chromatin at this time. The cytoplasmic bodies are fused and bulky. In the upper layers of the Malpighian stratum, nuclei having peripherally displaced (marginated) chromatin and containing large inclusion bodies which measure from $\frac{1}{3}$ to $\frac{1}{2}$ the diameter of the nucleus are found. These are considered to be at stage D (Fig. 5). With the further progress of the infection, (stage E, Fig. 6) the inclusion body may grow to occupy most of the distended nucleus, whose membrane can be recognized by the densely marginated chromatin deposited against its inner aspect. Stages A, B, and C have been described in some detail in a previous communication (4). Both cytoplasmic and intranuclear inclusion bodies of stages D and E are colored red with the Macchiavello stain, in contrast to the blue staining chromatin disposed against the nuclear membrane. Cells in stage E are relatively infrequent as compared with the earlier and later stages. Stage F represents the final evolution of the nuclear lesion: the nucleus has lost its identity, and the enlarged (5 to 8 μ) inclusion body lies free within the cytoplasmic remnant of a cell located in the stratum corneum (Fig. 7). The large naked inclusion body, which somewhat resembles a pyknotic nucleus, is strongly colored by the Feulgen test, stains blue with the Macchiavello method, and stains strongly with hematoxylin.

All of the visibly infected cells are considerably larger than normal cells (4). Scattered among these cells in the hyperplastic prickle cell layer which constitutes the bulk of the papillomatous tumor, are large cells with abundant amphophilic cytoplasm and voluminous nuclei, lying singly or in small groups. Their maturation is apparently arrested. These cells bear none of the evident stigmata of viral infection which have been described, such as inclusion bodies. Their chromatin is disposed in the same manner as that of normal appearing cells, and they possess 2 to 4 nucleoli. Such cells, although differing from those recognizably infected, must be considered in some way affected by the morbid process, and are designated X (Fig. 8).

Mitotic figures, while moderately frequent in cells of the basal layer of the stratum germinativum and in the hyperplastic and normal stratum spinosum are not encountered in the affected cells (4, 19).

Microphotometric Determinations of DNA

Normal and Hyperplastic Epithelium.—The relative content of Feulgenstaining DNA in the nuclei of cells of both basal and spinous Malpighian layers
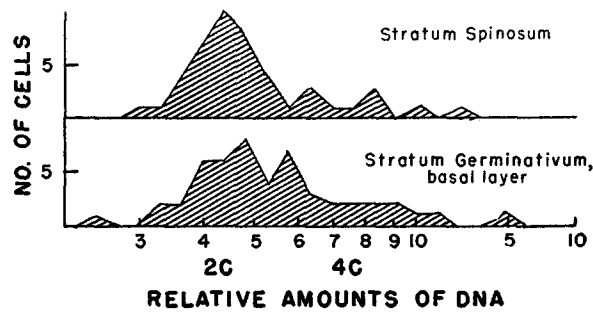
TEXT-FIG. 2. Frequency distribution curves of relative amounts of Feulgen-stained DNA in normal cells of the basal and spinous Malpighian layers of strip of unaffected skin adjacent to the viral papilloma. These data were obtained with the plug method. (See text). Abscissa is logarithmic; ordinates are linear.
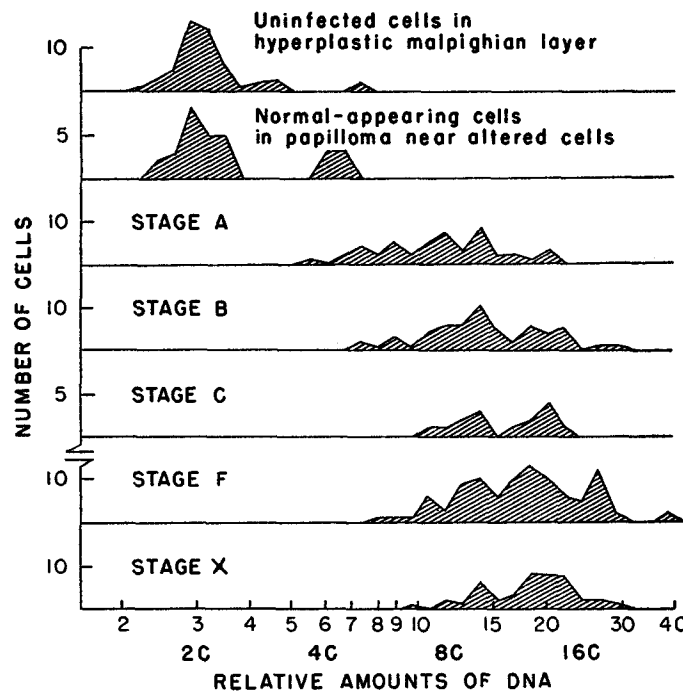
TEXT-FIG. 3. Frequency distribution curves of relative amounts of Feulgen-stained DNA in cell of some of the cytopathogenic stages and in normal appearing cells in viral papilloma of human skin. These data were obtained with the plug method described in the text. Abscissa is logarithmic; ordinates are linear.

was measured in apparently *normal epidermis* removed with the verruca. In most instances the normal appearing cells in the hyperplastic Malpighian epithelium of enlarged rete pegs containing no visibly infected elements were also measured. The results are plotted in Text-figs. 2 and 3. Text-fig. 2 shows

the distribution of the amounts of DNA per cell in basal and spinous Malpighian layers of normal human skin. The majority of these interphase nuclei are in the 2C category and possess the diploid amount of DNA per nucleus, characteristic of most mitotically quiescent cells. Much smaller numbers of cells are found with double this amount of DNA (4C category) in both basal and Malpighian layers. Relatively fewer such 4C cells are present in the latter. Between the 2C and the 4C peaks there are moderate numbers of nuclei whose DNA contents range between the 2C and 4C classes. The intermediate interphase cells are assumed to be in the midst of the process of synthesizing DNA in preparation for their next division, the cells of the 4C category having doubled their DNA, but not yet entered into mitosis. These histograms are characteristic of growing tissues. The distribution plots show larger number of diploid cells in the spinous Malpighian layer than in the basal, and a smaller number of spinous Malpighian cells in the intermediate and 4C groups.

The DNA contents of normal-appearing prickle cells of the hyperplastic epithelium adjacent to the papilloma, but which does not itself contain infected cells, are entirely similar to those of the Malpighian layer of intact skin. The histograms (Text-figs. 2 and 3) show a similar major group of diploid (2C) nuclei, a few premitotic cells with double the amount of DNA (4C), and a number in the intermediate range, still in process of synthesis.

Early Cytopathologic Stages in Viral Infection.—These are characterized by the presence of a well defined nucleus containing a Feulgen-negative inclusion body, and comprise stages A to D. The relative amounts of DNA per nucleus in the nuclei of stage A, containing the earliest recognizable inclusion body, fall within a range spanning 4C to 8C classes (Text-fig. 3). No cells of group A contain less than tetraploid amounts of DNA, and most of them have approximately octoploid (8C) amounts. Some cells of this stage approach the 16C category. At stage B, most of the nuclei still have amounts of DNA approximating the 8C class; there are more nuclei with amounts of DNA approaching or within the 16C category, and few with amounts somewhat closer to the 4C mark, but greater than tetraploid. In stage C of the infection there are very few or no nuclei with relative quantities of DNA in the 4C class: most of the stage C cells have from nearly octoploid (8C) to approximately 16-ploid quantities. The same is true of stage D, in which no 4C nuclei are represented (Text-fig. 4).

It is apparent that cells infected with the virus causative of papilloma have much greater average amounts of DNA than growing uninfected cells. The different pattern of distribution of amounts of DNA of uninfected and normal appearing cells, and those bearing cytopathologic stigmata is evident in both histograms (Text-figs. 3 and 4). The number of cells in any DNA class at any point in time is an index of the length of time that cells which have syn-

thesized the amount of DNA in question remain in that phase of their cycle. In the usual frequency distribution diagram of a proliferating tissue, the presence of peaks at the 2C and 4C class marks, with relatively fewer cells containing amounts of DNA intermediate between them reflects the rapidity of the synthetic process in the premitotic interphase. Many of the infected
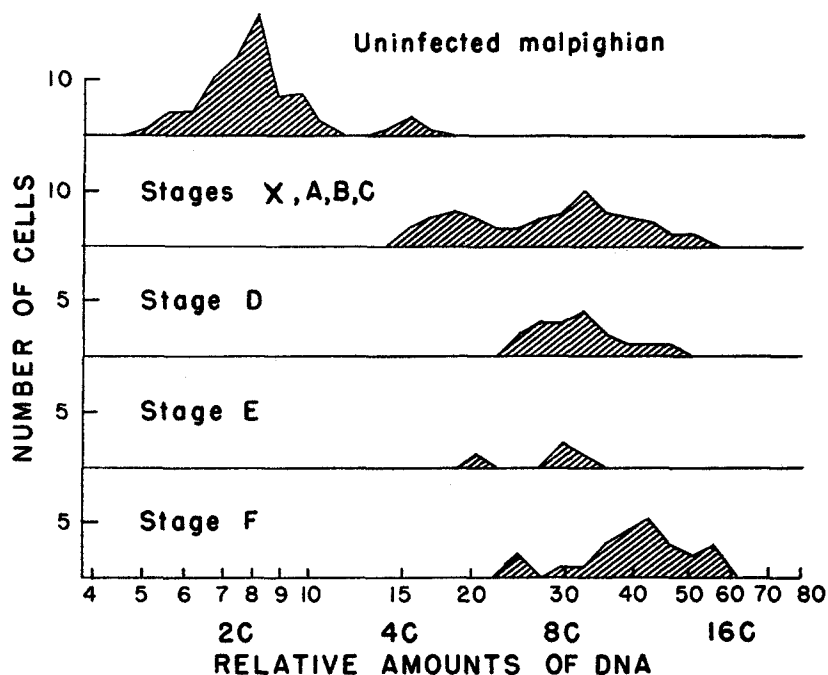

TEXT-FIG. 4. Frequency distribution curves of relative amounts of Feulgen-stained DNA in all the cytopathogenic stages of viral infected cells of human skin papilloma, and of normal appearing cells in adjacent hyperplastic Malpighian layers. No distinction is made between cells of the earlier stages (A–C) or the enlarged cells (X) which occur among them. These data were obtained using the two wave length method (see text). Abscissa logarithmic; ordinates linear.

nuclei contain amounts of DNA intermediate between the 4, 8, and 16C groupings. There is, however, little well defined accumulation of cells within these categories as compared with the intermediate ranges between them, hence no sharp and distinct peaks. The distribution curves suggest that DNA synthesis in infected cells, at least after stage A, may not occur in the interval fashion of dividing cells (Text-fig. 5). The synthesis from 4C to 8C amounts which occurs in the early stages of infection appears to be more regular, however, since no 4C nuclei occur after stage A, and no nuclei with less than 8C amounts occur after stage B. Since the number of cells in any stage reflects

the duration of that stage, stages *D* and *E* would appear to be of relatively shortest span.

Later Cytopathologic Stages in Viral Infection.—At stage *E* the hitherto Feulgen-negative inclusion body becomes strongly Feulgen-positive, and remains so through the final stage of its evolution (stage *F*). This important change in localization of DNA in the nucleus is not accompanied by corresponding quantitative alterations of DNA content. Although measurements of nuclei in stage *E* (Text-fig. 4) are hampered by the paucity of cells in this stage and the larger distributional error involved, they nevertheless reveal
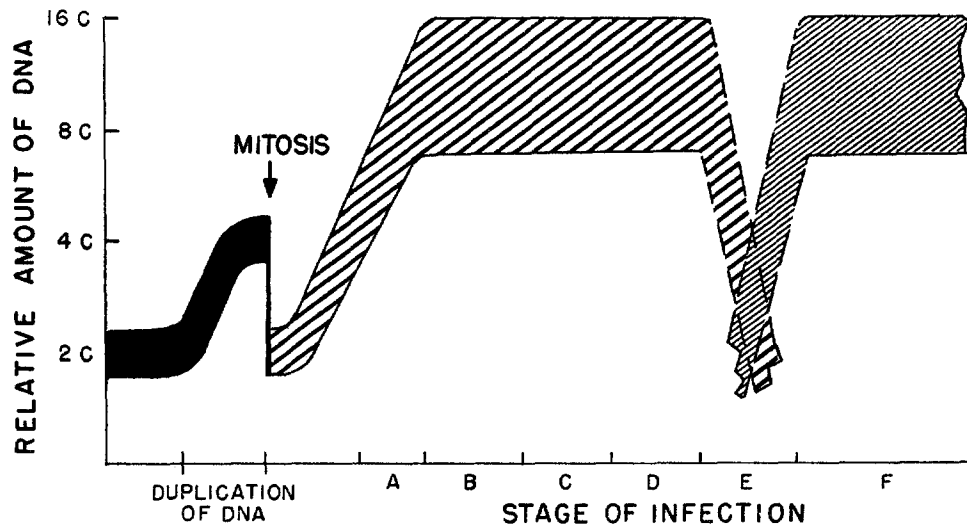
TEXT-FIG. 5. Schematic representation relating the amount of DNA per cell to the mitotic cycle and to the stages of infection with papilloma virus. The widely barred curve shows the DNA of the entire nuclear content. The close hatched curve is the DNA of the mature inclusion.

that most of the cells contain about 8*C* amounts of DNA. The naked inclusion bodies of stage *F*, on the other hand, are numerous, and being fairly regular and homogeneous can be measured with minimal inherent error. The frequency distribution curves for stage *F* show a range of DNA contents per body of from 8 to about 16*C* (Text-figs. 3 and 4) and are entirely similar to those of the antecedent stages *D* or *C*, except for greater numbers in or near the 16*C* class.

Other Cells within the Papilloma.—Lying between the infected cells of any of the stages described, there are normal-appearing prickle cells of the Malpighian layer, and the hypertrophic cells which have been designated X. The relative amounts of DNA contained in the *unaltered prickle cells of the papilloma* fall into two classes, 2*C* and 4*C* (Text-fig. 3), in a distribution simi-

lar to that of homologous cells of the hyperplastic or normal Malpighian layer. However, there is a greater number of papilloma prickle cells with the tetraploid amount of DNA. The *hypertrophic X cells*, on the other hand, all have elevated DNA contents, which vary in amount from just under 8C up to and including 16C (Text-fig. 3). The pattern of the frequency distribution curve of the X (hypertrophic) cells is the same, in effect, as that drawn for any of the later stages of the infection.

Protein of the Inclusion Bodies

Qualitative Test for Histone.—Application of the alkaline-fast green method for basic protein of the histone type results in specific staining of nuclear histone in normal cells and staining of carefully identified bodies of stage F (Fig. 8). This is believed to indicate the presence of a protein of high isoelectric point similar to nuclear histone.

DISCUSSION

Discrepant reports of the morphological tinctorial and histochemical properties of the inclusion bodies in a number of diseases caused by animal viruses appear to have been due to inadequate recognition of the fact that these bodies evolve through a series of stages with the development, multiplication, and dispersal of virus, each of which may possess different characteristics. The intranuclear bodies of yellow fever, cytomegalic inclusion disease, and especially herpes simplex may be cited as examples (20–35). Wolman (25) has concluded that inclusion bodies resulting from infection with most of the animal viruses contain DNA at some time in their development. However, during the stages in which inclusions are Feulgen-negative, they have been confused with various cell organelles, and particularly with large eosinophilic nucleoli (23). The latter difference in identification appears to have occurred in the case of the earlier developmental stages of the intranuclear bodies of the human viral papillomas (4, 6, 19). Conclusive reasons have been presented for regarding the eosinophilic intranuclear bodies as inclusion bodies resulting from viral infection of epidermal cells (4, 9).

The pattern of development of these inclusion bodies, beginning with a nucleic acid-free matrix and evolving into a body which subsequently comes to contain DNA, is reminiscent of the intranuclear cycle of some strains of RI-APC virus (26).[2] In herpes, on the other hand, the final type A inclusion body is alleged to be a matrix containing no detectable nucleic acid (20–22) but the inclusion bodies of putative earlier stages of development are reportedly Feulgen-positive (21).

The human skin papilloma has been the first animal lesion within which

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[2] The term adenoviruses has more recently been proposed for this group of viruses by a committee on nomenclature (*Science*, 12, 119, 1956).

viral particles have been found in crystal-like formation (8). These have not yet been localized with sufficient precision, but the findings of close packed arrays of particles in the stratum corneum suggests that they may prove to be identical with the Feulgen-positive bodies of stages E and F. A correlated study of the same structures with both electron and light microscopes, like that made by Bloch *et al.* (26) would be required to establish this fact. Since the crystalline arrays reported by Bunting (8), which we regard as represented by stages E-F bodies, are composed predominantly of close-packed viral particles (8, 9), it may tentatively be considered as likely that the viral particles contain DNA. Large crystals composed of particles of some types of RI-APC virus in the nuclei of infected HeLa cells have been studied more recently with the electron microscope using the improved techniques of preparation presently available (27). In a correlated electron microscopic and histochemical study, these crystals and hence viral particles have been shown to contain DNA (26). The appearance of a Feulgen-negative matrix (inclusion body) in the nucleus of RI-APC–infected cells, prior to the development of viral particles, may be analogous to the occurrence of a Feulgen-negative inclusion body which precedes the development of large DNA-containing inclusions in the infected cells of human skin papilloma.

The red staining of the inclusion body with Macchiavello's stain of stages up to and including E, in contrast to the blue staining chromatin, has been taken as an indication of the presence of a matrix material (9). By stage F, the inclusion appears to have lost this tinctorial property and stains blue, perhaps owing to loss of some presumably proteinaceous matrix. The occurrence in the bodies of the F stage of a protein which, after removal of nucleic acid is sufficiently basic to accept the alkaline-fast green stain of Alfert and Geschwind (10) is somewhat unexpected. This protein has the tinctorial properties of a histone, which it may in fact be. It is not known whether this represents residual nuclear material or viral protein. In the latter case, it would differ markedly from the proteins of purified animal viruses which have so far been analyzed in which acidic amino acid residues predominate (28–30).

It is obvious that infection with the causative virus of the human papilloma stimulates marked intranuclear synthesis of DNA. The synthesis begins very early in the course of infection of cells of the lower stratum Malpighii, before recognizable morphological stigmata appear, and progresses rapidly. No inclusion bodies are formed until after DNA synthesis has proceeded at least to the 4C stages, and in most nuclei beyond that to 8C and even greater amounts. The rapidity of synthesis is attested to by the fact that practically all the DNA destined to be formed in the ultimate development of the lesion, has been produced at stage B. No significant DNA synthesis appears to occur thereafter. The frequency distribution curves of the relative amounts of

DNA in all subsequent stages, however structurally altered they may appear, are quite similar. Neoformation of DNA would appear to require a nucleus relatively little altered, and does not proceed when more profound structural changes become evident. The extreme changes in nuclear organization, and the occurrence of marked enlargement, basophilia, and Feulgen staining in the inclusion bodies which characterize the transition from stage D to E and F are concomitants of a process of relocation of the DNA, without change of amount. These changes are represented schematically in Text-fig. 5; the crossing of the curves indicates "transfer" of DNA to the inclusion body.

Accurate information on the location, number, and form of viral particles in the nucleus is crucial to an understanding of the processes described. Observations by Bunting with the electron microscope (8, 9) seemed to show that there is no virus within or in spatial relation to the inclusion bodies in presumably earlier stages of infection. Virus has not been identified in such nuclei (9). In the lower corneum, where F stages abound, viral masses, sometimes in close packed crystalline array, have been seen. It is reasonable to assume that the association of viral particles with the intranuclear inclusion body is heralded by the development of basophilia and a positive Feulgen reaction in it, and takes place in stage E. The evidence, while suggestive is however, not complete.

The relationship of the viral particles to the enhancement of endocellular DNA production is of great interest. It would be especially desirable to know whether complete viral particles are present and in what amount in the apparently unaltered prickle cells within the papilloma, as well as in stage A. Does accelerated DNA production in the nucleus begin during an eclipse period, or is it associated with recognizable viral particles? When does virus first become recognizable and is there an actual increase in the number of viral particles (amount) per nucleus, or only in their local concentration, in the later stages of infection?

It seems likely that the increased DNA in the earlier stages is predominantly nuclear (host), and that ultimately all of the host DNA is broken down and contributed to virus for reassembly as viral DNA. The seemingly more regular or stepwise pattern in the synthesis of from $4C$ to $8C$ amounts of DNA in stages A through B suggests that at least in the early phases of infection, DNA synthesis by the usual nuclear mechanism is stimulated, and that formation of new viral DNA occurs subsequently and at the expense of the increased host DNA. This would be analogous to the recorded utilization of products of host DNA for bacteriophage production (31, 32). It is also possible that the increased DNA being measured in stages A to D is both host and viral. The absence of distinct DNA classes and the wide range of amounts of DNA per nucleus may merely reflect the presence of varying amounts of virus per nucleus.

On the basis of cytochemical studies utilizing ultraviolet microscopy on the cells of verruca vulgaris, Hydén (6) concluded that viral infection caused a marked stimulation of the "nucleo-protein-forming parts" of the host nucleus, with increase of first protein and then DNA which then aggregated in inclusion bodies. Blank *et al.* (7), concurring in Hydén's thesis, found that the basophilic intranuclear inclusion material of some verrucae was "newly formed DNA" which they believed to represent virus. It is not clear that all the warts studied by Hydén (6) and by Blank *et al.* (7) had the cytological characteristics of those described by Bunting as yielding viral particles (4). The identification of inclusion bodies and of nucleoli in the cells used by Hydén (6) and Blank *et al.* (7) differs from Bunting's (4, 9) and ours. This makes it difficult to relate their findings to those reported in this paper. The conception that the papilloma virus exploits the DNA forming parts of the cell, however, is in agreement with the facts obtained from all these studies. In this respect infection with the skin papilloma virus may differ from herpes simplex viral infection, in which the DNA content per cell of hyperplastic chick embryo organs is alleged to be unchanged (33). Leuchtenberger and Lund (34) measured the DNA content of cells of "verruca vulgaris" microphotometrically. They found it to vary in distinct multiples, with definite classes. Since the histopathology of the verrucae studied by these authors was not detailed, their findings cannot be compared with those presently reported for viral papillomas. The differences in the data of Leuchtenberger and Lund (34) and those of the present report may be an indication of a relatively normal mode of DNA synthesis in the common non-infective verruca, and the accelerated DNA production related to virus formation in the infected cells of the viral papillomas of the kind dealt with in this paper.

Epidermal cells with morphological stigmata of infection, *i.e.* inclusion bodies, have lost their capacity for mitotic proliferation and normal differentiation and probably all go on to die (4, 19). The bulk of the tumor is composed of the normal appearing cells in the hyperplastic Malpighian layer adjacent to the infected foci and interspersed among the obviously infected cells of the papilloma. Mitoses are encountered among them. The presence of a prominent 4C group in these cells may reflect either a large population of cells that have doubled their DNA prior to entry into mitosis, or may constitute the first sign of virus-induced stimulation of DNA production prior to the appearance of the stage *A* inclusion body.

The stimulus to the hyperplastic proliferation of the cells that constitute the papillomatous tumor is unknown. It may result from liberation of growth-promoting substances from cells damaged by virus, as Beveridge and Burnet (35) believed to occur in the plaques of the chick chorioallantoic membrane infected with destructive vaccinia or herpes simplex viruses. The growth in this case would be regenerative and consequent on destruction of cells. Conceivably it might also result from masked or inapparent viral infection of

some cells, or may be a prior stage of infection, in which only a stimulating action rather than the usual destructive effect is manifested.

Of particular interest are the hypertrophic cells designated X. Except for enlargement and apparent arrest of differentiation in them, they show none of the stigmata of infection that have been described. They contain polyploid amounts of DNA, most falling between the $8C$ and $16C$ categories and the pattern of their frequency distribution curves is similar to those of the infected cells of stages $B - E$ and the F bodies. Until such cells have been visualized with the electron microscope, several conjectures may be invoked to explain their occurrence. It is possible that the X cells represent early stages of infection, prior to the formation of inclusion bodies. However, in view of the numerous cells of higher polyploid classes in the X cell population, this would seem to be less likely an explanation than aberrant infection, or infection with masked virus or provirus in which DNA synthesis is stimulated by virus but no inclusion body is formed. It may also be supposed that the X cells are not themselves infected, but that either DNA synthesis is stimulated by substances arising from infected cells, or mitotic division is inhibited, or both, leading to enhanced DNA content. The absence of distinct peaks at regular class marks in the histogram, characteristic of polyploidy, weakens this last hypothesis.

Extensive microphotometric measurements of the DNA content of the individual cells in the different layers of normal human skin do not appear to have been made, but Leuchtenberger, Leuchtenberger, and Davis (36) reported a single DNA class in human epidermal epithelium. This is at variance with our finding of distinct diploid and presumably premitotic tetraploid DNA classes in both basal and spinous layers (Text-fig. 2). Our data are generally in agreement with the mitotic indices in the layers of human epidermis counted by Thuringer and Cooper (37), who found that proliferation occurs in both basal and spinous layers.

SUMMARY

The morphological appearances and some tinctorial properties of the cells of the infectious (viral) papilloma of human skin are described. Pathological alterations of the nuclei of affected cells occur in a definite sequence of stages. In the earliest, and acidophilic Feulgen-negative intranuclear inclusion body is recognizable. Subsequently the inclusion body and nucleus enlarge, there is progressive disorganization of nuclear structure, and the inclusion body then becomes basophilic and stains with the Feulgen reaction. The nucleus finally disappears, leaving the inclusion body in the cell remnant.

The relative amounts of Feulgen-colored DNA per cell at each stage in

the evolution of the lesion in the infected cells, as well as in normal appearing and hypertrophic cells of the papilloma, were measured microphotometrically in Feulgen preparations. Determinations were made using the "plug" and "two wave length" methods. These are compared with measurements of DNA in cells of the basal and spinous layers of normal human skin.

The frequency distribution curves of relative amount of DNA in cells of normal skin, and normal-appearing cells in hyperplastic epithelium, show the bimodal diploid and tetraploid peaks characteristic of growing tissues.

Infection of the epidermal cell entails prompt synthesis of DNA in the nucleus. Increased amounts of DNA (tetraploid to 16 ploid levels) are found in the earliest recognizable cytopathological stages of infection and do not increase appreciably during the subsequent evolution of the cellular lesion. At a relatively late stage, all the cellular DNA is relocated ("transferred" or "reassembled") in the inclusion body, and is not further significantly increased in amount. Active formation of DNA in affected cells appears, from these measurements, to occur only in relatively intact nuclei.

The inclusion bodies of infected cells are found to contain a relatively basic protein which stains with the alkaline-fast green method for histone.

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EXPLANATION OF PLATES

All of the specimens of Figs. 1 to 8 were stained with hematoxylin and eosin.

PLATE 15

FIG. 1. Normal appearing prickle cell of hyperplastic Malpighian layer in viral papilloma of human skin. The nucleolus is eccentrically located. × 1250.

FIG. 2. Stage *A*, the first recognizable manifestation of infection in a cell of the lower stratum Malpighii. The eccentric inclusion body (arrow), is eosinophilic and surrounded by a narrow halo. × 1250.

FIG. 3. Stage *B*, showing larger centrally located intranuclear inclusion body, (arrow), which is eosinophilic, and some cytoplasmic masses. × 1250.

FIG. 4. Stage *C*. A still larger intranuclear inclusion body, surrounded by halo and confluent cytoplasmic masses. × 1250.
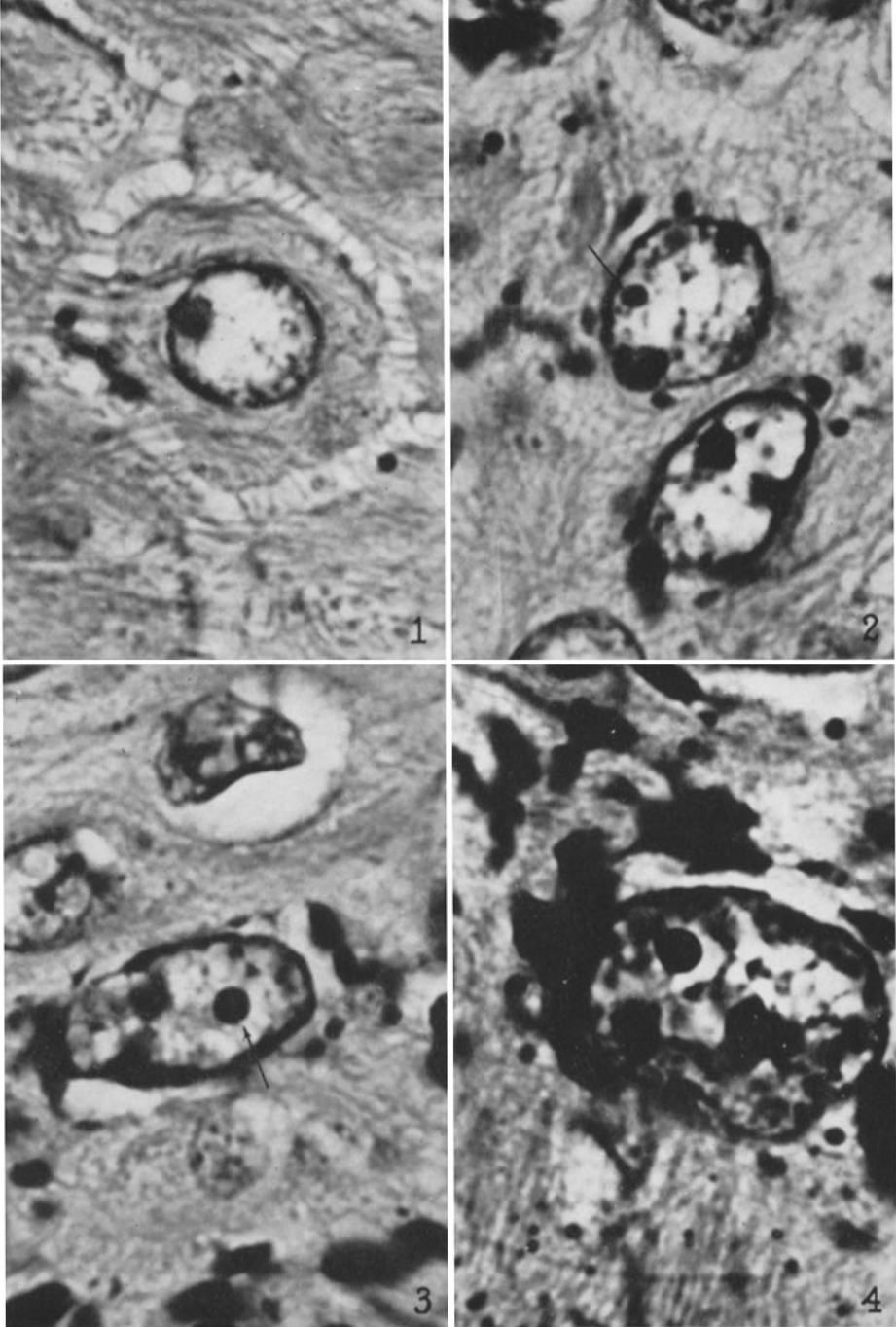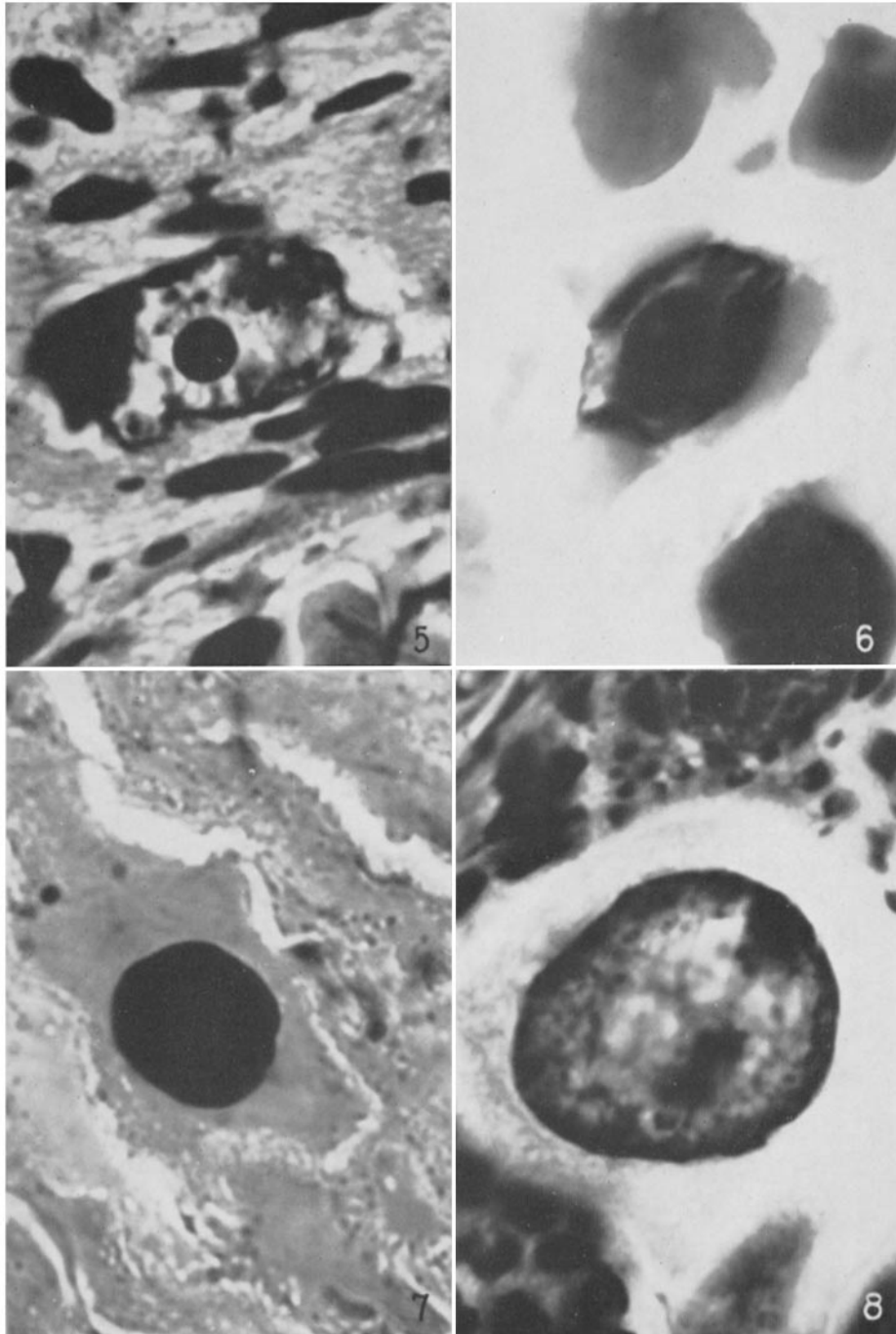
(Bloch and Godman: Viral papilloma of human skin)

Plate 16

Fig. 5. Stage *D*. The inclusion body has further enlarged. Some disorganization of the nuclear contents is apparent. × 1250.

Fig. 6. Stage *E*. The inclusion body almost fills the nucleus, which becomes a containing sac bordered by marginated chromatin. At this stage the intranuclear inclusion begins to stain with the Feulgen test. × 1250.

Fig. 7. Stage *F*. The nucleus has disappeared, leaving a large naked inclusion within the cytoplasmic remnant. × 1250.

Fig. 8. Hypertrophic cell with markedly enlarged nucleus whose chromatin pattern is relatively normal. Such cells, designated X, are found among infected cells bearing inclusion bodies. X cells have no inclusions. × 1250.
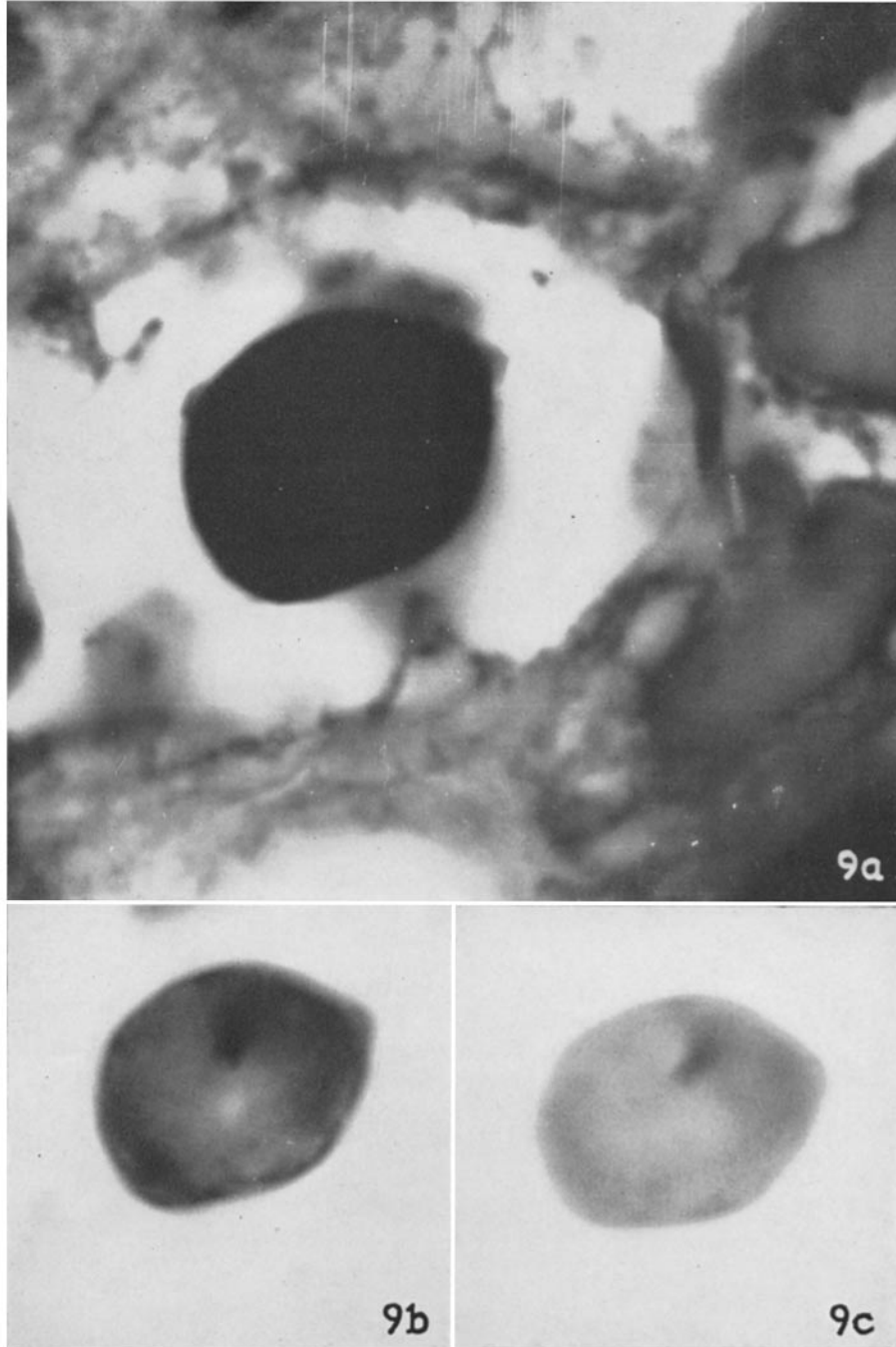
(Bloch and Godman: Viral papilloma of human skin)

FIGS. 9 *a* to 9 *c*. Stage *F*. The same object stained successively with:

FIG. 9 *a*. Hematoxylin-eosin. Note the inclusion body and the adjacent cyto-plasmic masses. × 3600.

FIG. 9 *b*. Feulgen reaction for DNA: only the inclusion stains. × 3600.

FIG. 9 *c*. Alkaline-fast green for basic protein of the histone type: only the in-clusion stains. × 3600.

(Bloch and Godman: Viral papilloma of human skin)