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Desoxyribose Nucleic Acid from Isolated Chromosome Threads in Experimental Epidermal Methylcholanthrene Carcinogenesis in Mice*

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(Received for publication August 15, 1946)

INTRODUCTION

In the major cancer research project conducted at this hospital, cytological, biochemical and biophysical changes that suprervene in the epidermis of mice as a result of applications of methylcholanthrene have been followed. This approach to the problem of induction of malignancy has resulted in the discovery of distinct cellular and intercellular transformations leading to an early establishment of a new chemical equilibrium in the altered epidermis, which is maintained, substantially without further modifications, throughout the "latent period" of carcinogenesis. The steps by which these transformations are effected have been integrated and embodied in reports published by Cowdry (12-14).

The biological importance of nucleic acids in growth and development has recently been reviewed by Davidson and Waymouth (20) and by Davidson (19). There is increasing recognition of a causal connection between the processes connected with growth and the spatial distribution of nucleic acids in the cell. Thus disturbances in the cellular nucleic acids accompanying neoplasia have been noted by Caspersson and Santesson (7), Koller (24) and Stowell (33, 35). Stowell and Cooper (34) measured the relative amounts of Feulgen stained thymonucleic acid in normal epidermis, hyperplastic epidermis and epidermoid carcinoma with a microphotometric apparatus. They detected in general a fall in the thymonucleic acid content per unit volume of hyperplastic epidermis, and a rise in the carcinomas as compared with the normal epidermis. Carruthers and Suntzeff (6) have recently made quantitative determinations of desoxyribose nucleic acid in epidermal cells during carcinogenesis. They observed a progressive decrease in the amount of this substance from normal to malignant stages through the intermediate hyperplasia. The observations of these last investigators are in harmony with their earlier findings on the trend of biochemical changes in epidermal carcinogenesis. Our objectives have been (a) to isolate chromosomes from the epidermal and carcinomatous cells and collect them as free as possible from other cellular constituents; (b) to determine quantitatively desoxyribose nucleic acid in these chromosomes during epidermal carcinogenesis.

MATERIAL AND METHODS

Swiss mice were treated with methylcholanthrene, as already described by Cowdry (12). Epidermis was separated from the underlying dermis at room temperature using the method devised by Suntzeff and Carruthers (36), which involves stretching the skin tightly over a board covered with transparent plastic and scraping the epidermis off the dermis. This technic is a distinct improvement over the one adopted earlier by Baumberger, Suntzeff and Cowdry (1) in that while the latter method employed a temperature of 50°C, the critical temperature for biological activities, for softening the collagenic fibers and thus to separate the epidermal layers from the corium, the new procedure is carried out at room temperature, thus eliminating the attendant dangers of water loss and deleterious effects on the cells.

The separated epidermis was immediately dropped

* Aided by grants from the National Cancer Institute and an anonymous donor.
into vials containing physiological saline surrounded by freezing mixtures or dry ice. In the same manner solid pieces of transplanted squamous cell carcinomas, free from visibly necrotic areas, were dropped into similar containers. The lines of transplantable skin carcinomas of mice, induced by methylcholanthrene, employed by us were started nearly five years ago by Cooper, Firminger and Reller (11), and to date have been carried through 40 passages. Healthy and non-necrotic tumors, kindly donated by Drs. Cooper and Suntzeff, were cut up into small pieces, approximately 2 mm. in size, and were introduced subcutaneously into mice 6 weeks old. Some mice of the same age were also injected subcutaneously with fine fragments of the tumor suspended in physiological saline. The rate of growth of the transplanted tumor in the mice was at least double that of the injected population judged by the time lapse between introduction of the material and expression of optimum growth. Best results were obtained with tumors harvested 2 weeks after implantation. Although there were individual variations between tumors of this age, the majority of them showed little necrosis or cellular degeneration. The frozen tissues showed no visible structural alterations before isolation of chromosomes.

EXPERIMENTAL PROCEDURE

The technic of isolation of the chromosomes was that of Claude and Potter (9) and Mirsky and Pollister (25, 26) slightly modified. The basis of isolation was recognition of the fact that disruption by mechanical breakage of the nuclear and cell membranes releases the diverse components of the cellular system into the surrounding medium. As demonstrated by Bensley (2), Claude and Potter (9) and Mirsky and Pollister (25, 26) these components can be segregated, concentrated and purified by differential centrifugation. The final fraction readily lends itself to further study and chemical analysis. By using 1.5 per cent citric acid at about pH 3 as employed by Dounce (22, 23) and Ziegler (38), the nuclei were loosened but at this low pH nucleoproteins were undoubtedly denatured. Moreover it was thought that the cells were subjected to a milieu antagonistic to normal cellular activities. Hence this experimental procedure was abandoned.

Following the technic of Claude and Potter (9), who used leukemic cells of the C58 strain of mice and leukemic Wistar rats, the epidermal tissues were ground with sand in a mortar before centrifugation, but the results were unsatisfactory because it was extremely difficult to eliminate the fine particles of sand from the final fractions. The method that was finally developed consisted of grinding the separated epidermis, or the fragments of tumor, in a coarse mortar at a temperature of 1° to 2° C., until most of the cells and nuclei were crushed. The crushed mass was frozen, suspended in physiological saline at a temperature of about 1° to 2° C., and further disintegrated by subjection to the action of a high speed Waring blender which had been cooled in dry ice before use.

Although it was comparatively easy to disintegrate cancer cells by this method, both normal and hyperplastic epidermal cells offered a great deal of resistance and longer treatment, both in the mortar and blender, was necessary to rupture them. Even so, some of the epidermal cells remained unbroken.

For a satisfactory separation, control of two variables was found to be of paramount importance, namely: the maintenance of a temperature between 0° and 5° C. and the regulation of pH from 7.2 to 7.4 throughout the course of the experiment. Under these conditions, the chromatin threads that are in suspension retain their distinctive fibrous appearance. The schedule of differential centrifugation was in all essential respects similar to that outlined by Claude. The instrument used was a model PR1 International Refrigerated Centrifuge adjusted to maintain a temperature of 0° C.

Two experimental errors are not completely eliminated: (a) Loss of some extremely fine chromatin strands near the limits of microscopical resolution because of their failure to sediment with the rest of the chromatin material, and (b) contamination of the chromatin threads by small amounts of cytoplasmic particles and mitochondria. Because these defects in the method are applicable more or less equally to all three types of tissues studied, they were not deemed serious enough to vitiate the results.

The chromosomes, thus isolated, are ready for microscopical examination and biochemical analysis. They are Feulgen-positive and give a distinct blue color when subjected to the diphenylamine reaction of Dische (21) as employed by Seibert (32), Dounce (23) and Car ruthers and Suntzeff (6). In quantitative evaluation of the desoxyribose nucleic acid the Dische reaction was applied to isolated chromosomes dried in vacuo, and the percentage transmission of the characteristic blue color at 600 m/ was measured with a Coleman spectrophotometer. The standard curve was made by Dr. Car ruthers from pure sodium desoxyribonucleate by plotting the concentration of this substance against the percentage transmission. The actual nucleic acid concentration was derived by multiplying the concentration of the sodium salt by 0.944.

OBSERVATIONS

Morphology.—Since most of the epidermal nuclei were in the resting stage and only a small proportion of them in various phases of mitoses, the bulk of the isolated chromosomes was evidently derived from the
resting nuclei. The position of the primary constriction is clearly recognizable in many of the chromosomes, especially in the stretched out condition. The centromeric regions appear as Feulgen-negative gaps between the long and short arms of the chromosomes (Figs. 1 to 3). The final purified sample appeared white and had a viscous consistency. Under dark field illumination the chromatin appeared as distinctly fibrous, rod-shaped or granular bodies of varying length but of more or less uniform diameter, the latter being usually from 0.5 to 2μ. Many of the thicker threads were presumably derived by an elongation and partial despiralization of the heterocentric or chromocentric regions so clearly seen in intact resting nuclei. The thicker threads were more heavily charged with thymonucleic acid, as revealed by the Feulgen stain, than the finer strands. This does not necessarily mean that there is inherently a greater concentration of deoxyribonucleic acid per unit length of these chromosomes. It is not at all unlikely that a more tightly coiled state exists in them, as shown by Coleman (10) in the case of the differentially condensed X-chromosomes of the Acrididae and by White (37) in the heteropycnotic X-chromosomes of the Tettigonidae and Acrididae.

On strictly morphological grounds it is difficult, if not impossible, to distinguish between chromosomes derived from normal, hyperplastic, or epidermal cells (Figs. 1 to 3). This is hardly remarkable since microscopic examination of the normal, hyperplastic and malignant cells shows the same types of nuclear architecture—chromatin strands and heteropycnotic masses on the so-called "net work" with the diameters of the intra-nuclear strands corresponding with that of the isolated fibrous chromatin. However, the chromosomes isolated from squamous cell carcinoma appear to be more densely stained with the Feulgen stain that those of the other tissues. This agrees with the cytochemical findings described below. Because of his failure to observe loose ends in intact resting nuclei, Claude and Potter (9) came to the conclusion that the chromatin threads were oriented end to end to form a continuous filament. We cannot agree; for chromosome threads are distributed in random fashion throughout the nuclear space and allowance has to be made for the fact that they represent structures spatially distributed in three dimensions. It would, therefore, be extremely difficult to detect free ends. Moreover, the weight of cytological evidence, gained by a study of the mitotic cycle, militates strongly against a continuous end to end alignment of chromosomes.

Cytochemistry.—The formed elements easily go into solution in 1 molar sodium chloride and in distilled water, leaving highly refractile granules. They may be reprecipitated in the form of fibrous material by the addition of water. Other physicochemical properties detected in fibrous nucleoproteins by Minsky and Pollitzer (25, 26) and Minsky (27) have also been observed but will not be described here.

Significant differences were found in the deoxyribonucleic acid content of the chromosomes of normal, hyperplastic and carcinomatous cells (Table I). It will be seen that the average content of deoxyribonucleic acid in normal epidermal chromosomes is 0.129 mgm. per mgm. of dry weight or, roughly 13 per cent, whereas in mice painted thrice with methylcholanthrene it is 0.091 mgm. per mgm. of dry weight showing a 30 per cent drop in deoxyribonucleic acid as compared with the normal. Increasing the number of paintings to 6 has no significant effect on the nucleic acid content of the chromosomes.

**Table I:** Deoxyribonucleic Acid of Chromosomes in Experimental Epidermal Carcinogenesis

<table>
<thead>
<tr>
<th>No of mice</th>
<th>No of paintings</th>
<th>Time after first treatment to killing of mice: days</th>
<th>DNA* per mgm. dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>---</td>
<td>---</td>
<td>0.108</td>
</tr>
<tr>
<td>11</td>
<td>---</td>
<td>---</td>
<td>0.136</td>
</tr>
<tr>
<td>12</td>
<td>---</td>
<td>---</td>
<td>0.154</td>
</tr>
<tr>
<td>10</td>
<td>---</td>
<td>---</td>
<td>0.118</td>
</tr>
<tr>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 (Total)</td>
<td></td>
<td>Average</td>
<td>0.129</td>
</tr>
<tr>
<td>METHYLCHOLANTHRENE-TREATED MICE—HYPERPLASTIC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>10</td>
<td>0.098</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>10</td>
<td>0.083</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>11</td>
<td>0.090</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>11</td>
<td>0.092</td>
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<tr>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41 (Total)</td>
<td></td>
<td>Average</td>
<td>0.091</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>16</td>
<td>0.091</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>16</td>
<td>0.099</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>16</td>
<td>0.127</td>
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<tr>
<td>---</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>40 (Total)</td>
<td></td>
<td>Average</td>
<td>0.106</td>
</tr>
<tr>
<td>SQUAMOUS CELL CARCINOMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>---</td>
<td>---</td>
<td>0.171</td>
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<tr>
<td>10</td>
<td>---</td>
<td>---</td>
<td>0.211</td>
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<tr>
<td>6</td>
<td>---</td>
<td>---</td>
<td>0.203</td>
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<td>10</td>
<td>---</td>
<td>---</td>
<td>0.250</td>
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<td>10</td>
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<td>---</td>
<td>0.226</td>
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<tr>
<td>---</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>44 (Total)</td>
<td></td>
<td>Average</td>
<td>0.208</td>
</tr>
</tbody>
</table>

* DNA = Deoxyribonucleic acid.

In malignancy, however, there is an increase which, although it is variable in the different animals examined, nevertheless reaches spectacular heights compared with chromosomes of the normal and hyperplastic epidermises. If, for instance, we exclude the average amount (0.171 mgm. per mgm. dry weight) of deoxyribonucleic acid in squamous cell carcinoma obtained from the first 8 mice examined (Table I), the remaining instances...
Figs. 1-3
methylcholanthrene operates to produce an altered yet unequivocal evidence that breakdown products of equilibrium at the precancerous level. Nor is there as content of chromosomes for hyperplastic epidermis. methylcholanthrene, under investigation by W. L. Simpson, the causal agents in squamous cell carcinoma from that of normal epidermis appears to be in conformity with the altered economy of the cell. It is not clear how the altered desoxyribose nucleic acid content of normal, hyperplastic and carcinomatous epidermal tissues, as reflected in the newly established equilibrium, is sufficiently clear to warrant suggestive inferences. It must be pointed out, however, that in all such studies based on analyses of components of whole tissues, we are handicapped by not being able to recognize any differences that may be localized in the tissues. It is not inconceivable that a marked localized change in a few cells might be masked by including with them numerous others which are not altered in the same manner. Notwithstanding this, we believe that the general trend of changes in the desoxyribose nucleic acid content of normal epidermis is distinctly seen. Many of the chromosomes show a differentiated into long and short arms, separated by Feulgen-negative gaps, which correspond to the position of the centromeres.

**DISCUSSION**

The change that takes place in desoxyribose nucleic acid content of the chromosomes of hyperplastic epidermal cells and of squamous cell carcinoma from that of normal epidermis appears to be in conformity with the altered economy of the cell. It is not clear how methylcholanthrene operates to produce an altered equilibrium at the precancerous level. Nor is there as yet unequivocal evidence that breakdown products of methylcholanthrene, under investigation by W. L. Simpson in this hospital, are the causal agents in sequelae of chemical changes occurring in the cell. But there is a strong suggestion that in some manner the balance of euchromatin-heterochromatin is upset. In a consideration of present status of the part played by nucleic acids in tumor production, the work of Caspersson and Santesson (7) stands out. These investigators obtained quantitative data, by using ultraviolet spectrophotometric and cytochemical methods, on disturbances in endocellular chemical mechanisms in epithelial tumors and propound the thesis that the genetically inert heterochromatic segments of chromosomes have a significant role in carcinogenesis. They believe that, since heterochromatin is a special center for synthesis of cell protein, any quantitative disturbances in it would immediately affect the pace of cell growth and during mitoses cause cumulative disturbances in the division of euchromatic and heterochromatic segments. This in turn, is assumed to pave the way for further qualitative and quantitative changes leading in the direction of neoplastic growth.

In the light of the concepts formulated by Caspersson and Santesson, the early drop in desoxyribose nucleic acid in hyperplastic epidermal chromosomes may logically be explained in either of two ways. First, there may be a direct lowering of desoxyribose nucleic acid synthesis in the nuclei following methylcholanthrene treatment. The more probable alternative, however, is that the decreased desoxyribose nucleic acid is a consequence of its partial conversion into cytoplasmic ribose nucleic acid. Biese's findings (3) on the pronounced increase of cytoplasmic ribose nucleic acid, even as early as half a day after methylcholanthrene treatment, bear this out. In the case of squamous cell carcinoma the desoxyribose nucleic acid content is conspicuously intensified. This again suggests several interpretations. First, there may be less conversion of the desoxyribose nucleic acid into the ribose form. Alternatively, it may mean a greater synthesis of the desoxyribose nucleic acid connected with more intense mitotic activity.

Whether the altered desoxyribose nucleic acid equilibrium in hyperplastic cells is the first step that sets in train a sequence of events in the cellular system on its way to malignancy, or whether it is merely an accompanying phenomenon along with the other occurrences, such as decreases in calcium, iron, copper, zinc and lipids, cholesterol, etc., is not evident. But in view of the importance of desoxyribose nucleic acid in chromosomal autonomy and heredity and since a cancer cell is, genetically speaking, distinct from a normal one, any event that modifies this hereditary constituent is likely to have far-reaching consequences. It is, therefore, not out of place to examine in this connection the work of other investigators who have analyzed this problem in the light of the euchromatin-heterochromatin balance. The case of Sorghum purpureo-tericen investigated by Darlington and Thomas (17) is particularly interesting on the theory that nucleic acid imbalance causes malignancy. In normal plants, according to these workers, the first division of the pollen grains gives a vegetative and a generative nucleus, the latter of which gives rise to the two male nuclei. But in plants where heterochromatic supernumerary B-chromosomes are

**DESCRIPTION OF FIGURES 1 TO 3**

The photomicrographs were made from Feulgen stained preparations and are magnified approximately 700 diameters. In the ringed areas, the morphology of chromosomes is distinctly seen. Many of the chromosomes show a differentiation into long and short arms, separated by Feulgen-negative gaps, which correspond to the position of the centromeres.

![Fig. 1](cancerres.aacrjournals.org) Chromosomes isolated from normal epidermis.

![Fig. 2](cancerres.aacrjournals.org) Isolated chromosomes from hyperplastic epidermis.

![Fig. 3](cancerres.aacrjournals.org) Isolated chromosome concentrate from squamous cell carcinoma.
present, the vegetative nucleus undergoes a succession of\npolymitotic divisions giving rise to several generative\nnuclei. To all intents and purposes the pollen grain\nbehaves like an “encapsulated tumor.” This exception-\ntional behavior of the vegetative nucleus is presumably\nbrought about by action of the supernumerary hetero-\ncromatic B-chromosomes. The activity of so-called\ninert chromosomes would, therefore, seem to indicate\nthat nucleic acid supply is a crucial variable in the\ncausation of neoplastic growth. From this viewpoint\nDarlington (18) visualizes in normal growth a correct\neuchromatin-heterochromatin balance. Morbid mitoses,\nhis thesis, are produced when this balance is upset.

From a detailed cytological analysis of chromosome\nbehavior in 565 human tumors (carcinoma of the skin,\nesophagus, colon, rectum, larynx, lung, cervix, uterus\nand breast) Koller (24) has come to similar conclusions.\nHe maintains that the quantitative change in nucleic\nacid metabolism revealed by chromosome behavior may be\none of the deciding criteria of distinction between\nnormal cells and tumor cells derived from them. Accord-\ning to him the normal functioning of a cell is con-\ntioned by a specific heterochromatin-euchromatin balance.\nSince the amount of nucleic acid and the rate of\nits production determine the frequency of division,\nKoller argues that there must be a causal connection\nabnormal high concentrations of deoxyribose\nnucleic acid and intensified division rate and malignancy.

That there is an increase in the amount of Feulgen-\npositive material in rat sarcoma as compared with nor-\nmal rat fibroblasts was histochemically shown by\nCowdry and Paletta (16) from a study of tissue culture of these\ncells. Stowell (35) devised a photometric histochemi-\nal method for the determination of thymonucleic acid\nin normal, hyperplastic and neoplastic epidermis of mice\nand men, and established the order of decreasing mean\namount of thymonucleic acid per unit volume of tissue\nand per cell. Hyperplastic epidermis in which the cells\nwere large, contained less thymonucleic acid per unit\nvolume of tissue than the normal epidermis and the\ncarcinomas contained more deoxyribose nucleic acid per\nunit volume of tissue than the normal epidermis. Using\nhomologous normal and tumor tissues (rat liver, rat\nhepatoma, mouse lung and mouse lung tumors)\nSchneider (30) discovered that the deoxyribose nucleic\nacid content of both tumors was much higher than that\nof their normal homologues.

In contrast to the findings of the previous workers\nDounce (23) found no increase in the deoxyribose\nnucleic acid of nuclei isolated from Walker carcino-\nsarcoma 256, normal liver and rat hepatoma 31; but\nactually observed a decrease in the hepatoma cells. It\nis difficult to explain this apparent discrepancy unless\nwe admit that it is due to differences in technic, and
type described by Koller (24) and Darlington and Thomas (17). From the cytochemical evidence at hand the conclusion that there is a constant association of increased desoxyribose nucleic acid with squamous cell carcinoma is thus inescapable and because nucleic acids are so intimately bound up with the genetic structure of the cellular system, it would, therefore, seem that quantitative changes in their attachment or detachment from the chromosomes assume more than trivial interest. It is irrelevant for our argument whether this augmentation in carcinoma is the result of greater storage or greater synthesis of desoxyribose nucleic acid or lesser conversion of the latter into the ribose type.

We hav e remarked earlier that there are not sufficiently discernible features between chromosomes isolated from normal and abnormal tissues to warrant a distinction on a purely morphological basis. This does not necessarily mean that there are no enlarged chromosomes in neoplastic tissues, as observed by Biese le and Cowd ry (4). Since only a few cells are in mitosis in the hyperplastic and carcinomatous cells and fewer still in metaphase, it would not be a simple matter to detect enlarged chromosomes among the enormous number of threads presented by chromosome concentrates. However, the earlier observations of Biese le on chromosomal changes in epidermal carcinogenesis and the assumption of polytene (multiple strand) structure of neoplastic chromosomes need to be revised. A valid criterion of distinction between chromosomes isolated from different states of epithelium would be the recognition of quantitative differences in their desoxyribose nucleic acid content.

SUMMARY

1. A mechanical method of separation and isolation of chromosomes from the tissues of mouse epidermis in normality, hyperplasia and squamous cell carcinoma is described.

2. Cytochemical determinations of desoxyribose nucleic acid from the three stages in carcinogenesis are given. Soon after the application of methylcholanthrene there is seen a sharp drop in the concentration of desoxyribose nucleic acid in the chromosomes, which is maintained at a new equilibrium level until the onset of carcinoma. In this state there occurs a striking increase in the desoxyribose nucleic acid content of the chromosomes, far above that of chromosomes in normal and hyperplastic epidermis.

3. The bearing of the disturbances of nucleic acid equilibrium on carcinogenesis is discussed.

ACKNOWLEDGMENT

We wish to express our grateful thanks to Dr. C. Carruthers of this Hospital for his generous cooperation in the cytochemical aspect of this investigation. We are also appreciative of the many helpful suggestions offered by Dr. W. L. Simpson.

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