

# FEULGEN HYDROLYSIS OF NORMAL CELLS AND MOUSE ASCITES TUMOR CELLS

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## ABSTRACT

The effect of HCl hydrolysis on the dye content (Feulgen reaction) of normal cells and mouse ascites tumor cells was examined by means of cytophotometric measurements. After 11 min of hydrolysis, 16-day-old tumor cells showed a hypotetraploid DNA line with doubling peaks. The DNA values were in the ratios of 1:2:4:8 during all the tested hydrolysis times (3 to 21 min). The size of the nucleus and the DNA concentration did not influence the hydrolysis and the dye content. However, the time of the hydrolysis considerably influenced the dye content of normal and tumor cells. The course of the curves obtained by plotting dye absorption against hydrolysis time showed an inflection of the curve at 9 min' hydrolysis time in tumor cells, whereas the inflection occurred at 8 min in mitotic cells. These inflections were statistically significant. The DNA stem-line<sup>1</sup> for tumor cells shifted during different hydrolysis times when compared to normal cells. The possibility is discussed of two types of DNA which differed in their acid sensitivity and which yielded atypical hydrolysis curves.

## INTRODUCTION

The Feulgen reaction together with cytophotometric measurements has recently been used to determine the DNA contents of normal and tumor cells (3, 9, 12). In these investigations, the DNA contents of tumor cells and normal cells were compared. It was assumed that the Feulgen reaction occurred identically in different cells. Furthermore, on the basis of population averages the dye content was considered to be proportional to the DNA content. This relation, however, has not yet been verified on a cell-to-cell basis.

We examined the effects of the time of hydrolysis on the dye content of different types of normal cells and of cell populations from mouse ascites tumor. The significance of the size of the nucleus

<sup>1</sup> The expression "DNA stem-line" is derived from the word "chromosome stem-line" and means a frequency distribution of the DNA contents of tumor cells in a certain area (DNA-class).

was explored. Differences in the hydrolysis curves of normal and tumor cells during interphase and mitosis are also presented in this communication.

## MATERIALS AND METHODS

Cellular smears were prepared from the Ehrlich mouse ascites tumor 4 and 16 days after inoculation (in two further preparations, after 14 days) and from thymic lymphocytes and peritoneal mesothelial cells. Smears of tumor cells and normal cells were always spread on the same slide.

### *Preparation*

Slides were air-dried for 10 min, fixed for 10 min in neutral formalin (10%), and washed briefly in distilled water. Hydrolysis in 1 N HCl (60°C) was carried out for 3 to 21 min in a water bath, thermostatically controlled ( $\pm 0.5^\circ\text{C}$ ) with stirring apparatus. Slides were then incubated for 1 hr in Schiff's reagent (pH 1.4) (7), prepared with Pararo-

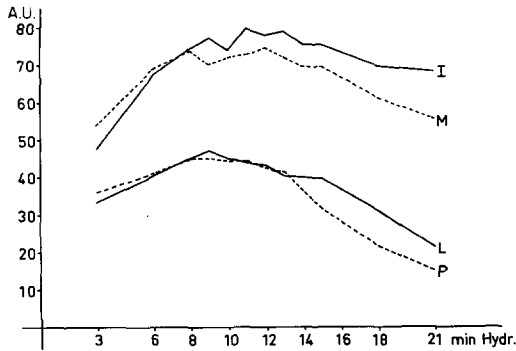


FIGURE 1 Hydrolysis time curve of mouse ascites tumor cells: I, interphase cells on 16th day; M, mitotic cells on 4th day; L, thymic lymphocytes, and P, peritoneal mesothelial cells. A.U. = arbitrary units. Every point on the curve is represented in I by 100 cells, in M by 50 cells, and in L and P by 20 cells.

sanilin (Bayer, Leverkusen), part K 44 564. Slides were washed in SO<sub>2</sub> water, three times for 10 min each, washed in distilled water, dehydrated in 70%, 96%, and absolute alcohols and xylol, each for 5 min, and mounted and embedded in Eukitt ( $n_D = 1.494$ ), while the refractive index of the cells was about  $n = 1.53$ .

#### Estimation of the Error Caused During Preparation and Measuring

The light-scattering error caused mainly by the differences in the refractive indices was estimated by measuring unstained controls. For this purpose, another set of slides was prepared, hydrolyzed, and measured in the same way as those described above, the only difference being that they were not incubated in Schiff's reagent. At every time of hydrolysis, 10 of the slightly visible shadows of the nuclei were measured in comparison to the background. The registered arbitrary units (A.U.) were found between 0 and 4 (independent of the time of hydrolysis); the average was at 1.15 A.U. (1.48% of 77.3 A.U. at 9 min hydrolysis in the stained set of slides). This means that the error caused by light scattering or by the difference of 0.04 units in the refractive index of the mounting medium is very small and nearly the same for all times of hydrolysis and, therefore, may be disregarded. Similar results were found by Srinivasachar and Patau for the two-wavelength method (14).

#### Cytophotometry

1. Deeley's integrating microdensitometer (5) was used for the measurements (Messrs. Barr & Stroud, Glasgow, Scotland).

CONDITIONS FOR MEASURING: Interference

filter  $\lambda$  560 m $\mu$ ; condenser NA 0.3. Objective NA 1.25; ocular  $\times 10$ ; size of the scanning diaphragm, 0.7  $\mu$ . Reproducibility of the measurements,  $\pm 1.5\%$ .

2. Since the integrating microdensitometer cannot be applied for the separate estimation of the nuclear size and the extinction, a registering cytophotometer (self-constructed) was used. Parts and conditions for measuring: Monochromator (Zeiss PM Q II,  $\lambda$  560 m $\mu$ ), condenser NA 0.32, objective NA 0.85, ocular  $\times 10$ . Measuring point, 0.7  $\mu$ . Movement of the object with a highly precise mechanical stage from Zeiss (Präzisionsfeinverschiebetisch) (1  $\mu$ /sec). Integral registration of the extinction was made with the Honeywell recorder. Distance of the scanning lines was 1  $\mu$ . The reproducibility of the measurements was  $\pm 2\%$ . The area was calculated from the individual scanning lines. The statistical calculations were made by the t-test and the Duncan test (6).

## RESULTS

### 1. The Dye Content (A.U.) as a Variable of the Time of Hydrolysis

The hydrolysis curves for normal cells, such as thymic lymphocytes and peritoneal mesothelial cells, show a typical course with a slow rise, broad plateau in the region from 9 to 12 min, and a gradual decline (Fig. 1). The curves of tumor cells are different. Tumor cells in interphase at the 16th day after inoculation show a fall and subsequent rise to a maximum.

The resting cells have a lower dye content (74.3 A.U.;  $s = 5.05$ ) after 10 min of hydrolysis than after 9 min (77.3 A.U.;  $s = 5.74$ ;  $d = 3.03$ ;  $t = 3.96$ ) or 11 min (79.6 A.U.;  $s = 5.19$ ;  $d = 5.37$ ;  $t = 7.41$ ). In mitosis, the fall in the curve is shifted to the left about 1 min with a minimum at 9 min (70.2 A.U.;  $s = 4.99$ ) and relative maxima at 8 min (74.0 A.U.;  $s = 3.28$ ;  $d = 3.78$ ;  $t = 4.06$ ) and 11 min (72.9 A.U.;  $s = 4.02$ ;  $d = 2.63$ ;  $t = 3.08$ ). ( $d$  = difference of the means,  $s$  = standard variation.)

This decrease in the dye content at 9 and 10 min, respectively, is statistically significant ( $P < 0.05$ ). The same inflection of the curves occurs in the different DNA classes (I to III) of resting cells and mitotic cells (Fig. 2).

In two additional experiments (cells in interphase), an average decrease of 4.4% A.U. was found at 10 min when compared with the A.U. values at 9 min, and an average decrease of 6.5% A.U. when compared with the 11 min in one

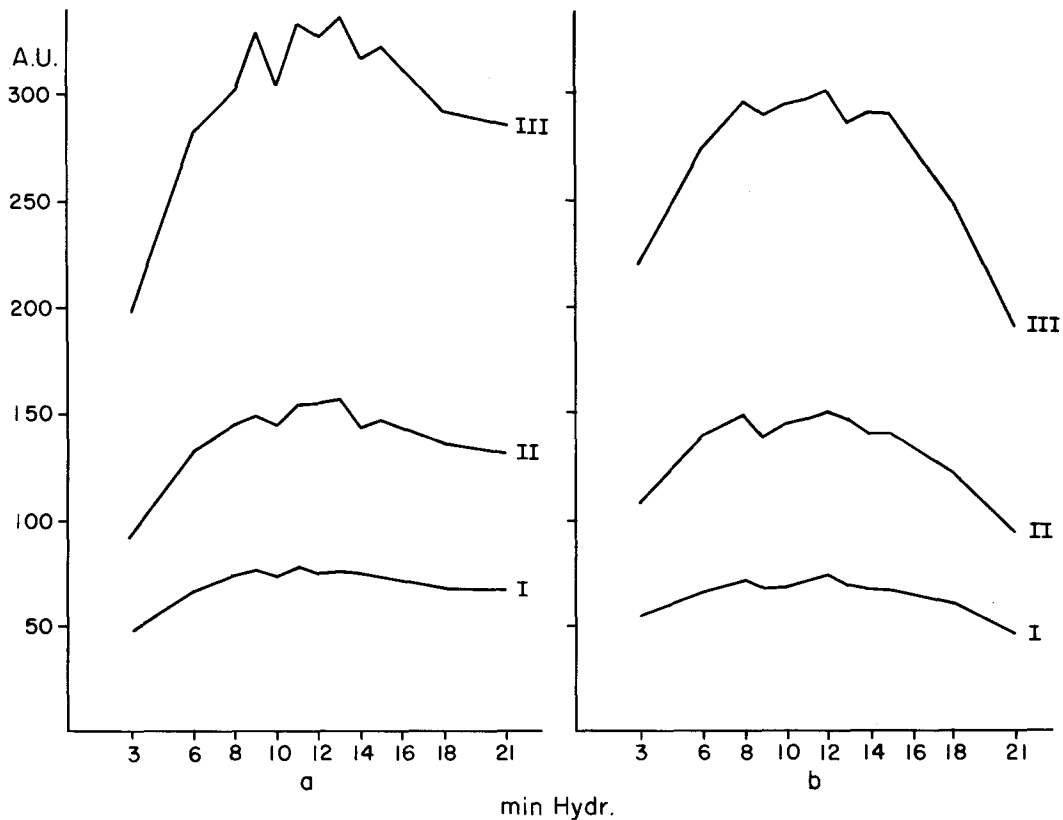


FIGURE 2 Hydrolysis time curves of mouse ascites tumor cells: *a*, interphase cells; *b*, mitotic cells. Divided into DNA classes: *I* = DNA-class I which corresponds with the stem line. *II* = DNA-class II. *III* = DNA-class III.

curve, respectively, 12 min in the other curve (A.U. value at 9 min = 100%).<sup>2</sup>

Very recent experiments with interphase cells of the same tumor under somewhat changed conditions of hydrolysis (N/10 HCl, 37°C, lasting from 3 to 51 hr) showed a very deep and obvious decrease in the course of the hydrolysis curve.

## 2. Stem-Line and Time of Hydrolysis

In comparison to normal cells, the measured tumor cells have a hypotetraploid value after 11 min of hydrolysis. As shown in Fig. 3, the diploid value for lymphocytes is 44 A.U.; the so called "DNA stem-line" of a 16-day-old tumor is 79 A.U. (DNA-class I = hypotetraploid stem-line). Corresponding polyloid values are found at 155

<sup>2</sup> H. Krug (Department of Pathology, University of Leipzig) found an inflection of the hydrolysis curve in a mouse ascites tumor at 11 min to be about 10% (personal communication).

A.U. (DNA-class II), 334 A.U. (DNA-class III) as well as at 650 A.U. (DNA-class IV) (Fig. 3 *a*). The stem-line of this tumor is more distinct on the 16th day of growth than on the 4th. 4 days after inoculation the tumor grows rapidly with a high mitotic rate. This results in a large number of cells in DNA synthesis, so that many DNA values are found between the polyloid peaks (Fig. 3 *b*).

A displacement is found when the position of the tumor DNA stem-line is compared with the diploid lymphocytic values during different times of hydrolysis. As shown in Fig. 4, the average value of the first frequency maximum of the tumor measuring values (stem-line) is 44% higher than the diploid value of the lymphocytes during 3 min of hydrolysis. After 6 min of hydrolysis, it is 67% as opposed to 77% for 8 min, 68% for 9 min, 59% for 10 min, 79% for 11 min, 78% for 12 min, and 68% for 13 min. At 3 min hydrolysis time, the tumor stem-line would have a triploid value, and

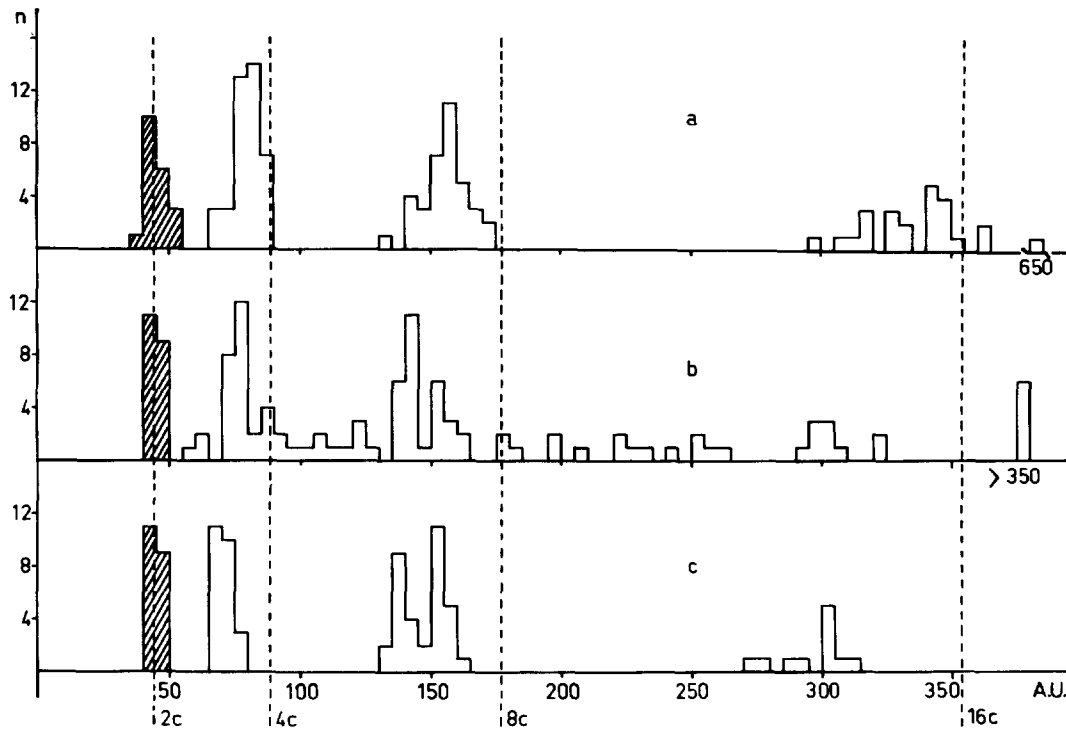


FIGURE 3 DNA distribution curves of lymphocytes (striped) and mouse ascites tumor cells: *a*, on 16th day; *b*, on 4th day after inoculation; *c*, measured values of mitosis on the 4th day. At *c*, the first maximum represents values of a single telophase or an anaphase chromosome plate. The 2nd maximum at 150 A.U. contains both telophase nuclei, both anaphase chromosome plates, and the prophases and metaphases and anaphases and telophases of the next ploidy step. *h*, number of measured nuclei. *2c*, diploid values; *4c*, tetraploid values; *8c*, octoploid values; etc. Hydrolysis time, 11 min.

at 11 to 13 min, a hypotetraploid stem-line would be found. The difference in the course of the hydrolysis time curves of normal and tumor cells results in this displacement of the DNA stem-line.

The modalities of the different DNA classes in the tumor cell population change with the different times of hydrolysis, in the same way that the tumor stem-line does. Table I *a* shows the relation of the DNA classes I to IV with regard to the stem-line (DNA-class I). The proportions remain 1:2:4:8 in all classes.

### 3. Cells in Mitosis and Time of Hydrolysis

The added DNA values for the two daughter chromosome plates of ana- and telophase as well as the pro- and metaphase nuclei are always found, as may be expected, in the next higher DNA class, while the separately measured values of ana- and telophase are in a class which corresponds to the interphase cells. (Fig. 3 *c*). However, the arbitrary units are slightly lower than the corresponding

interphase values on the 16th day. After 11 min of hydrolysis, the daughter chromosome plates of anaphase cells and telophase cells have a dye content of 73.5 A.U. (stem-line 79.1 A.U.). The mean of both daughter chromosome plates and of the pro- and metaphase is 149.8 A.U. (interphases 155.3 A.U.).

In class III, the mitoses measure about 300.0 A.U. (interphase cells 334.1 A.U.). The difference of the measured values is statistically significant for the stem-line ( $d = 5.6$ ;  $t = 7.3$ ;  $P < 0.01$ ). The fact that the hydrolysis curve of mitotic cells is shifted to the left by 1 min (Fig. 1) may explain this effect.

As in interphase cells, the DNA classes of mitotic values have ratios of 1:2:4:8 (see Table I *b*).

### 4. Size of Nucleus and Extinction of Tumor Cells as a Function of Time of Hydrolysis

The deviations between the hydrolysis curves for tumor cells and normal cells may be due to

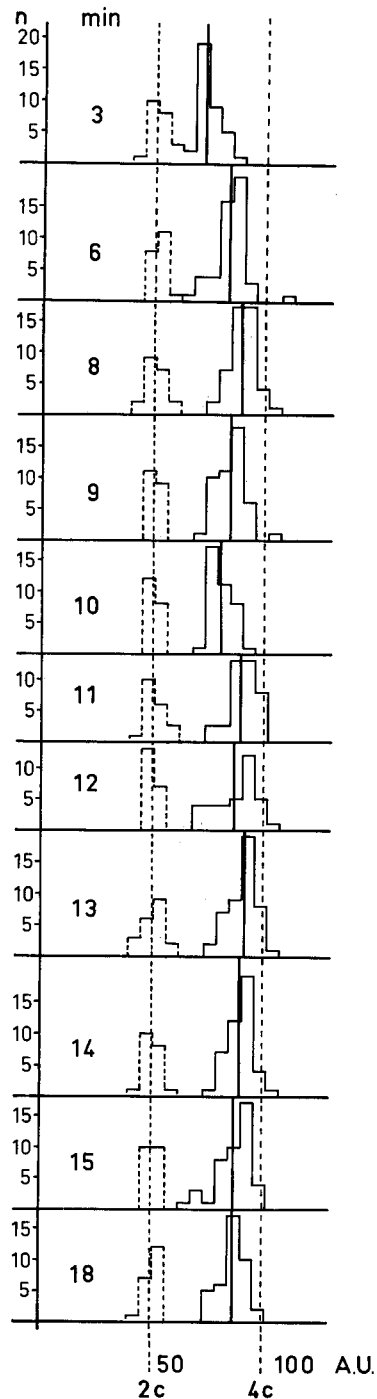


FIGURE 4 Diagram of the position of the tumor stem-line (DNA stem-line) in relation to the diploid value of lymphocytes during different hydrolysis times. The diploid DNA value after 9 min of hydrolysis was chosen as reference value.  $n$ , number of measured nuclei.

different nucleic acid concentrations and differences in the sizes of the nuclei. The sizes of the nuclei in the different DNA classes vary. The DNA values are distributed into regular classes. Therefore, the sizes of the nuclei cannot be a critical factor for the dye content. To show this relation, the tumor cells are grouped according to their sizes, and the average extinction at different times of hydrolysis is related to a given time. In relation to the standard value, all nuclei of different sizes are expected to show the same percentage deviation at the same hydrolysis time. As shown in Fig. 5, the DNA content during different times of hydrolysis is a certain percentage lower than the chosen standard values at 9 min of hydrolysis (=100%). However, after similar times of hydrolysis, the percentages of decrease are nearly equal for the different nuclear sizes (26% for 3 min, 6% for 12 min, 42% for 15 min, 64% for 21 min). These findings indicate that the size of the nucleus does not influence hydrolysis and dye content.

#### DISCUSSION

Purine bases are liberated from DNA during acid hydrolysis in the Feulgen reaction, depending on time and temperature, and aldehyde groups are set free, which can be demonstrated by the Schiff reagent (8). The presence of proteins may conceivably influence the development of the dye content (4, 10, 13, 15). Therefore, a different protein content or protein composition could change the dye content of different cells. The size of the nucleus could also be a limiting factor.

Our measurements of tumor cell populations with different DNA contents show that, even during different times of hydrolysis, constant relations are found in the different ploidy steps. It may be concluded that under cytophotometric conditions the Feulgen reaction on intact cells, after a certain time of hydrolysis in 1 N HCl, produces a dye product which is proportional to the DNA content despite differences in size, DNA concentration, and protein amount. Alfert et al. (2) and Sandritter et al. (11) could also show that the dye content of activated cells from endocrine organs (thyroid, islet cells of the pancreas) after the Feulgen reaction is not influenced by the non-histone protein content of the cell nuclei.

Comparative studies of mesothelial cells, lymphocytes, and tumor cells show that, always in respect to the diploid value of the lymphocytes,

TABLE I

Results of Measurements (A.U.) of Interphase and Mitotic Cells

*a*, Interphase cells on 16th day. *b*, Mitotic cells of mouse ascites tumor cells on 4th day. Measurements made as a function of hydrolysis time (Hydr.). All values were referred to class I (stem-line).

Hydr.	Cl.I	Cl.II	Cl.III	Cl.IV	Hydr.	Cl.I	Cl.II	Cl.III	Cl.IV
<i>min</i>					<i>min</i>				
3	1	1.95	4.15		3	1	1.99	4.07	7.96
6	1	1.93	4.24		6	1	2.12	4.17	
8	1	1.94	4.04		8	1	2.09	4.15	
9	1	1.93	4.25		9	1	2.05	4.30	
10	1	1.95	4.08		10	1	2.14	4.37	
11	1	1.96	4.22	8.22	11	1	2.07	4.19	
12	1	2.05	4.32		12	1	2.04	4.08	
13	1	2.04	4.38		13	1	2.13	4.14	8.30
14	1	1.90	4.18		14	1	2.09	4.34	
15	1	1.99	4.34		15	1	2.11	4.34	
18	1	1.99	4.24		18	1	2.01	4.10	7.75
21	1	1.94	4.17		21	1	2.05	4.13	7.88

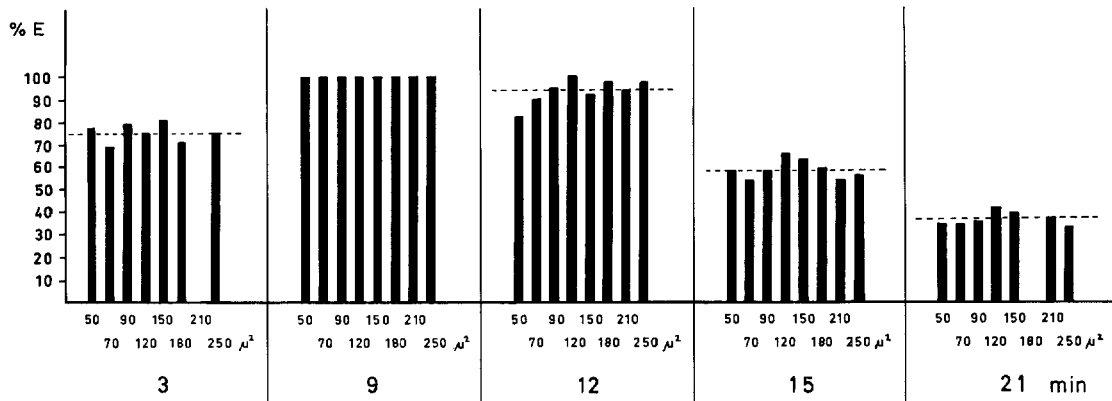
*a**b*

FIGURE 5 Diagram of the relationship between size of nucleus and extinction during different times of hydrolysis of tumor cells (interphase cells). *E*, extinction. 9 min of hydrolysis time was taken as 100%.

the dye content of the tumor cell population (stem-line) varies during different times of hydrolysis from triploid values after 3 min to hypotetraploid values after 8 min and 9 min, respectively (Fig. 4). However, apparently the real DNA content of the tumor cannot change its ploidy with hydrolysis time. This error, which should be considered during such examinations, may be caused by the relatively less liberation of aldehyde groups during short times of hydrolysis. The curve inflection after 10 min of hydrolysis (i.e. 9 min for mitosis) also changes the position of the DNA stem-line. Both facts point to differences in acid sensitivity of DNA of tumor cells and DNA of other

cells. Above all, the two peaks in the hydrolysis curves of the tumor cells may be explained by the superimposition of two curves with peaks at different times. This would suggest the existence of two different nucleoproteins, one being more sensitive to acid hydrolysis than the other, in the liberation of aldehyde groups as well as in degradation and solubilization of the nucleoprotein complex, which causes the subsequent decrease of the curves after longer times of hydrolysis. Agrell and Bergquist (1) also found hydrolysis curves with more than one maximum in embryo cells of *Rana temporaria*, and Woods (16), in experiments with lily anthers, concluded that the DNA is

composed of acid-sensitive and relatively acid-resistant components in a ratio of 3:1.

The difference in acid sensitivity could be attributed to nucleoproteins with different protein components. Further investigations have convinced us that these differences are due to different contents of euchromatin and heterochromatin.<sup>3</sup> For mitotic cells, we observed a shift of the hy-

drolysis curve to shorter times for about 1 min. Whether this effect is a reflection of a change in the nonhistone protein content or loss of nuclear membrane, or other factors, remains to be demonstrated.

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<sup>3</sup> See Sandritter et al., *Histochemie*, 1965, 4, 420.

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