CONSTANCY OF DNA CONTENT IN ADRENAL MEDULLA NUCLEI OF COLD-TREATED RATS

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ABSTRACT

Reports of changes in DNA content of certain types of cells following exposure to conditions of stress has led to the suggestion that two kinds of DNA may be present. One is genetic DNA, and the other is called "metabolic" DNA. In a further attempt to investigate the possibility of this phenomenon, determinations of DNA content were made on Feulgen-stained nuclei of adrenal glands and kidneys in cold-treated rats. Feulgen-stained nuclei were measured by two-wavelength microspectrophotometry. Particular attention was given to the handling of the smears in hydrolysis and staining. Mean values of Feulgen-DNA contents in a total of 720 nuclei demonstrated (*a*) a constancy of DNA content within 2% in individual nuclei both in adrenal medulla and kidney cortex, (*b*) no more than an average of 2% difference in DNA content between control and experimental nuclei, and (*c*) no more than an average of 1.5% difference in DNA content between normal kidney cortex nuclei and normal adrenal medulla nuclei. These results confirm the view that the more precise the measurement, the more accurately the constancy rule is obeyed. Moreover, there is no support for the concept of a metabolic DNA in the rat adrenal medulla.

INTRODUCTION

In attempting to explain several experimental observations of changes in DNA contents resulting from imposed physiological stress, or, in some cases, morphogenetic activities, the concept of "metabolic" DNA has been proposed (12). The hypothesis embodies the view that there are two forms of DNA in certain types of cells: one that is stable with a genetic function, and the other that is unstable with a physiological role (13). Low temperature is one of the extrinsic factors that has been reported to induce changes in nuclear DNA content. Darlington and LaCour (2), for example, indicated a loss of chromatin in the heterochromatin of cold-treated *Fritillaria* chromosomes, and LaCour et al. (6) confirmed this result with their report of a loss of Feulgen-DNA in the root cells of *Vicia*, *Trillium*, and *Paris*. On the basis of these and other observations, the suggestion has been made that heterochromatin might be the site of the metabolic DNA, and euchromatin the site of genetic DNA. However, more recent experiments of the kind described by Woodard et al. (18) do not support the view that cold treatment results in a loss of DNA from chromosomes.

Changes of a similar nature have been described in animal tissues following application of certain hormones or other substances (11). The effect of cold treatment on the nuclei of rat thyroid and adrenal medulla was exhibited as an increase in Feulgen-DNA content up to 15% (7, 10, 16). On the other hand, Viola-Magni (17) has presented data showing a decrease in the DNA content of the adrenal medulla nuclei in cold-treated rats. It was presumed that the difference in procedure accounted for the contrasting results; Leeman (7) employed continuous cold exposure, whereas Viola-Magni exposed the rats intermittently to cold. In the latter case, with a total experimental

time of 300 hr, as much as a 40% decrease in DNA content of adrenal medulla nuclei was detected. Several of the criticisms raised by a number of investigators to the various, and often conflicting, results showing changes in DNA content have been related to the technical problems encountered in spectrophotometric measurements. The two-wavelength method of cytophotometry is one that avoids or minimizes several of the difficulties of measurement, for example, those of distributional error and stray light. In a further attempt to examine the question of DNA loss from cold-treated nuclei, determinations of DNA content were made on Feulgen-stained nuclei of rat adrenal glands and kidneys. Whereas the handling of the animals was according to the experimental procedures followed by Viola-Magni, who used the technique of scanning integration, our measurements were made with a two-wavelength cytophotometer. These investigations have revealed no significant loss of DNA from the nuclei of cold-treated rats.

MATERIAL AND METHODS

Animals

Male albino rats from the stock maintained in the Laboratory for Pathology (Leiden) and derived from the inbred strain RT of The Netherlands Cancer Institute, Amsterdam were used. The rats were fed on a standard diet, and were raised in separate cages for the duration of the experiment. They had an average weight of 250–300 g and were of average age between 100 and 200 days. Six rats were kept at room temperature (±20°C) for the experimental period (300 hr). Six other rats were placed in a cold room at 4°C for 15 hr/day, and at room temperature, in the same location with the control rats, for 9 hr/day. Thus the experimental rats were in the cold intermittently for a total of 195 hr. At the end of the final cold period, the rats were sacrificed by stunning and exsanguination.

Cytological Procedure

The medulla of the adrenal gland from each rat was carefully dissected from the cortical tissue, and gently smeared on clean slides. A small slice of kidney cortex was smeared in a similar manner, and air dried. The dried smears were quickly placed in a mixture of nine parts methanol: one part 35% formaldehyde for overnight fixation. They were then washed thoroughly in methanol (three rinses and overnight), and stained according to the Feulgen procedure. A large volume of 1 N HCl was employed for hydrolysis, and the temperature was carefully maintained at 60°C throughout the 12 min period. Smears from the tissues of all the rats, control and experimental, were stained simultaneously in the same container with fresh Schiff's reagent. As a control for the staining procedure and subsequent cytophotometry, smears of chicken erythrocytes were hydrolyzed and stained with the rat smears. The slides were made permanent with a mounting medium, Caedax (Merck), with a refractive index of 1.55.

Spectrophotometric Procedure

The Feulgen-stained cell nuclei were measured with a microspectrophotometer based on the two-wavelength principle. The instrument is described by van der Ploeg and van Duijn,[1] and is basically similar to the one described by Mendelsohn (8).

Manual movement of a 1 mm diameter aperture through the 10 mm macrofield stop[1] facilitates the test for homogeneity. After very careful alignment, the deviations from complete homogeneity were not more than 4% for a field which was larger than that actually used in the measurements. The diameter of the limiting diaphragm is ¼–½ the illuminated field.

This homogeneity test was carried out at the beginning and at the end of each series of measurements. Stray light was tested with iron filings embedded in Caedax, and was found to be below ½%.

The spectra of Feulgen-stained nuclei were measured with a 0.6 μ diameter measuring point and an illuminated field of 1 μ in diameter, with Zeiss Ultrafluar optics. The wavelength with an extinction half that at the maximum of absorption at 565 mμ was found to be 506.0 ± 0.6 mμ.

A 10 × objective with a numerical aperture of 0.30 was used as the condenser. The test described by Chamberlain and Walker (1) revealed that the 0.6 μ aperture received some stray light from its outer edges but none outside the area with a diameter of 1 μ. This resulted in an effective measuring point of 1 μ-free of stray light.

RESULTS

The data derived from the spectrophotometric measurements are summarized in Tables I and II. Each value, in arbitrary units of extinction, represents the mean ± the standard error of the mean of measurements on 10 cells, for a total of 30 cells on three slides. The average represents mean values for the three slides of each group, and

[1] van der Ploeg, M., and P. van Duijn. Quantitative cytophotometric determination of PAS-positive material in neutrophilic leucocytes. Data in preparation.

TABLE I

Feulgen-DNA Contents of Nuclei of Kidney Cortex and Adrenal Medulla of Normal Rats

Rat No.	Slide	Kidney cortex	Rat No.	Slide	Adrenal medulla
		mean SEM			
	1	0.130 ± 0.001		1	0.139 ± 0.001
1	2	0.139 ± 0.002	1	2	0.137 ± 0.001
	3	0.136 ± 0.001		3	0.141 ± 0.001
	avg.	0.135 ± 0.003		avg.	0.139 ± 0.001
	1	0.138 ± 0.001		1	0.136 ± 0.002
2	2	0.144 ± 0.001	2	2	0.148 ± 0.002
	3	0.136 ± 0.002		3	0.137 ± 0.002
	avg.	0.139 ± 0.003		avg.	0.140 ± 0.004
	1	0.138 ± 0.001		1	0.141 ± 0.002
3	2	0.139 ± 0.001	3	2	0.142 ± 0.001
	3	0.136 ± 0.001		3	0.129 ± 0.001
	avg.	0.138 ± 0.001		avg.	0.137 ± 0.004
	1	0.142 ± 0.001		1	0.134 ± 0.002
4	2	0.135 ± 0.001	4	2	0.131 ± 0.002
	3	0.136 ± 0.002		3	0.142 ± 0.002
	avg.	0.138 ± 0.002		avg.	0.136 ± 0.004
	1	0.134 ± 0.002		1	0.134 ± 0.001
5	2	0.138 ± 0.001	5	2	0.141 ± 0.001
	3	0.133 ± 0.001		3	0.132 ± 0.001
	avg.	0.135 ± 0.002		avg.	0.136 ± 0.003
	1	0.143 ± 0.001		1	0.144 ± 0.001
6	2	0.141 ± 0.001	6	2	0.138 ± 0.002
	3	0.131 ± 0.001		3	0.153 ± 0.002
	avg.	0.138 ± 0.003		avg.	0.145 ± 0.005
	Total	0.137 ± 0.001		Total	0.139 ± 0.001

the figure in the "Total" line is the mean of these mean values. It was, therefore, possible to determine the degree of variation from cell to cell on one slide, the variation between slides, the variation among rats, and of course, any differences between control and experimental conditions. All such variations can be observed from the data in the tables. Application of the *t* test to the total mean values for each of the four major categories demonstrated no significant differences at the 5% level between control kidney nuclei and control adrenal nuclei, between control and experimental adrenal nuclei, or between experimental kidney nuclei and experimental adrenal nuclei. Between experimental and control kidney nuclei the differences in values were statistically significant at the 5% level but not at the 1% level. However, the biological significance of this difference is greatly minimized by the other correlations. Acceptance of this difference as significant still represents only a 2% "loss" of Feulgen-DNA, a value far below any reported by Viola-Magni. In fact, this difference is noted in the kidney, whereas Viola-Magni reports a loss only in the adrenal medulla.

The constancy of the amount of Feulgen-dye found in different nuclei on one slide is quite high. Standard deviations did not exceed, on the average, 3%, and were thus slightly lower than those reported for the earlier version of the instrument. In the previous experiments the total variation between nuclei was not more than 4%, and the difference in the mean values for 10–15

TABLE II

Feulgen-DNA Contents of Nuclei of Kidney Cortex and Adrenal Medulla of Cold-Treated Rats

Rat No.	Slide	Kidney cortex	Rat No.	Slide	Adrenal medulla
		mean SEM			
	1	0.133 ± 0.001		1	0.130 ± 0.001
7	2	0.142 ± 0.001	7	2	0.134 ± 0.001
	3	0.132 ± 0.002		3	0.132 ± 0.003
	avg.	0.136 ± 0.003		avg.	0.132 ± 0.001
	1	0.131 ± 0.001		1	0.142 ± 0.003
8	2	0.140 ± 0.002	8	2	0.137 ± 0.001
	3	0.125 ± 0.001		3	0.136 ± 0.002
	avg.	0.132 ± 0.005		avg.	0.138 ± 0.003
	1	0.136 ± 0.001		1	0.140 ± 0.002
9	2	0.134 ± 0.001	9	2	0.143 ± 0.001
	3	0.137 ± 0.001		3	0.131 ± 0.001
		0.136 ± 0.001			0.138 ± 0.003
	1	0.133 ± 0.001		1	0.136 ± 0.001
10	2	0.131 ± 0.001	10	2	0.141 ± 0.001
	3	0.133 ± 0.001		3	0.133 ± 0.001
	avg.	0.132 ± 0.001		avg.	0.137 ± 0.002
	1	0.133 ± 0.001		1	0.127 ± 0.001
11	2	0.133 ± 0.001	11	2	0.140 ± 0.002
	3	0.129 ± 0.001		3	0.135 ± 0.002
	avg.	0.132 ± 0.001		avg.	0.134 ± 0.004
	1	0.137 ± 0.002		1	0.138 ± 0.002
12	2	0.142 ± 0.002	12	2	0.134 ± 0.002
	3	0.132 ± 0.001		3	0.140 ± 0.002
	avg.	0.137 ± 0.003		avg.	0.137 ± 0.002
Total		0.134 ± 0.001	Total		0.136 ± 0.001

nuclei among such different cells as kidney cells, liver cells, and leukocytes was less than 1%. The present experiment confirms the position that the more precise the measurement, the more accurately the constancy rule is obeyed.

Differences in response to the stain and/or hydrolysis may be considered on the basis of measurements of the chicken erythrocyte nuclei, all of which were from one blood sample. Six slides of smeared erythrocytes were placed at random in the staining containers along with the smears of rat cells. The results of the measurement of Feulgen-DNA in the erythrocytes are presented in Table III, and indicate that the main variation is between slides. Apparently there is a factor which differs between slides and which results in a 3% variation. This may be due to some uncontrolled variable factors in the staining or in the optical quality of slides and embedding. Since these random factors are averaged in the mean values of the three slides from one animal, it turns out that the variation between animals is in the 2% range. Whatever the reason for such differences, the fact that they are found in both types of preparations strongly supports the conclusion that the differences found in the experimental material are not due to the conditions of treatment. Moreover, because of the differences found between slides, it is unlikely that the variations have a biological basis.

The data, taken as mean values, demonstrate (*a*) a constancy of DNA content, with a variance

of about 2%, of individual nuclei both in adrenal medulla and in kidney cortex, despite the fact that nuclei differing in diameter by a factor of about 2 were measured without any selection, (*b*) no more than an average of 2% difference in DNA content between control and experimental rat nuclei, and (*c*) no more than an average of 1.5% difference in DNA content between normal kidney cortex nuclei and normal adrenal medulla nuclei. These results do not agree with those of Viola-Magni (17), who found as much as a 40% difference for adrenal medulla nuclei in case (*b*), and a 16% difference in case (*c*). Thus her findings of a loss in DNA from adrenal medulla nuclei in cold-treated rats and a higher average DNA content in adrenal medulla nuclei, compared with kidney cortex nuclei, have not been confirmed. Our preliminary studies have demonstrated similar results when rats were exposed for a total of 100 hr to intermittent cold treatment; there were no significant differences in DNA contents between control rats and cold-treated rats, or between normal adrenal medulla nuclei and kidney nuclei. Furthermore, measurements of Feulgen-DNA in adrenal cortex nuclei were comparable with those in adrenal medulla nuclei and kidney cortex nuclei following 100- or 300-hr experiments (unreported data).

TABLE III

Feulgen-DNA Content of Chicken Erythrocyte Nuclei

Each value is the mean of 20 nuclei ± SEM

Slide	1	2	3	4	5	6	Average
Mean ± SEM	0.284 ±0.006	0.274 ±0.002	0.297 ±0.003	0.280 ±0.002	0.261 ±0.002	0.270 ±0.002	0.278 ±0.003

DISCUSSION

Richards (9) has recently discussed the criteria necessary to establish an exception to the rule of DNA constancy. These criteria are (*a*) that chromosome number and variations thereof are known, (*b*) that no increase in DNA has occurred through synthesis, and (*c*) that errors in measurement, involving staining procedures and instrumentation, are absent. The first of these is difficult to satisfy for every cell to be measured; the second can be checked by combined radioautographic and cytophotometric methods (see Gall and Johnson, reference 3); and the last can be carefully controlled by routine tests for staining and regular tests of the instrument. It is the third criterion that has received particular attention in the investigations reported here. Considerable care was taken in the handling of the smears for both hydrolysis and staining. The hydrolysis time of 12 min, the control of the temperature (60°), and the use of a large volume of 1 N HCl with constant agitation were important features of the procedure. Of particular importance are the design and operation of the spectrophotometer, described earlier. The instrument was tested according to the criteria of Swift and Rasch (14), and twice-daily tests of homogeneity of illumination of the measuring field produced consistently reproducible values with a variation of 2–4%.

Comparison of the results of our experiments with those of Viola-Magni suggests that the disagreement is most likely due to differences between the instruments used for measurement of the nuclei. Whereas we employed two-wavelength spectrophotometry, Viola-Magni used a scanning integrating histophotometer based on the principle of the apparatus described by Jansen (5). At least one major and critical factor that can account for false values is that of stray light. This can be considerable since, in Jansen's design, the diaphragm in front of the multiplier has to remain open at least for the whole field to be scanned. In addition, the image of an aperture in a moving Nipkow disk, demagnified to about 1 μ, is inherently distorted by diffraction phenomena. This results in too low values for strongly stained chromatin particles and consequently in a distributional error, depending on the type of distribution of the chromophores in the object. For this reason, Jansen[2] changed the position of the Nipkow disk to the image plane in front of the multiplier.

Since the experimental procedures were otherwise identical to those of Viola-Magni, the tech-

[2] Jansen, M. T. Personal communication.

nical features of the histophotometer seem the most logical target for criticism. Moreover, some of the inconsistencies extant in the literature on the subject of metabolic DNA may very well be resolved by a careful examination of the type of cytophotometry employed for the Feulgen-DNA determinations in a given investigation.

The present investigation confirms the view that when Feulgen-stained nuclei are measured by a reliable cytophotometric principle, properly applied, variations either between cells or between tissues decrease. Nevertheless, the results of Hale (4), for example, who used an apparatus well controlled against error, showed a deviation from constancy for leucocyte DNA. Even this deviation could not be confirmed by den Tonkelaar and van Duijn (15). The use of fixation in methanol-formaldehyde by the latter authors instead of the freeze-substitution in ethanol as used by Hale may account for the different results in this case.

It is evident from our studies that, at least in the rat adrenal gland, there is no support for the concept of a metabolic DNA. This conclusion is in agreement with reports on other organisms as well (3, 18).

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