CHANGES IN THE DNA CONTENT OF ADRENAL MEDULLA NUCLEI OF RATS INTERMITTENTLY EXPOSED TO COLD

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ABSTRACT

In the adrenal medulla of rats exposed intermittently to cold (+4°C) for 100 and 300 hours, a considerable decrease (24 to 40 per cent) of the DNA content per nucleus was observed, followed by restoration to normal or above normal values within 10 days after the withdrawal of the stimulus. The findings were obtained with a scanning integrating histophotometer, and confirmed by microinterferometric investigations (on the basis of the measurement of total dry mass of nuclei isolated in aqueous medium before and after treatment with DNase) and by microchemical determinations, combined with the count of the nuclei in the homogenates. The observed decrease of DNA content cannot be attributed to errors of the methods used, nor to consequences of cellular degeneration. The available evidence seems to indicate a real decrease rather than a change in the state of a part of DNA in the nucleus *in vivo* whereby it becomes extractable by aqueous solutions. The restoration cannot be due to mitotic processes, which were actually never detected even with the use of colchicine, since the adrenal medulla cells in the adult rat are known to be irreversible, postmitotic cells. A correlation between the functional activity of the adrenal medulla cells and the content or state of DNA in their nuclei is demonstrated.

INTRODUCTION

The advent of visible light microphotometry, preceded by ultraviolet microphotometry, has provided considerable information on the amount of DNA contained in individual nuclei, on its relationship with the number of chromosomes, and on its variations in physiological and pathological events of the life of cells.

Some problems about the constancy of the DNA content per nucleus, however, remain unsolved as yet. Boivin (1) and Boivin *et al.* (2) maintain, on the basis of chemical investigations, that identical amounts of DNA are associated with a chromosome set of a species and that the DNA content can be assumed to be a biological constant. This concept has been supported also by results of histophotometric investigations (3–6).

However, several authors have pointed out that the DNA content per nucleus may increase or decrease under several conditions capable of inducing functional changes in the cell and not related to multiplication processes. Lison and Pasteels (7–9) first observed variations using Lison's histophotometer; with the same instrument other authors (10–29) observed changes in the DNA content of cells of various tissues. However, the accuracy of the Lison's histophotometer has been questioned (30). Similar results have been obtained with the use of the Pollister histophotometer (31) or similar instruments (32–33) and with more rigorous meth-

ods (34); variations have also been observed in various experimental situations, using integrating histophotometric apparatuses (35–37). The reported variations of the DNA content never exceed 10 to 15 per cent. Only in a restricted number of investigations have such variations been observed or confirmed by biochemical determinations (38–42).

In spite of the rather large number of these observations, the conclusions of which have been accepted by Brachet (43), several objections to their interpretation have been raised (36–44) which have not been conclusively rejected so far. These objections deal primarily with the technical approach used (apparatus and staining procedures) and with the possible interference of growth and multiplication processes on the data obtained.

The experiments reported in the present paper show that in the cells of the rat adrenal medulla, which are incapable of multiplication in adult life, the DNA content per nucleus decreases considerably (24 to 40 per cent) upon intermittent exposure of the animals to low temperature. The decrease is followed by recovery upon withdrawal of the stimulus. These variations have been measured with microphotometric, microinterferometric, and chemical methods.

A preliminary report of this work has been published elsewhere (45).

MATERIAL AND METHODS

1. *Animal Technique*

Male albino rats of the Italico strain, bred in this Department, have been used. They were kept on a standard balanced diet. Their weight ranged between 250 to 350 gm and their age was between 100 and 200 days.

The animals were divided into 4 groups: the first group was kept at a temperature of 4°C for 15 hours a day over a total period of 100 hours; the second group was subjected to the same treatment over a total of 300 hours; the third, after exposure to 4°C for 300 hours as before, was kept again at room temperature for a period of 10 days; a fourth group was kept at room temperature and served as control.

Animals were killed by stunning and exsanguination and the adrenal glands were quickly dissected out.

2. *Histochemical Procedure*

A. FIXATION AND EMBEDDING: The adrenal glands were fixed in 10 per cent neutral formalin for 24 hours, washed in tap water overnight, then quickly dehydrated and embedded in paraffin wax. Embedded together in the same block were an adrenal gland of a control animal, an adrenal gland of an experimental animal, and a piece of kidney of a control animal.

B. FEULGEN REACTION: The Feulgen method was used to stain DNA, on 15-μ-thick sections, according to the technique described by Leuchtenberger (46). The hydrolysis time was set at 12 minutes since it had been experimentally determined that such a duration of hydrolysis gives the maximum liberation of aldehydic groups. It was ascertained that cytoplasmic staining was always absent.

C. NAPHTHOL YELLOW S STAINING: In order to detect variations of the nuclear proteins, naphthol yellow S staining was used. According to Deitch (47), this substance stains in a stoichiometric way the basic groups of some amino acids (lysine, arginine, histidine). For this reaction, sections 3 μ thick were used, in which full-thickness sections of nuclei were chosen, in order to avoid interference from the basic cytoplasmic proteins. The extinction ratio between Feulgen dye and naphthol yellow S stain was measured (see below).

3. *Histophotometric Measurements*

In order to avoid the distributional error (48–50), extinction measurements on nuclei were made with a scanning integrating histophotometer. This apparatus was designed and built in this Department on the principle of the one described by Jansen (51). The light source is a stabilized mercury vapour lamp (Osram HBO 200), the wave length of 546 mμ being selected by a Zeiss interference filter. A report on the description of this apparatus is in preparation.

An histophotometer, also built in this Department and described by Bonucci (34), was used for the determination of the extinction ratio 480/546 mμ and for the absorption curves of Feulgen dye. Light source was a tungsten ribbon 100-w, 6-v, 18-A lamp. The desired wave length was isolated by a grating monochromator Bausch and Lomb 250 mm, with input and output slit openings of 1 mm, thus determining a band width of less than 10 mμ. In order to eliminate as much as possible flare light from the histophotometer, which gives rise to the Schwarzschild-Villiger effect (52), the original condenser of the monochromator was substituted by a special condenser, composed of a converging lens and of an objective $\times$ 40, NA 0.65 and containing an iris diaphragm. For the same purpose, the microscope condenser was replaced with an objective $\times$ 20, NA 0.40, supplied with an iris diaphragm, which was closed so as to give NA $\sim$ 0.3. Because of the use of this condenser system and with Köhler illumination, the illuminated area was reduced to that of the portion of the nucleus being measured (approximately $\frac{1}{3}$ the diameter of the latter). The extent of microscope

flare light was tested with opaque objects, according to Swift and Rasch (53), and found to be less than 0.3 per cent.

The extinction measurements for the calculation of the ratio of naphthol yellow S protein complex to Feulgen dye were made at wave lengths of 480 and 546 mμ, respectively. In the adrenal medulla nuclei the concentration of material staining with naphthol yellow S is very high, much higher than in the hepatocyte nuclei (54), and the measurements at 435 mμ, as indicated by Deitch (47), are not feasible. The wave length of 480 mμ was therefore used, in which the extinction of naphthol yellow S dye-protein complex is lower than at 435 mμ, the extinction due to Feulgen dye being practically similar to that at 435 mμ.

Besides the measurements on normal and experimental medulla nuclei, measurements were made also on kidney cortex nuclei used as a test object (34).

Some animals which had been exposed to cold for 300 hours and at room temperature for 2, 5, and 10 days were injected subcutaneously with colchicine (0.1 ml per 100-gm body weight of a solution containing 10 mg of colchicine in 1 ml of 80 per cent ethanol and 9 ml of H_2O (55)).

4. *Dry Mass of the Nuclei*

For the measurement of their dry mass, the nuclei were prepared with two different methods: *(a)* in an aqueous medium from fresh tissue; *(b)* in anhydrous glycerol from freeze-dried tissue. The nuclei prepared according to the former method undergo a notable loss of the soluble proteins (56), and their dry mass values correspond substantially to the content of insoluble proteins. In contrast, the nuclei prepared according to method *(b)* possess their full complement of proteins. The difference between the values obtained with the two methods gives an indication of the nuclear content of soluble proteins (57).

A. PREPARATION OF NON-AQUEOUS NUCLEI: The whole adrenal gland was frozen and dried, according to the method described by Pellegrino and Tongiani (58), and embedded in paraffin wax under vacuum. The block was sectioned in a microtome until the medulla was apparent on the surface. Small fragments of medulla were then taken out with the aid of sharp needles and were freed from paraffin wax with anhydrous xylene on a glass slide. A drop of anhydrous glycerol was added and the nuclei were freed from the cytoplasm by careful rotation and compression between slide and coverglass. The edges of the coverglass were sealed with paraffin wax, in order to prevent hydration under the microscope.

B. PREPARATION OF NUCLEI IN AN AQUEOUS MEDIUM: The adrenal gland was dissected out, freed from the surrounding fat tissue, cut semicircularly under a dissecting microscope (magnification, × 40) and opened flat. The medulla appeared as a prominent whitish nodule in the middle of the gland and could be completely freed from the cortical tissue. The medulla tissue was then transferred to 0.5 ml of the saline solution of Barnes *et al.* (59) in a glass micro-homogenizer of the Potter-Elvehjem type, kept in ice and dispersed by hand with 4 to 5 rotatory movements of the piston. Preliminary trials had demonstrated that, with this technique, the maximum number of free nuclei was obtained.

After centrifugation in a refrigerated centrifuge (Eispyrouette, Phywe) which was rapidly brought to the acceleration of 3,000 *g* and then left to decelerate until it stopped, the sediment was resuspended in 0.5 ml of the solution of Barnes *et al.* (59).

By this method, nuclei free of cytoplasmic remnants as ascertained by phase contrast microscopy were obtained.

C. REMOVAL OF DNA BY DNASE: One volume of a solution of DNase (Sigma Chemical Co., St. Louis) in Barnes *et al.* (59) fluid (1 mg/ml) was added to one volume of the suspension of the nuclei, obtained according to the above procedure. The mixture was incubated at 37°C for 20 minutes, stirring every 3 minutes, then centrifuged as described above. The sediment was resuspended in 2 ml of Barnes *et al.* (59) solution, left at +3°C for 15 minutes, and washed again twice in the same fluid.

The extinction at 260 mμ of the supernatant fluid was measured with a Unicam SP. 500 spectrophotometer after each washing, in order to get an indication of the amount of DNA lost from the nuclei. The extinction values were high after the first washing, then showed a very sharp decrease in the following one, and reached zero by the last one. Complete removal of DNA by this treatment was ascertained by Feulgen staining of the nuclei, which was negative.

Another part of free nuclei suspended in Barnes *et al.* (59) fluid was treated in the same way, except that the DNase was substituted by a volume of the saline solution, and served as controls.

D. MICROINTERFEROMETRY: The nuclear dry mass was determined with the interference microscope Baker-Smith ("half-shade" eyepiece and objective × 100 "shearing," water immersion) (60).

Köhler illumination was used with a 100-w tungsten lamp, and light at 546 mμ was selected by an interference filter (Zeiss).

As the dry nuclei had been compressed during their preparation, they could be considered cylindrical. In this case, the formula used for the calculation of the total dry mass was:

$$M = \frac{\Delta A}{100\alpha} \tag{1}$$

where M is the total dry mass; Δ, the optical path

TABLE I

Comparison of Dry Mass Values Obtained Respectively with "Half-shade" Eyepiece and Integrating Apparatus on Samples Taken from the Same Preparation of Aqueous Nuclei of Adrenal Medulla after DNase Treatment*

Method	Average mass value with S.E.M.	t	P
"Half-shade"	14.06 ± 0.6 (50)	—	—
Integrating apparatus	13.85 ± 0.5 (50)	0.0085	>0.9

Parentheses indicate numbers of nuclei measured. t and P show significance of the difference of the two means.
* See text.

difference; A, the area of the nucleus; α, the specific refractometric increment for proteins in anhydrous glycerol = 0.00095 (61).

Areas were obtained measuring the diameter of the nuclei with a filar eyepiece micrometer (Reichert) and applying the formula

$$A = \pi r^2$$

The nuclei suspended in aqueous medium were observed in a microchamber, so that they could maintain their spherical shape (62). In this case, the following formula was used (63):

$$M = \frac{2\Delta A}{3.100\alpha} \qquad (2)$$

where the symbols have the same meaning as in formula (1), except that α is the specific refractometric increment for proteins in water = 0.0018 (64, 65).

This formula is based on the fact that a sphere has a volume equal to that of a cylinder, with base equal to the cross-sectional area (πr^2) of the sphere and height equal to ⅔ of the sphere diameter.

The reliability of this measuring method has been tested by comparison with an integrating interferometer based on the model of Mitchison, Passano, and Smith (66). The results (Tables I and II) show that no significant differences in mass values were found between the two methods.

The optical path difference for aqueous nuclei did not exceed 0.25 λ and that for non-aqueous ones 0.15 λ. Both types of nuclei were of a fairly homogeneous appearance.

TABLE II

Comparison of Dry Mass Values Obtained Respectively with Half-shade Eyepiece and Integrating Apparatus on Aqueous Nuclei of Adrenal Medulla

Nucleus No.	Half-shade	Integrating apparatus
1	16.9	16.8
2	16.9	16.8
3	26.3	26.6
4	18.6	18.2
5	17.6	16.8
6	24.1	25.2
Mean	20.06 ± 1.66	20.06 ± 1.86

The same nucleus was first measured with the half-shade and subsequently with the integrating apparatus.

5. *Chemical Estimation of DNA and RNA*

The micromethod of Steele *et al.* (67) has been used with suitable modifications. The adrenal medulla was weighed with an accuracy of ±0.1 mg in a tared homogenizer containing 0.1 ml of Barnes *et al.* (59) fluid, in order to avoid desiccation of the tissue.

After homogenization (see above 4, B), 0.4 ml of Barnes *et al.* (59) fluid was added and 0.1 ml was drawn out. Of these, 0.02 ml was placed on a coverslip and, after drying and fixation with 95° ethanol, were stained with the Feulgen method; the remaining 0.08 ml was used for the count of the nuclei.

To the remaining part of the homogenate, 1.0 ml of 10 per cent trichloroacetic acid (TCA) was added and the mixture was centrifuged in the cold; for a complete account of the extraction technique, see Steele *et al.* (67).

After extraction of the nucleic acids, performed with 5 per cent TCA at 90°C, the liquid was kept at 4°C for 3 hours before centrifugation, so that most of the DNA and RNA could be found in the supernatant of the first washing (1st fraction of Steele *et al.* (67)). A second and a third washing with 5 per cent TCA were then performed (2nd and 3rd fractions of Steele *et al.* (67).

Deoxyribose and ribose were determined on separate 0.05-ml aliquots of each fraction, using, respectively, the micromodifications of the diphenylamine method of Dische (68) and of the orcinol method of Mejbaum (69).

Extinctions were measured in a Unicam SP. 500 spectrophotometer, equipped with diaphragms, using a microcell (0.1 ml, 1.0 cm light path) at a wave length of 595 mμ for DNA and of 640 mμ for RNA.

Preparations of DNA (Na salt) from thymus nuclei (Sigma Chemical Co., St. Louis) and of RNA (Na salt) from yeast (also from Sigma Chemical Co., St. Louis) were used as standards. Their atomic extinction coefficients (εP) were 8742 for DNA and 5638 for RNA.

Determinations were simultaneously carried out on experimental and control adrenals. The amounts of DNA and RNA given are the sum of those found in the three fractions analyzed.

6. *Count of the Nuclei*

In order to calculate the amount of DNA per nucleus and RNA per cell, the count of the nuclei in the homogenate was performed in parallel with the chemical determinations.

To one volume of homogenate (0.08 ml as described above in 5), one volume of a 1.0 per cent solution of gentian violet in 1.0 per cent aqueous acetic acid (*v/v*) was added and mixed thoroughly. Nuclei were counted in a Bürker camera, and counts were repeated twice. About 800 nuclei were counted each time.

7. *Nuclear Volumes*

Nuclear volumes were determined from the measure of the diameter obtained by a filar eyepiece micrometer (Reichert). The volumes were calculated by the formula $\frac{4}{3}\pi r^3$.

RESULTS

1. *DNA Content of Nuclei*

A. HISTOPHOTOMETRIC DETERMINATIONS: The histograms of the DNA values of the normal adrenal medulla nuclei show that the great majority of the data are grouped in a single class (Fig. 5). This distribution is typical of the values obtained with a scanning integrating histophotometer in comparison with the greater variability of data obtained with conventional apparatus. In comparison with the DNA values of the nuclei of the kidney tubules, which are almost exclusively diploid (70) (with the exception of a few higher values described by Pisi and Cavalli (18) Becker and Ogawa (71) and Franck (72) that were never encountered in our determinations), those of the adrenal medulla nuclei were on the average higher (16.4 per cent).

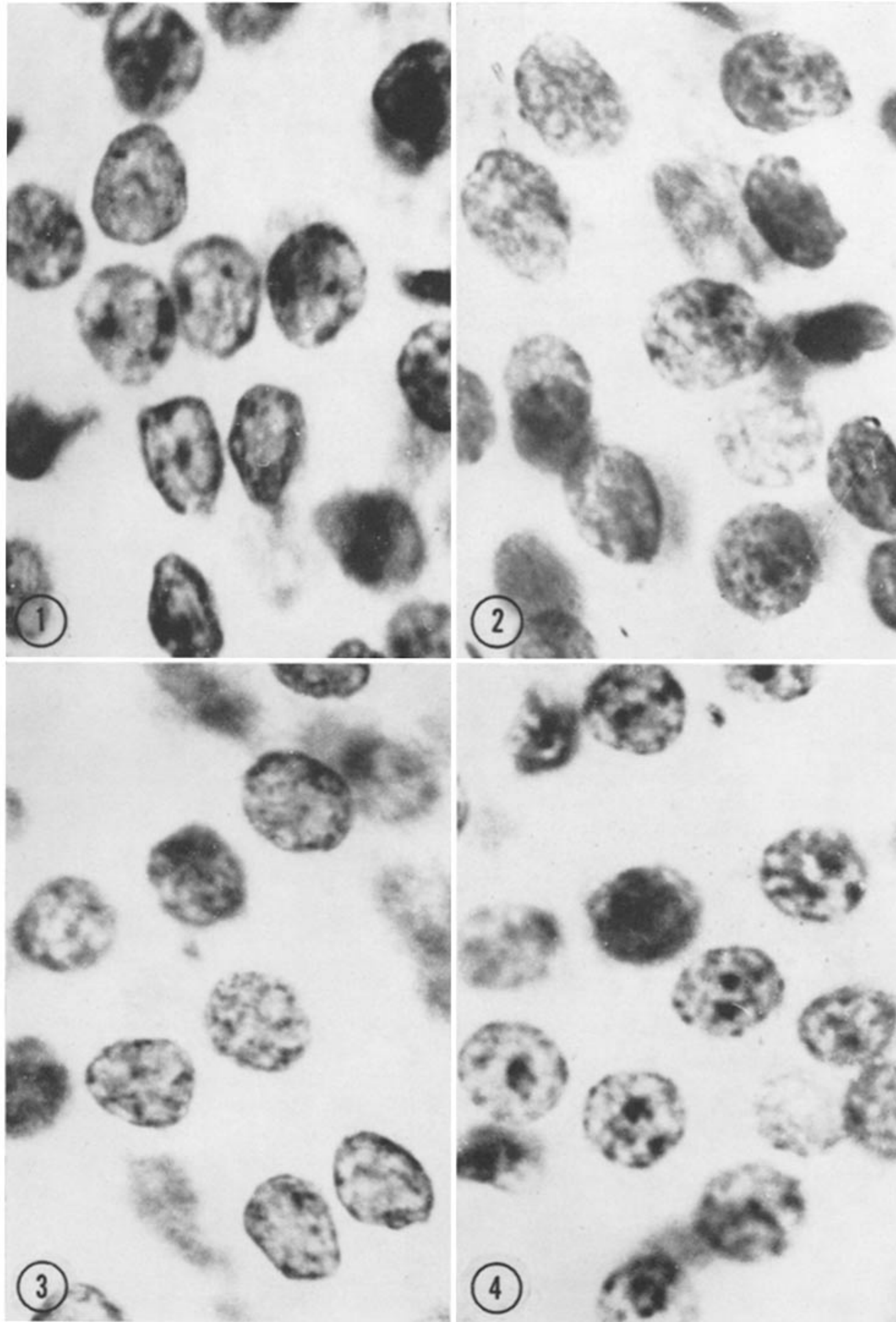
In the adrenal medulla nuclei of rats exposed intermittently to 4°C over a total period of 100 hours, the values of DNA per nucleus are decreased on the average by 24.0 per cent (Fig. 5 *A*; Table III); on exposure to 4°C for 300 hours, the diminution reaches an average of 40.1 per cent (Fig. 5 *B*; Table III). The variability of the values, however, is scarcely increased, indicating that the DNA decrease is fairly equally distributed over all the nuclei.

If, after exposure to low temperature, the rats are brought back to room temperature for 10 days, the DNA per nucleus increases up to normal and above normal values (average increase above normal 17.5 per cent, Table IV). The histogram of Fig. 5 *D* shows that the majority of the nuclei are distributed with respect to the normal between the diploid and the diploid premitotic value, some reaching the latter. Therefore, the increase in DNA content in the recovery phase takes place in every nucleus, but to a different extent from one to another.

The change in the DNA content of a nuclear population, revealed by histophotometric measurements, can be considered as an expression of the functional state of the cells only if certain criteria detailed by Walker and Richards (44) are fulfilled. These are: (i) no process of DNA synthesis should be present, as a consequence of chromosome replication; (ii) the number of chromosomes be known; (iii) no errors in the measurement, arising from the instrument and/or the staining method, should occur.

As far as the first condition is concerned, Mitchell (55) could not detect mitoses in the rat adrenal medulla from the 30th day of life on. We made an extensive search for mitoses in our material also with the use of colchicine. In this case, in normal conditions and after 2, 5, and 10 days of recovery, numerous microscopic fields were scanned, and we observed a total of about 150,000 nuclei without being able to detect a single mitosis. An identical result, but without use of colchicine, was obtained in the examination of cold-exposed adrenal medullas.

For the second condition, although it is not possible to make a direct count of the chromosomes in the adrenal medulla cells, their number can be assumed to be identical with that in cells having the same degree of ploidy. It is difficult to admit that during the DNA decrease, the number of chromosomes changes in the same way in almost all adrenal medulla cells. We would also think



FIGURES 1 THROUGH 4 Feulgen staining of rat adrenal medulla cells. Fig. 1, cells of normal animals; Fig. 2, cells of rats exposed intermittently to cold for 100 hours; Fig. 3, cells of rats intermittently exposed to cold for 300 hours; Fig. 4, cells of rats kept for 10 days at room temperature after 300 hours of intermittent exposure to cold. × 2,300.

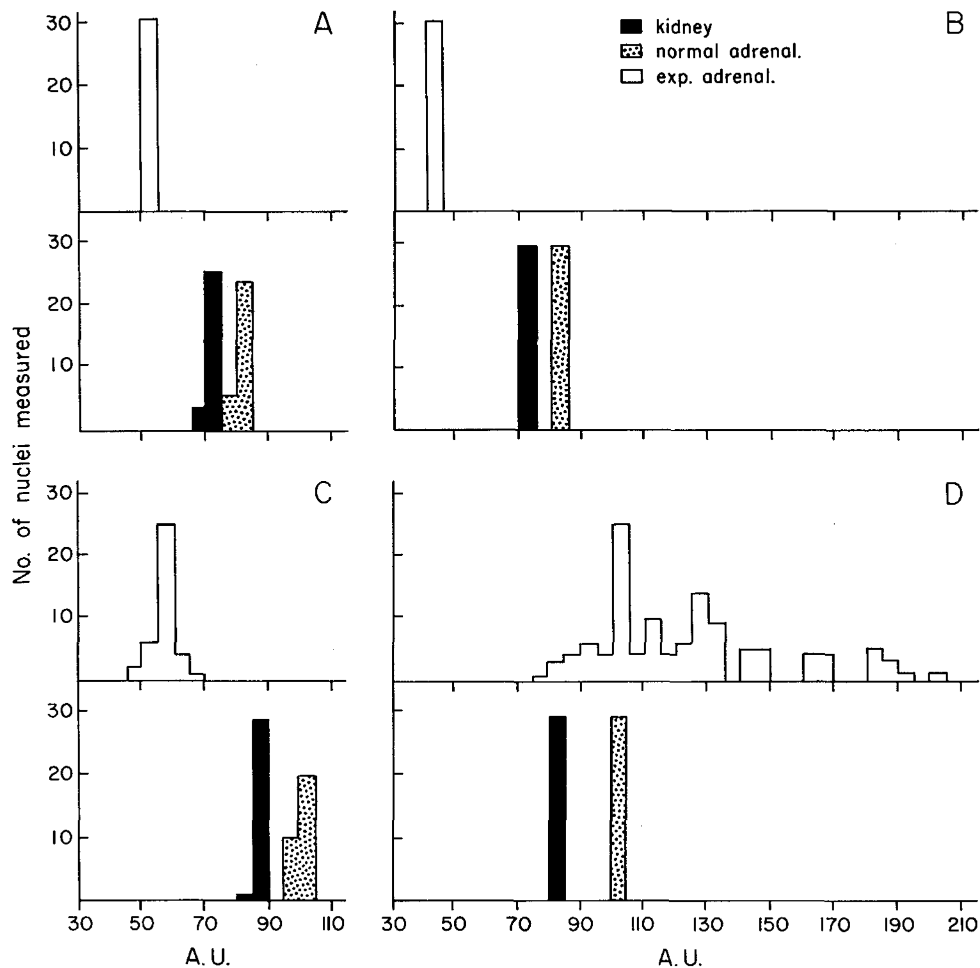


FIGURE 5 Histograms of DNA content obtained by histophotometry in arbitrary units (*A.U.*). Treatments: *A*, 100 hours of cold exposure; *B*, 300 hours of cold exposure; *C*, 300 hours of cold exposure (smears); *D*, 300 hours of cold exposure +10 days at room temperature.

The experiments were chosen for illustration, since they were representative of the given experimental situation.

that their number does not vary during the recovery period.

As far as the third criterion is concerned, kidney, control, and experimental adrenal medullas have always been subjected together to the same treatment. Therefore, possible differences in the fixation (4) or staining (such as variations in the pH or SO_2 content of the Schiff reagent (36)) could not interfere with our results.

Although it is difficult to rule out completely the possibility of proportionality failure (between amount of Feulgen dye measured and amount of DNA present) which can be due to a number of causes, some of these have been tested as follows.

First, it could be supposed that the nuclei of the cold-exposed adrenal medulla have an optimum hydrolysis time for the Feulgen reaction different from that of nuclei of the control adrenal and of the kidney. This variable has been tested: no differences have been observed either in the normal or experimental nuclei. As Fig. 6 *A* shows, the maximum stain development always occurred after 12 minutes of hydrolysis.

The DNA decrease observed could be due to

TABLE III

Effect of Cold Exposure and of the Subsequent Recovery on DNA Content of Adrenal Medulla Nuclei, Determined by Histophotometry

(Arbitrary Units).

Kidney (± S.E.M.)	Adrenal medulla		
	Control (± S.E.M.)	Experimental (± S.E.M.)	Average variation
			per cent
	+ 4°C for 100 hrs.		
77.6 ± 0.6 (15)	90.1 ± 0.3 (16)	67.0 ± 0.8 (21)	−25.6
72.8 ± 1.0 (13)	90.0 ± 0.3 (20)	89.4 ± 0.4 (24)	− 0.7
79.9 ± 0.5 (30)	90.1 ± 0.1 (30)	65.7 ± 0.7 (30)	−27.1
70.2 ± 0.2 (30)	90.1 ± 0.5 (30)	68.1 ± 0.8 (30)	−24.4
70.2 ± 0.2 (30)	80.1 ± 0.3 (30)	50.2 ± 0.1 (30)	−37.3
66.0 ± 0.2 (30)	80.2 ± 0.2 (30)	60.0 ± 0.2 (30)	−25.0
		Mean	−24.0
	+ 4°C for 300 hrs.		
80.0 ± 0.2 (30)	94.7 ± 0.4 (30)	58.7 ± 0.6 (30)	−38.0
95.1 ± 0.1 (30)	103.6 ± 0.2 (30)	63.2 ± 0.2 (30)	−39.0
80.1 ± 0.4 (30)	95.1 ± 0.2 (30)	58.2 ± 0.9 (22)	−38.8
70.1 ± 0.1 (30)	80.0 ± 0.3 (30)	39.6 ± 0.2 (30)	−50.5
	Smeared nuclei		
86.0 ± 0.1 (30)	96.9 ± 0.2 (50)	57.0 ± 0.4 (50)	−41.2
84.2 ± 0.1 (31)	99.4 ± 0.3 (30)	56.9 ± 0.6 (38)	−33.7
		Mean	−40.1
	+ 4°C for 300 hrs. + 10 days at room temperature		
84.0 ± 0.9 (30)	89.0 ± 0.9 (30)	105.2 ± 8.4 (39)	+18.2
81.7 ± 0.7 (30)	89.1 ± 0.4 (25)	103.1 ± 9.0 (42)	+15.7
67.5 ± 1.4 (30)	78.1 ± 1.3 (24)	96.7 ± 2.2 (36)	+23.8
93.0 ± 0.4 (30)	101.7 ± 0.5 (30)	108.3 ± 3.1 (30)	+6.4
80.2 ± 0.2 (30)	100.5 ± 0.2 (30)	120.5 ± 2.7 (120)	+19.9
85.3 ± 0.2 (30)	110.3 ± 0.2 (30)	124.8 ± 4.8 (30)	+13.4
		Mean	+17.5

Control and experimental adrenal and kidney were embedded, sectioned, and stained together. Figures in parentheses are the numbers of nuclei measured. The mean values are expressed in arbitrary units. The last column represents the mean per cent variation of experimental *vs* control adrenal medulla nuclei. Each line represents a different animal.

differences of the absorption curve of the Feulgen dye between cold-treated and control medulla cells. However, no such differences were detected (Fig. 6 *B*).

Also, differences in refractive index between the nucleus and surrounding medium could cause an error due to scatter and not specific light loss (Swift and Rasch, 53). To avoid this, the sections were mounted in thick Canada balsam. It was ascertained that only very faint Becke lines were visible in the nucleus when the microscope tube was racked upwards. As a further precaution, I_0 readings were always made on the cytoplasm surrounding the nucleus to be measured.

As regards the apparatus, an integrating histophotometer (scanning type) was used. This gives a measure over the whole nucleus and avoids the distributional error (48, 49) as well as the errors arising from an irregular nuclear shape.

Another possible error could arise from part of

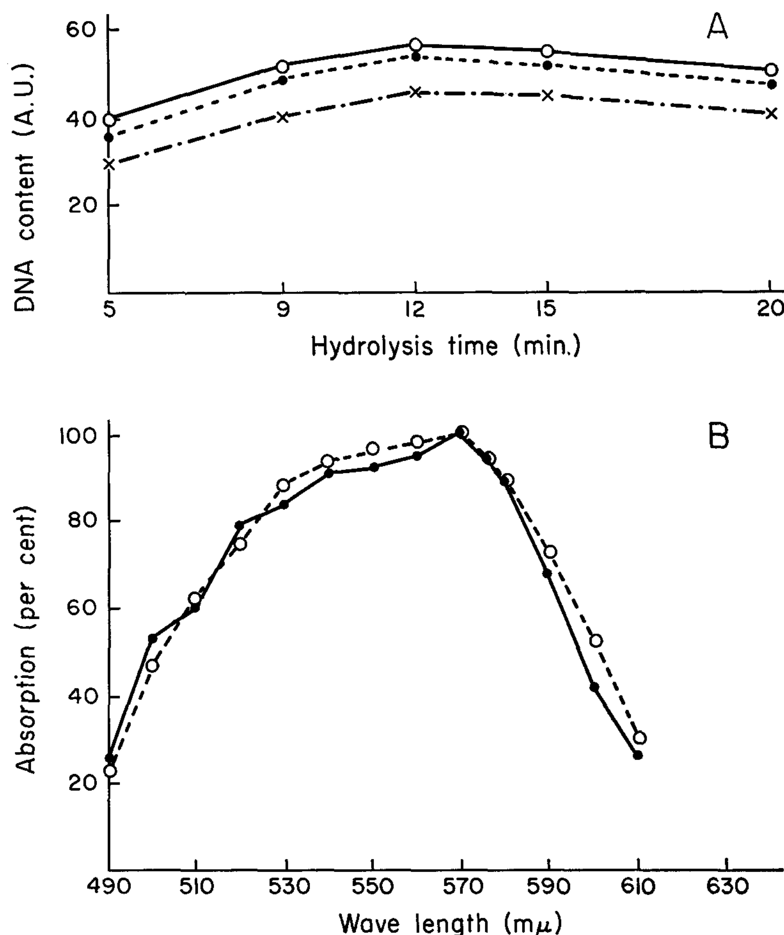


FIGURE 6 *A*, Hydrolysis time curves on the tubular cells of kidney (--●--) and of the adrenal medulla cells: control (—○—), after exposure to low temperature for 300 hours (-·-·×·-·). The average total amount of Feulgen dye for samples of 10 cells of each type is plotted against the hydrolysis time in minutes. *B*, The spectral absorption curve of Feulgen dye of adrenal medulla cells of normal rats (—●—●—) and of rats exposed to cold for 300 hours (--○--).

the specimen's being out of focus, since no crushing condenser was used (48). However, as the histogram of Fig. 5 *C* shows, identical results were obtained on smears of isolated nuclei, which are flattened, and on sections. Finally, errors due to high extinction values can be excluded, since the maximum observed values in the more condensed zones measured with the "plug" method did not exceed 0.46, with an average of 0.37.

Although most errors in the histophotometric determinations could be excluded, in view of the criticism often directed at this technique (4, 36, 44) it was deemed desirable to test the DNA variations observed by other independent methods.

B. MICROINTERFEROMETRIC DETERMINATIONS: The amount of DNA contained in single nuclei was calculated by subtracting the nuclear dry mass after DNase treatment from the dry mass of nuclei suspended in an aqueous medium (Fig. 7).

Control experiments, performed on isolated kidney tubule nuclei, showed that the DNase treatment caused a dry mass loss of 6.7 pg(picogram or 10^{-12} gm) per nucleus (Table IV). This value corresponds exactly to the DNA content of kidney nuclei, obtained in chemical determinations made by several authors, and reported by Davidson (73).

In the nuclei of the normal adrenal medulla a dry mass decrease of 9.3 pg has been found upon treatment with DNase (Tables IV and VII). It is probable that this value corresponds substantially to the DNA content of the nuclei, since chemical determinations reported below (see Table V) show a value of 8.4 pg. In this case, the difference between the two methods is 11 per cent. It seems possible to conclude, therefore, that the loss of substances other than DNA, upon DNase treatment, is very small, if any at all.

Moreover, it is evident also with this technique that the nuclei of adrenal medulla contain an amount of DNA-like substances (removable by DNase) larger than that of the kidney nuclei (on the average +39 per cent).

In the adrenal medulla of animals exposed to 4°C for 300 hours, the average dry mass loss per nucleus upon DNase treatment was 6.4 pg (Tables IV and VII), 31.2 per cent lower than that in the normal medulla.

When the rats were kept at room temperature for 10 days after the cold exposure, the average dry mass difference under the action of DNase returned to slightly greater (+16 per cent) than normal values (Tables IV and VII).

Thus, also by this method, it appears that the DNA content per nucleus is reduced by the action of low temperature with respect to the normal cells. The extent of the decrease found is slightly less than that determined with the histophotometric technique.

C. CHEMICAL RESULTS: The DNA content per nucleus has been estimated by dividing the value of DNA chemically determined on the homogenate of adrenal medulla by the number of nuclei contained in the homogenate.

In the normal medulla the average DNA content per nucleus is 8.4 pg (Table V). This amount is lower than that obtained with the interferometric method. This discrepancy might be accounted for by the presence of connective cell nuclei (40 per cent of the total adrenal medulla nuclei).

In the adrenal medulla of animals exposed to low temperature for 100 hours, this value is 6.2 pg and falls to 5.9 pg after an exposure of 300 hours (Table V). The reduction in the DNA content has been found, accordingly, to be 26 per cent after 100 hours and 30 per cent after 300 hours of exposure to cold. The amount of DNA decreases after 100 hours fits well with the data obtained with the other methods; however, after 300 hours, the decrease in DNA as determined by chemical methods agrees with that obtained with the interferometric method, but is lower than that obtained with the histophotometric method.

After 10 days of recovery at room temperature, the DNA values came back to normal, but no "overshoot," such as that observed with interferometric and histophotometric methods, has been detected.

It has been suggested that the DNA diminution in liver nuclei under the action of the cortisone is only an apparent one (42, 70, 74), because part of the DNA might be present in a depolymerized

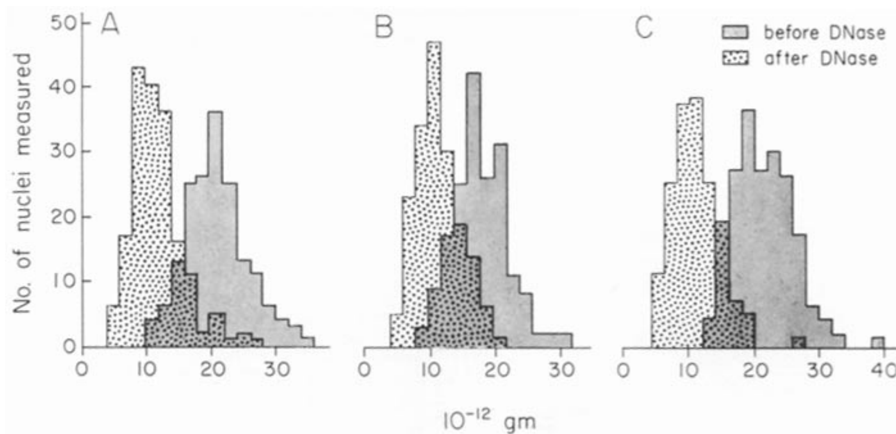


FIGURE 7 Histograms of total dry mass of aqueous nuclei of adrenal medulla before and after DNase treatment: *A*, Control. *B*, Experimental, 4°C for 300 hours. *C*, Experimental, 4°C for 300 hours and room temperature for 10 days.

form in the nucleus. Also, in our case it could be supposed that, as an effect of the exposure to cold, the state of a part of the DNA would be changed and that the latter, although present in the nuclei *in vivo*, could be extracted by cold TCA in the chemical method, before extraction of nucleic acids with TCA at 90°C, thus escaping detection.

In order to test this hypothesis, the reaction of Dische (68) was performed on the cold acid-soluble fraction of the homogenates of adrenal medulla. The dye development was found to be proportional (about 4/25) to the amount of deoxyribose present in the TCA extracts at 90°C, both in the control and in the experimental animals; whereas, if the hypothesis were true, a marked increase of dye development should have been observed in the extracts of the experimental medulla as compared with the controls. These results should, therefore, be taken as an indication against the depolymerization hypothesis.

2. *RNA Content of Medulla Cells*

In order to correlate the observed variations in DNA with the RNA cellular content, the RNA content was chemically determined in the homogenates of adrenal medulla.

The RNA content was found to be 59.8 pg per cell in the control animals, and 59.0 and 45.9 pg per cell in the experimental animals after 100 hours and 300 hours, respectively, of exposure to cold (Table V). Thus, the RNA content is reduced by 23 per cent only after 300 hours of exposure to 4°C, no changes being observed after 100 hours. In the period of recovery at room temperature, the RNA content also came back to above normal values.

3. *Nuclear Volumes*

The volumes of aqueous nuclei of the adrenal medulla, determined in the interference microscope, do not show any modifications during cold exposure and recovery.

4. *Total Dry Mass of Nuclei*

The total dry mass of non-aqueous nuclei of adrenal medulla of normal animals is not different from that of experimental animals (Table VII). It is unlikely, because of the rigorous technique used,

TABLE IV

Comparison between Total Dry Masses, Obtained by Microinterferometry, of Aqueous Nuclei of Adrenal Medulla Cells, before and after Treatment with DNase

Material	No. Exp.	A Dry mass of aqueous nuclei before DNase (± S.E.M.)	B Dry mass of aqueous nuclei after DNase (± S.E.M.)	A − B
		10^{-12} gm	10^{-12} gm	10^{-12} gm
Normal kidney	1	20.8 ± 1.7 (30)	13.4 ± 0.8 (30)	7.3
	2	17.9 ± 0.5 (49)	11.7 ± 0.7 (50)	6.2
Normal adrenal medulla	3	23.4 ± 0.6 (30)	14.1 ± 0.8 (30)	9.3
	4	21.7 ± 1.0 (25)	12.0 ± 0.9 (25)	9.7
	5	22.3 ± 0.7 (20)	12.5 ± 0.5 (25)	9.8
	6	21.8 ± 0.6 (50)	12.4 ± 0.5 (50)	9.4
	7	19.7 ± 0.6 (49)	11.0 ± 0.5 (49)	8.7
Experimental adrenal medulla; 4°C for 300 hrs.	8	19.9 ± 0.7 (25)	13.5 ± 0.5 (25)	6.4
	9	18.0 ± 0.6 (30)	13.4 ± 0.7 (30)	4.6
	10	19.2 ± 0.9 (20)	12.0 ± 0.6 (25)	7.2
	11	18.6 ± 0.5 (50)	11.4 ± 0.4 (50)	7.2
	12	17.2 ± 0.5 (50)	11.2 ± 0.5 (49)	6.0
Experimental adrenal medulla; 4°C for 300 hrs. + 10 days at room temperature	13	23.3 ± 1.0 (40)	12.9 ± 0.4 (40)	10.4
	14	21.8 ± 0.7 (31)	12.6 ± 0.8 (29)	9.2
	15	21.2 ± 0.5 (50)	9.8 ± 0.4 (49)	11.4
	16	22.1 ± 0.6 (50)	11.1 ± 0.4 (50)	11.0

Parentheses indicate numbers of measurements made.

TABLE V

Effect of Exposure to Cold on the Number of Cells, and Content of DNA and RNA of Adrenal Medulla Tissue (Chemical Determinations)

Condition	No. of animals studied	Cells 10^3/mg fresh tissue (± S.E.M.)	t	p	DNA μg/mg fresh tissue (± S.E.M.)	t	p	DNA pg/nucleus (± S.E.M.)	t	p	RNA μg/mg fresh tissue (± S.E.M.)	t	p	RNA pg/Cell (± S.E.M.)	t	p
Normal	12	287.9 ± 23.5			2.41 ± 0.21			8.4 ± 0.4			18.1 ± 1.7			59.8 ± 2.82		
+ 4°C for 100 hrs.	4	297.9 ± 45.5	2.040	0.1 > P >0.05	1.83 ± 0.30	1.413	0.2 > P >0.1	6.2 ± 0.7	2.741	0.02 > P >0.01	17.3 ± 1.7	0.256	0.9 > P >0.8	59.0 ± 1.59	0.143	0.9 > P >0.8
+ 4°C for 300 hrs.	8	288.4 ± 22.1	0.148	0.9 > P >0.8	1.72 ± 0.17	2.359	0.05 > P >0.02	5.9 ± 0.2	3.466	0.01 > P >0.001	12.4 ± 0.8	2.712	0.02 > P >0.01	45.9 ± 1.47	3.809	0.01 > P >0.001
+ 4°C for 300 hrs. + 10 days at room temperature	2	279.0 ± 46.0	1.406	0.2 > P >0.1	2.40 ± 0.53	0.019	P > 0.9	8.5 ± 0.5	0.106	P > 0.9	21.0 ± 3.5	0.732	0.5 > P >0.4	75.3 ± 0.2	2.381	0.05 > P >0.02

The t tests show the significance of the difference with respect to the controls.

TABLE VI

Effect of Exposure to Cold on the Volumes of Aqueous Nuclei of Adrenal Medulla Cells

Condition	Volumes	(± S.E.M.)	t	P
	μ^3			
Normal				
+ 4°C for 300 hrs.	187.3 ± 6.6	(174)		
+ 4°C for 300 hrs.	184.1 ± 1.5	(172)	0.384	0.8 > P > 0.7
+ 10 days at room temperature	173.7 ± 4.5	(170)	0.560	0.6 > P > 0.5

Parentheses indicate the number of measurements. The t tests show the significance of difference with respect to the controls.

TABLE VII

Comparison between Total Dry Masses, Obtained by Microinterferometry, of Non-Aqueous and Aqueous Nuclei of Adrenal Medulla Cells

Condition	A Dry mass of non-aqueous nuclei (± S.E.M.)	t	P	B Dry mass of aqueous nuclei (± S.E.M.)	t	P	A − B	C Dry mass of aqueous nuclei after DNase (± S.E.M.)	t	P	B − C
	10^{-12} gm			10^{-12} gm			10^{-12} gm	10^{-12} gm			10^{-12} gm
Normal	35.2 ± 0.8 (60)			21.6 ± 0.3 (174)			13.6	12.3 ± 0.3 (179)			9.3
+ 4°C for 300 hrs.	35.6 ± 0.3 (66)	0.035	P > 0.9	18.4 ± 0.3 (175)	7.273	P < 0.001	17.2	12.04 ± 0.2 (179)	0.769	0.5 > P > 0.4	6.4
+ 4°C for 300 hrs. + 10 days at room temperature				22.2 ± 0.4 (170)	1.600	0.2 > P >0.1	—	11.4 ± 0.3 (168)	0.714	0.5 > P >0.4	10.8

Parentheses indicate the number of measurements performed. The t tests show the significance of difference with respect to the controls.

that transport of some substances from the cytoplasm to the nucleus has occurred during the preparation and isolation of the freeze-dried nuclei. It is thus clear that *in vivo*, not considering the lipids which are extracted by this procedure, the total dry masses of normal and experimental nuclei are similar. This conclusion apparently does not agree with the histophotometric, interferometric, and chemical results, which show a marked decrease in DNA content.

On the other hand, the dry mass of the nuclei isolated in an aqueous medium (in which they lose water-soluble substances) is smaller in the experimental animals than in the controls (Table VII). By comparing the values of the dry mass of non-aqueous and aqueous nuclei, it is apparent that the aqueous nuclei of the experimental medulla have lost, on the average, 17.2 pg of soluble material, whereas the controls have lost 13.6 pg. The difference of 3.6 pg practically corresponds to the difference in DNA content between normal and experimental nuclei, as determined by microinterferometry with the use of DNase (2.9 pg). After this treatment, normal and experimental nuclei have the same dry mass, showing that, except for the decrease of DNA, no modifications in the water-insoluble components of nuclei take place (Table VII).

It is concluded, therefore, that the adrenal medulla nuclei of rats exposed to low temperature are not different from the normal as to the total content of dry substances, and also as to the content of insoluble proteins. The only difference is an increased content of soluble material, corresponding in amount to the lost DNA. The meaning of this result will be dealt with in the Discussion.

5. *Proteins of Nuclei*

A further attempt to investigate the behaviour of nuclear proteins was performed by determining

histophotometrically the extinction ratio naphthol yellow S stain/Feulgen stain.

This ratio showed a significant decrease after 100 hours of exposure to cold, a marked increase after 300 hours, and an amount still higher than normal after 10 days of recovery (Table VIII). In view of the variations of the DNA, this indicates that proteins decreased in the first period of cold exposure at a faster rate than DNA, whereas later their decrease was lower than that of the latter. The persistence of a high ratio during the period of recovery, in which DNA was markedly increased, indicates an even greater increase of the proteins.

Because these determinations were performed on sections, and the extraction of the various nuclear constituents in the course of the histological preparation is not known, it is difficult to correlate these findings with the data obtained by microinterferometry.

TABLE VIII

Effect of Cold Exposure and of Recovery on the Ratio Naphthol YellowS/Feulgen Dye (E 480/E 546 mμ) Determined on 3-μ-Thick Sections of Adrenal Medulla Nuclei

Slide No.	Normal (±S.E.M.)	Experimental (± S.E.M.)	t	P
1	1.03 ± 0.032 (30)	100 hrs. of cold 0.87 ± 0.037 (30)	3.239	0.01 > P > 0.001
2	1.00 ± 0.029 (30)	100 hrs. " " 0.86 ± 0.025 (30)	3.589	0.001 > P
3	0.97 ± 0.049 (30)	300 hrs. " " 1.31 ± 0.055 (28)	4.843	0.001 > P
4	0.99 ± 0.031 (30)	300 hrs. " " 1.14 ± 0.063 (30)	2.137	0.05 > P > 0.02
5	0.94 ± 0.023 (30)	300 hrs. + 10 days r.t.* 1.02 ± 0.033 (30)	1.923	0.1 > P > 0.05
6	0.94 ± 0.023 (30)	300 hrs. + 10 days r.t. 1.17 ± 0.026 (28)	6.800	0.001 > P

* r.t. = room temperature.
Parentheses indicate the number of measurements performed. The t tests show the significance of the difference with respect to the controls.

DISCUSSION

The changes in nuclear DNA content observed in our experiments show some peculiarities with respect to those reported in the literature.

In our experiments, the DNA variations take place in two phases: namely, a decrease during the intermittent exposure to low temperature, and a recovery to normal or above normal values on subsidence of the action. These two phases show a distinctive feature. The decrease is approximately equal in all nuclei, as if the reaction to the stimulus had been similar in extent in every cell; it takes place more rapidly in the first 100 hours of exposure to cold than in the following 200 hours. The recovery, on the other hand, does not occur simultaneously and to the same degree in every cell. Increases above the diploid value and up to the tetraploid value have been observed.

Hale (36) points out that in several cases in which an increase in DNA has been reported (in the thyroid treated with thiouracil (16, 20), after exposure to cold (20), in young rats (20); in the kidney in vicarious hypertrophy (17); in the interstitial cells of the testis treated with testosterone (75)) the presence of multiplication processes cannot be excluded. As a consequence, in those cases a relationship between DNA variations and functional activity of cell cannot be demonstrated. In our case, Hale's (36) objection might apply only to the recovery phase. Our results show that no mitoses were detectable, even with the use of colchicine, 2, 5, and 10 days, respectively, after bringing the animals back to room temperature. It is known that the adult adrenal medulla cells are irreversible, postmitotic cells (76). Also their nuclei show, by autoradiographic technique, a very poor incorporation of thymidine-H^3, a precursor of DNA (Diderholm and Hellman, 77, on 22-day-old rats). This does not rule out the possibility of a synthesis of DNA during the recovery, and this will be dealt with later on.

As far as the first phase is concerned, regressive phenomena can be excluded, since, in the histological preparations, no sign whatever of necrosis or any other cellular damage was noticed.

The decrease in DNA content found in our experiments reaches large values, amounting, after intermittent exposure to cold for 300 hours, to 40.1 per cent on microphotometric determinations, to 31.2 per cent on microinterferometric

measurements, and to 30.0 per cent on chemical analysis, matched only by very few other observations reported in the literature.

In general, the decreases reported so far are less: *e.g.*, 10 to 15 per cent (thyroid upon treatment with thyroxin (16) and Lugol (20) and after hypophysectomy (20); adrenal cortex upon treatment with deoxycorticosterone (24) and pituitary hormones (78); maturation of osteocytes (34); leucocytes in comparison with gastric and kidney cells (36)). Moreover, the significance of some of the decreased values has been put in doubt because the determinations have been made with apparatuses objectionable on technical grounds.

The only values that approximate ours are those of Leeman (26), also on the adrenal medulla following section of the splanchnic nerves (31 per cent decrease), and those of Birge *et al.* (33) on bovine spermatozoa during *in vitro* storage.

La Cour *et al.* (35) found a marked decrease of DNA in the cells of the roots of *Vicia*, *Trillium*, and *Paris* exposed to low temperatures, but their results have not been confirmed by Woodard and Swift (79). According to Lowe and Rand (42) and Lowe *et al.* (74), liver nuclei following treatment with cortisone show a notable decrease in DNA content. However, these observations apply to a material quite different from ours, *i.e.* vegetal cells, on the one hand, and reversible, intermitotic cells of the liver, an organ which is endowed with several nuclear classes in dynamic equilibrium, on the other.

In our case, as stated in the Results, several possible causes of error in the histophotometric measurements can be ruled out.

Also, the possibility that our results may be due to a decreased affinity of DNA to the Feulgen stain (35) can be, in part, excluded on the basis of the tests performed (Fig. 6). In addition, the chemical determinations of DNA performed on the whole homogenate of the tissue show that our results cannot be due to transfer of DNA from the nucleus to the cytoplasm, an hypothesis suggested by Heyes and Shaw (40) to explain the difference between histophotometric and chemical determinations of DNA in the roots of plants exposed to cold (35). However, some quantitative discrepancies between the three methods used for DNA determinations have been constantly observed. From the information obtained, it is not possible at present to decide what can be their cause.

Careful consideration is deserved by the hypothesis that the DNA decrease may be only apparent and due to loss of depolymerized DNA during the manipulations preceding the histophotometric, microinterferometric, and chemical determinations. In particular, the comparison between the dry masses of non-aqueous and aqueous nuclei (Table VII), which shows that the medulla nuclei of cold-exposed animals contain less DNA and more soluble material than those of the controls (see Results, 1.) bears heavily on this point. However, the results of the Dische (68) reaction on the cold acid–soluble fraction of the homogenates indicate that no increase of cold-soluble deoxyribose-containing compounds takes place in the experimental cells. In view of the extent of the DNA decrease, this increase, if present, should have been detected easily. Furthermore, it is to be noted that in nuclei treated with saline solutions at neutral pH, 20 to 40 per cent (at most) of mononucleotides are extracted, at least in the case of thymus (80). Finally, it is apparent from our histophotometric data that, in the recovery phase, the DNA content in many nuclei increases well beyond the normal values, presumably indicating DNA synthesis. In fact, the evidence that no DNA synthesis takes place in the recovery period has been taken by Mirsky and Osawa (70), in the discussion of the data of Lowe and Rand (42) and Lowe *et al.* (74) on the decrease of DNA in the liver of rats under the action of cortisone, as supporting the depolymerization hypothesis. In summary, some indications exist that the DNA decrease here reported is real, and not due to changes in the properties of DNA. However, final proof of this can be obtained only by actual measurement of the incorporation of labeled precursors in the DNA in the recovery phase, a task now being undertaken.

A further point needs to be made. It appears from our histophotometric and microinterferometric data, as well as from the comparison between our chemical data on adrenal medulla DNA and those of others on kidney (73), that the normal adrenal medulla nuclei have a higher DNA content in comparison with the kidney nuclei, which are, in the great majority, diploid, and for this reason were chosen as a test object also in our histophotometric determinations. As pointed out before, the extent of this increase varies somewhat with the analytical method used, being on the average 16 per cent with the histophotometry, 39 per cent with the microinterferometry with the

use of DNase, and 25 per cent with the microchemical determinations.

However, from the point of view of its turnover, this part of the DNA does not seem different from the rest, on the basis of the lack of labeling of adrenal medulla nuclei with tritiated thymidine (Diderholm and Hellman, 77).

The existence of an extra amount of DNA in these nuclei might ease the interpretation of the marked DNA decrease observed in them under the influence of cold. In fact, it might suggest that the decrease is, at least in part, at the expense of this fraction of DNA, presumably not strictly connected with the main functions of the DNA associated with the chromosomal sets.

Thus, one of the main obstacles preventing, until now, the general acceptance of data on DNA variations would be removed, at least in this case.

The changes in RNA content of the cells show that these are of a less extent.

Variations in DNA content per nucleus have already been measured in adrenal medulla cells of rats exposed to cold. In the observations of Leeman (25) and of Verwoerd *et al.* (81), an increase of DNA, variable from 9 to 14 per cent, took place. In the same experimental conditions, Roels (82) found a 50 per cent increase in the dry mass of non-aqueous nuclei. However, it is to be pointed out that, in the above reported experiments, the exposure to low temperature was continuous, while in ours the exposure to 4°C was regularly interrupted every day by a period of exposure to room temperature (18–22°C). According to Cramer (83), an effective stimulation of the adrenal medulla is not accomplished by continuous exposure to low temperature, as confirmed by Des Marais and Dugal (84), but by intermittent exposures to cold, as in our experiments.

To quote: "As already stated, the efficacy of cold as a stimulus does not depend so much on the temperature itself as on the change in the temperature. Thus, the adrenal of a mouse which has been kept for a long time in a warm room at a temperature around 20°C will show active secretion as soon as the animal is subjected to a temperature of about 5°C. But when it is kept at that temperature for a week or 2 weeks, the adrenal will again assume more the appearance of a resting gland. The function of the adrenal in heat regulation enables the organism to adapt itself rapidly to sudden changes in the thermal environment. Once the organism has been tided over such a sudden change, it maintains its adaptation to the altered thermal environment by some other mechanism . . . " (83).

It is thus ascertained that hyperfunction of adrenal medulla cells causes changes in the state or content of DNA in their nuclei.

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