A RADIOAUTOGRAPHIC STUDY WITH H^3-THYMIDINE ON ADRENAL MEDULLA NUCLEI OF RATS INTERMITTENTLY EXPOSED TO COLD

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ABSTRACT

A considerable decrease (24 to 40%) of DNA content per nucleus previously observed in the adrenal medulla of rats exposed intermittently to cold is followed by restoration to normal and supranormal values. This phenomenon has now been studied by use of H^3-thymidine, which was given to normal rats, to rats exposed to cold, and to animals brought to room temperature after cold exposure. In the first two conditions, no significant labeling of nuclei was observed. In the third, labeling took place clearly in the 1st 3 days. The grain counts showed that the early labeled nuclei had more grains than those labeled later, indicating differences in the rate of DNA synthesis. A statistically significant correlation was found, on the same nuclei, between amount of Feulgen dye and number of grains. It is concluded that net synthesis of DNA takes place in the phase of recovery from cold. This fact is not related to cell division, as no mitoses could ever be detected, but rather to the cold-induced loss of DNA. Clear demonstration is thus given of a marked variation in the amount of DNA per nucleus in relation to the functional conditions of adrenal medulla cells.

INTRODUCTION

In a preceding investigation (1, 2), a strange and unexpected phenomenon was observed concerning the DNA content of the nuclei of the adrenal medulla cells. When rats were intermittently exposed to cold for a total period of 100 to 300 hr, the amount of DNA per nucleus decreased markedly (down to 40%). These data were obtained with three independent methods: (*a*) Feulgen photometry; (*b*) micro-interferometry (in which the DNA content per nucleus was calculated by the difference between the total dry mass of the nuclei isolated in an aqueous medium before and after treatment with DNase); (*c*) microchemical determinations of DNA (by the diphenylamine reaction) combined with the count of the nuclei in the homogenates.

With the same methods it was observed that, if the animals previously exposed to cold were kept at room temperature for 5 to 10 days, the amount of DNA per nucleus returned to normal and beyond (up to double the normal values).

These DNA modifications take place in cells certainly incapable of mitotic processes, as it appears from the survey of a large number of cells, particularly after treatment with colchicine (2).

As a consequence of these results, the problem arose whether the observed modifications of DNA content were real, i.e., reflecting the situation in vivo, or the result of artefacts arising during the analytical manipulations. Although many causes of error could be excluded on the basis of a critical examination of the methods employed

(2), a crucial demonstration of the reality of the observed modifications of DNA could be obtained only with the use of labeled precursors. In fact, the incorporation of these precursors into the DNA in the period of recovery (assumed as evidence of DNA synthesis) would have shown that during the preceding exposure to cold a real decrease in amount of DNA in vivo had occurred.

In the present paper the results of such a study, carried out by means of radioautographic and histophotometric techniques, are reported.

The data of this and of the previous work, taken together, show that following intermittent exposure to cold the amount of DNA per nucleus in the adrenal medulla cells decreases in vivo, as shown by the synthesis of DNA de novo taking place during the recovery. It is concluded that in the rat adrenal medulla the amount of DNA per nucleus can vary in relation to the functional activity of the cells.

MATERIAL AND METHODS

1. *Radioautography*

27 male albino rats, of the Italico strain, weighing about 200 g were used for the radioautography experiments. These rats were bred in our laboratory and fed a standard balanced diet. They were divided into three groups. Two groups were kept intermittently at a temperature of +4°C (15 hr at +4°C followed by 9 hr at room temperature, 18–20°C) for a total period of 100 and 300 hr. One group was not exposed to low temperature and served as control.

H^3-thymidine (Radiochemical Centre, Amersham, England; specific activity, 14.8 c/mM) diluted in distilled water was injected intraperitoneally, in amounts corresponding to 1 μc/g body weight.

The experimental procedures used are summarized in Table I.

TABLE I

Plan of the Radioautography Experiments

No. of animals	Cumulative exposure to + 4°C	Recovery at room temperature	No. injections of H^3-thymidine	Time of injections before sacrifice
	hr	*hr*		*hr*
3	0	0	1	8
2	0	0	3	56-32-8
3	0	0	4	8-6-4-2
2	100	0	1	15
1	100	8	1	8
1	100	9	1	9
2	300	0	1	15
1	300	8	1	8
3	300	8	4	8-6-4-2
1	300	9	1	9
1	300	10	1	10
1	300	32	2	32-8
1	300	33	2	33-9
2	300	56	3	56-32-8
2	300	104	1	8
2	300	224	1	8

The animals were killed by exsanguination. From each animal both adrenal glands were dissected out. One gland was fixed in 95° ethanol-acetic acid mixture (3:1, v/v), dehydrated, and embedded in paraffin wax. From the other gland the medulla was dissected out according to a technique previously described (2), smeared directly on slides, and fixed in 95°. These smears, as well as sections (12 μ thick) of the embedded adrenals, were stained with the method of Feulgen according to the technique described by Leuchtenberger (3); they were thereafter covered with a thin film of emulsion (G5 Ilford) or stripping film (A.R. 10 Kodak) and exposed for 10 days in a light-proof box kept at +4°C. The slides were developed with Kodak D 80 for 2 min (emulsion) or 5 min (stripping film), rinsed in a stopping bath, fixed in Kodak Rapid Fixer for 10 min, and then washed in tap water for 1 hr. For the general radioautographic procedure, see Caro (4). The background was low and amounted to 1 to 1.5 gr per nucleus. In each smear, 3,000 to 8,000 nuclei were examined and the percentage of labeled nuclei was determined, as well as the number of grains over each nucleus.

A count of labeled nuclei was made also on the sections. All the counts were corrected for background. Sections previously digested with DNase were used as controls and they always showed complete absence of labeling.

2. *Feulgen Photometry*

A. In some smears, already stained with the Feulgen method, before applying the emulsion histophotometric determinations were made. These determinations were made on the adrenal medulla of the animals, exposed to cold for a total period of 300 hr, after a recovery time of 0, 8, 9, 10, 32, 33, and 56 hr. In this case, the adrenal medulla was smeared on slides divided into three sections: one section for the control adrenal medulla, the second for the kidney, and the third for the experimental adrenal medulla. The contralateral adrenal gland was embedded in a paraffin wax block, together with a piece of kidney and with the control adrenal medulla, in order to ensure uniformity in the staining steps, as underlined in a previous paper (2).

The histophotometric determinations were made preferably on the smears. A scanning integrator microphotometer built in our Department (5) was used.

B. In order to compare the amount of DNA per nucleus with the level of H^3-thymidine incorporation, a number of labeled nuclei, of which the grain number had been counted, were selected from the smears and photographed. The position of these nuclei on the slide was determined on the basis of the coordinate system of a movable microscope stage. Nuclei with less than 10 gr were disregarded. The silver grains were then removed from the slide according to the method of Gall and Johnson (6), and, on the same nuclei, which were carefully relocated and identified, the amount of Feulgen dye was determined histophotometrically. By histophotometric measurements before and after removal of grains, it was ascertained that the procedure used did not affect the absorption of the Feulgen dye. Histophotometric determinations were made also on the nonlabeled nuclei on the same slides.

3. *Search for Mitoses*

Nine adult male albino rats, after exposure to cold for 300 hr, were kept at room temperature for 3, 5, 10, 16, and 23 days and given colchicine 8 hr before sacrifice (0.1 ml/100 g body weight of a solution containing 10 mg of colchicine (Merck) in 1 ml of 80% ethanol and 9 ml of H_2O).

The adrenal glands were dissected out, fixed in 10% neutral formalin, washed in tap water, dehydrated, and embedded in paraffin wax. Sections 5 μ thick were stained with the Feulgen method. The percentage of mitoses in 3,000 nuclei of the intestinal mucosa was determined in animals with or without colchicine treatment.

RESULTS

A. *Radioautography*

1. Percentage of Labeled Nuclei

a. CONTROLS: Many smears and sections of adrenal medulla of rats belonging to the control group were examined. No labeled nuclei in the adrenals of animals treated with a single injection were observed. This fact agrees with the radioautographic data of Diderholm and Hellman (7), who found no significant incorporation in the adrenal medulla of 22-day-old rats.

After repeated injections of H^3-thymidine (3 to 4), some labeled nuclei were noted (20 to 40 gr per nucleus). It has been possible to ascribe the great majority of these nuclei to the stromal tissue, although, in some cases, the nature of the nuclei remained uncertain, due to the staining procedure (Feulgen) used. However, the over-all percentage of labeled nuclei (average 0.3%) was 30 times lower than that found in the adrenals of the experimental animals treated with the same number of injections. Increasing the time during which the slides were exposed to the emulsion did not produce any increase in the percentage of the labeled nuclei.

b. EXPERIMENTAL ANIMALS: The animals exposed for a total period of 100 or 300 hr to +4°C injected with H^3-thymidine and subsequently exposed to cold for 15 hr showed no incorporation.

If the animals after the period of exposure to cold were kept at room temperature, the results varied depending upon i) the duration of the exposure to cold and ii) the number and timing of the injections. The results are fully summarized in Table II.

After 100 hr of cold exposure and 9 hr at room temperature, no incorporation occurred. If the period of cold was prolonged to 300 hr, incorporation of H^3-thymidine occurred during the period of recovery at room temperature.

The number of labeled nuclei was roughly proportional to the length of the recovery period. The incorporation was already evident after 8 hr of exposure to room temperature (the shortest interval studied) and continued progressively later (Fig. 1).

On smears of adrenal medulla, the percentage of labeled nuclei was 0.7 to 1.26% after 8 to 10 hr at room temperature (single injection); 1.41 to 2.47% after 32 to 33 hr (two injections, 24 hr apart); 2 to 7.60% after 56 hr (three injections, 24 hr apart). The sections showed the same trend, although some discrepancies with the values of the smears, attributable to the smaller number of nuclei that it was possible to examine on the section, were recorded.

These data indicate a greater velocity of incorporation during the first hours of recovery, in comparison with the subsequent periods. These percentages of labeled nuclei are low in comparison with those found with the use of H^3-thymidine in tissues in which the cells are actively multiplying, such as the epithelium of the esophagus (8). They are only slightly lower than those found in the seminal vesicles (8) and are definitely higher than those found in tissues, such as brain

TABLE II

Labeling of Adrenal Medulla Nuclei in Control and Experimental Animals

No. of animals	Time of exposure to + 4°C	Time of recovery at 18–20°C	No. of injections	Time of slide exposure	Smears			Sections		
					No. of nuclei counted	No. of labeled nuclei	Percentage of labeled nuclei	No. of nuclei counted	No. of labeled nuclei	Percentage of labeled nuclei
	hr	*hr*		*days*						
3	0	0	1	10	24,000	2*	0.008	15,000	3*	0.02
2	0	0	3	10	10,000	26*	0.26	2,780	6*	0.21
3	0	0	4	10	—	—	—	22,800	70*	0.30
2	0	0	4	30	—	—	—	13,700	26*	0.19
2	100	0	1	10	16,000	0	0	12,000	0	0
1	100	8	1	10	8,000	0	0	5,400	0	0
1	100	9	1	10	8,000	0	0	3,800	0	0
2	300	0	1	10	16,000	0	0	7,800	0	0
1	300	8	1	10	8,000	56	0.7	371	4	1.08
1	300	8	4	10	—	—	—	13,232	1,331	10.05
1	300	8	4	30	—	—	—	18,582	5,366	28.86
1	300	9	1	10	3,000	22	0.73	1,300	10	0.77
1	300	10	1	10	3,000	38	1.26	1,000	19	1.90
1	300	32	1	10	8,000	113	1.41	860	20	2.32
1	300	33	1	10	8,000	198	2.47	1,500	17	1.13
1	300	56	1	10	8,000	607	7.60	546	31	5.67
1	300	56	1	10	3,000	60	2.00	2,000	40	2.00
2	300	104	1	10	15,000	0	0	3,552	0	0
2	300	224	1	10	16,000	0	0	4,487	0	0

* Nuclei of stromal cells. The identification was uncertain in only 5% of the cases. In the counts of experimental animals the stromal cells have been subtracted.

and heart (9), where no cell multiplication is considered to take place.

In the adrenal medulla of animals repeatedly injected with H^3-thymidine during the 8 hr of the recovery period (1 injection every 2nd hr), the percentage of labeled nuclei becomes as high as 10%.

As described under Methods, the slides were routinely exposed to the emulsion for 10 days in a dark room. If this period is prolonged to 30 days the percentage of labeled nuclei is found to increase further (to 28.86%). This increase can be explained by the fact that the long exposure time allows low-labeled nuclei to produce blackening of the emulsion.

Count of Grains per Nucleus

The histograms of Fig. 2, which represent the distribution of the labeled nuclei with respect to the number of grains, show some differences in relation to the length of the recovery period. After a single injection in a recovery period of 8 to 10 hr, two groups of labeled nuclei can be identified: i) a larger group in which the number of grains per nucleus ranges from 4 to 5 up to 60, and ii) a second smaller group in which 60 to 120 gr per nucleus can be found (Fig. 2 *A*). If 4 injections of H^3-thymidine are given at 2-hr intervals during the recovery period (8 hr), the number of low-labeled nuclei (1st group) is greatly increased, whereas the number of high-labeled nuclei is virtually unchanged (not illustrated). The same happens if the exposure time of the slides is increased.

For recovery periods longer than 8 hr, the number of low-labeled nuclei increases considerably, whereas the number of high-labeled ones does not show significant variations (Fig. 2 *B* and *C*).

For comparison, the number of grains found in premitotic nuclei (e.g. those of the adrenal medulla of 10-day-old rats) after injection of a single dose of H^3-thymidine 8 hr before sacrifice is about 20 to 25 per nucleus (10), for an exposure time of the slides of 10 days.

The histograms of the grain counts, according

to Lajtha, Oliver, Berry, and Hell (11), can throw some light on the metabolic processes at the cellular level. The histograms of the first 8 hr of recovery can, therefore, be interpreted as an indication of a fast DNA synthesis, while in subsequent days the synthesis is slower. Moreover, it is necessary to consider that the histograms of the nuclei after 32 to 56 hr of recovery represent the summed amount of the incorporation during the first 8 hr and during the following hours. It is thus clear that DNA synthesis takes place at a much faster rate in the first 8 hr of recovery than in the following hours, in which it leads exclusively to the formation of scarcely labeled nuclei.

The experiments in which H^3-thymidine was injected after 104 and 224 hr of stay at room temperature (8 hr before sacrifice) have shown no incorporation of the labeled precursor into the DNA of the adrenal medulla nuclei. It can be concluded, therefore, that the synthesis of DNA is completed before 104 hr of recovery.

B. *Feulgen Photometry*

On the same slides on which the incorporation of H^3-thymidine was observed, the amount of Feulgen dye per nucleus had been measured before applying the emulsion. In each case, determinations were also made on the nuclei of

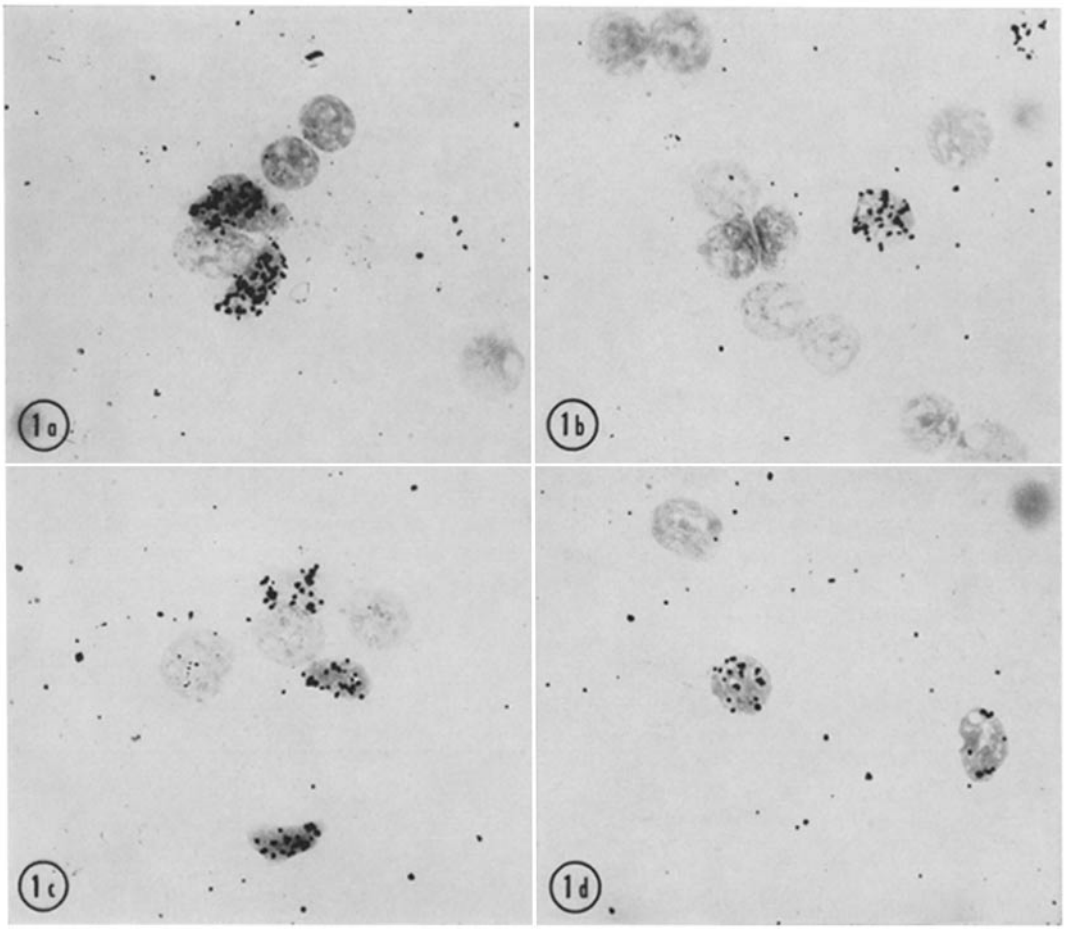

FIGURE 1 Labeled nuclei of the adrenal medulla of a rat exposed intermittently to cold for a total period of 300 hr, and subsequently at room temperature for 56 hr. Three doses of H^3-thymidine were injected, at 56, 32, and 8 hr, respectively, before sacrifice. The shape of some nuclei is distorted because of the smearing. × 1000.

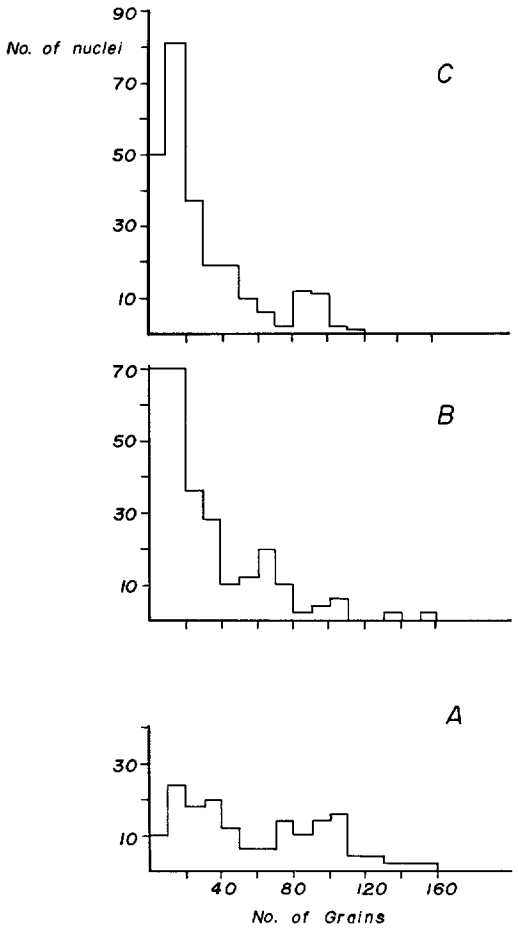

FIGURE 2 Histograms of the distribution of the label in the nuclei of adrenal medulla cells of rats exposed intermittently to cold for 300 hr and kept at room temperature for 8 to 10 hr (*A*), 32 to 33 hr (*B*), and 56 hr (*C*).

kidney and of the control adrenal medulla, mounted on the same slide. The kidney nuclei show a practically constant amount of Feulgen dye, with very small variations. This applies also to the nuclei of the control adrenal medulla, whose values are, on the average, 22% higher than those of the kidney nuclei. For the experimental adrenal medulla, the values are more scattered and hence the SEM is greater (Table III). In the animals kept at room temperature for 8 to 10 hr, the average amount of Feulgen dye per nucleus is always less than normal, but considerably larger than that of the animals sacrificed immediately at the end of 300 hr of cumulative cold exposure. As shown in Fig. 3 *B* and *C*, some nuclei already have made good the loss, and some have synthesized much more DNA, going well beyond the normal values.

After 32 hr of recovery, the average value of Feulgen dye per nucleus reaches the value of the control adrenal medulla and after 56 hr goes beyond it. The histogram shows that many nuclei have reached the normal values, while others have risen to almost double these values (Fig. 3 *D* and *E*).

C. *Correlation between Feulgen Dye Content and Grain Number of the Same Nuclei*

For the purpose of attempting a more detailed analysis of the relationship between grain number and amount of DNA of the nuclei, histophotometric determinations of the Feulgen dye content per nucleus were made on previously labeled nuclei whose grain number was known. Since the H^3-thymidine incorporation is faster at the beginning of the recovery, this study was

TABLE III

Feulgen Dye per Nucleus (Arbitrary Units) Determined by Histophotometry in Adrenal Medulla of Rats Exposed Intermittently to 4°C for a Total of 300 Hr and Kept at Room Temperature for Various Intervals

All values are averages of 100 nuclei on smears.

Time at room temp.	Kidney (± SEM) K	Normal adrenal medulla (± SEM) N	$\frac{N \times 100}{K}$	Experimental adrenal medulla (± SEM) E	$\frac{E \times 100}{N}$
hr					
0	55.4 ± 0.18	60.2 ± 0.19	+8	39.3 ± 0.34	−35
8	45.9 ± 0.24	52.8 ± 0.19	+15	46.0 ± 0.71	−13
10	40.0 ± 0.16	53.8 ± 0.32	+34	49.3 ± 0.88	−8
32	43.7 ± 0.43	52.2 ± 0.32	+19	50.5 ± 0.74	−3
33	42.1 ± 0.17	51.7 ± 0.31	+23	53.4 ± 0.63	+4
56	42.0 ± 0.22	50.2 ± 0.34	+19	58.6 ± 1.08	+16

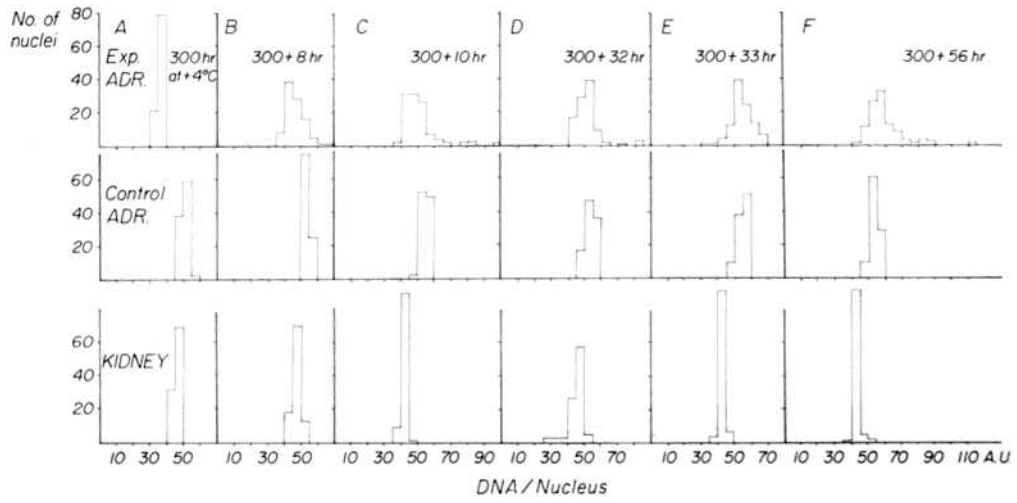

FIGURE 3 Histograms of Feulgen dye content of the nuclei of adrenal medulla after 300 hr of exposure to cold (*A*) and subsequently to room temperature for 8 hr (*B*), 10 hr (*C*); 32 hr (*D*), 33 hr (*E*), and 56 hr (*F*).

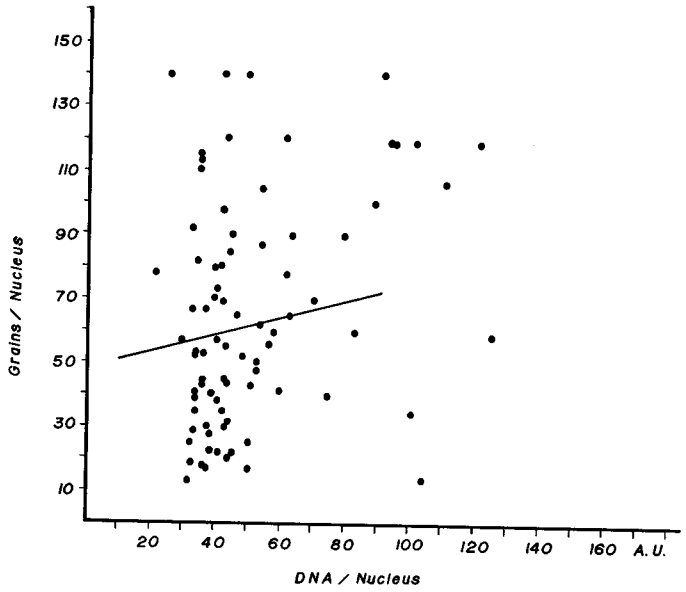

FIGURE 4 Plot of silver grains per nucleus (ordinate) against Feulgen dye content (abscissa) (rats after 8 to 10 hr of recovery from exposure to cold). The straight line is the regression of grain count on dye content.

limited to the animals sacrificed 8 to 10 hr after returning to room temperature and given a single injection of H^3-thymidine. The results show a significant correlation ($r = 0.2699$, $P < 0.02$) between Feulgen dye content and grain number. The regression of grain number on Feulgen dye content has been calculated and is shown by the slope of the line in Fig. 4. It is seen that the number of grains for a given dye content is widespread. Many factors can influence a quantitative relationship of this kind, as exemplified by Gall and Johnson (6). Furthermore it is clear that, other conditions being equal, the intensity of labeling of DNA will depend on the rate of synthesis during the period of availability of labeled precursors. The data of Hughes et al. (12),

Quastler and Sherman (13), and Koburg and Maurer (14) suggest that with thymidine the labeling of nuclei occurs only in the first 40 to 60 min after injection. On the basis of these considerations, it appears that synthesis of DNA in the period of recovery shows a wide inhomogeneity between individual nuclei, from the point of view of timing and rate. The comparison between the histograms of Feulgen dye content in radioactive and nonradioactive nuclei (Fig. 5) demonstrates a greater dispersion in labeled nuclei among which are nuclei with supranormal DNA values.

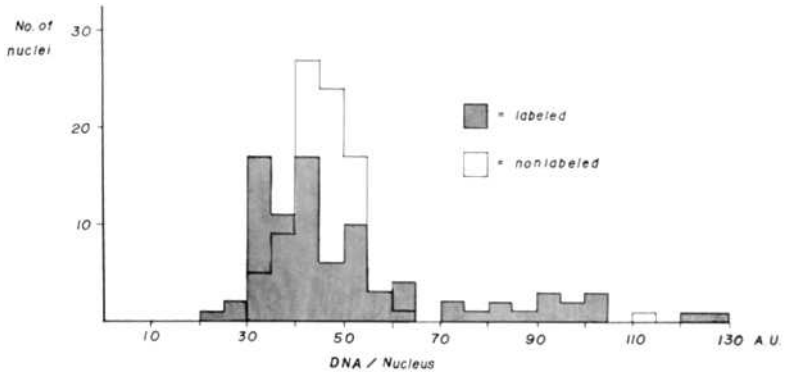

FIGURE 5 Histograms of Feulgen dye content in the radioactive and nonradioactive nuclei on the same slide.

D. Count of Mitoses

Many sections (about 10 for each rat) of adrenal medulla of animals treated with colchicine, each containing on the average 1,500 to 2,000 nuclei, have been scanned. Only rare mitotic figures were detected (0.03%), attributable to nuclei of stromal cells.

In the same rats, the effect of colchicine was tested on the cells of the crypts of the intestinal mucosa. Without colchicine, the mitotic index of these cells (calculated on 6,000 cells in two experiments) is 1%. After treatment with colchicine, the value of the index (calculated on 9,000 cells in three experiments) rose to 6%. This increase is in agreement with that reported by Pelc for the cells of the esophageal mucosa (8).

Mitoses of parenchymal cells have been observed in the adrenal medulla of young rats (10 days old) after treatment with colchicine. In this case, the mitotic index (1%) is good agreement with the percentage of labeled nuclei found after injection of H^3-thymidine (10).

DISCUSSION

These observations show that the adrenal medulla cells do not incorporate any appreciable amount of H^3-thymidine in the nuclei either under normal conditions, or during the period of exposure to cold, in which a considerable decrease of the amount of DNA per nucleus has been consistently detected. Also, no detectable incorporation takes place during the recovery period following 100 hr of exposure to cold; instead, a clear incorporation takes place in the period of recovery when the animals are brought back to room temperature after 300 hr of cold exposure. From the analysis of the percentage of labeled nuclei and of the intensity of labeling at various times during the recovery period, it appears that the rate of incorporation is high in the first 10 hr and declines rapidly thereafter. The process of DNA synthesis is completed before 104 hr.

H^3-thymidine is a specific precursor of DNA, being incorporated exclusively into the thymidylic acid moiety (15, 16). Its incorporation into DNA could indicate: (*a*) net synthesis of DNA followed, or not, by mitosis; (*b*) turnover of DNA without net synthesis, i.e., substitution of an "old" molecule of DNA by a newly synthesized one. In fact, while the first possibility has been exhaustively demonstrated, so that incorporation of H^3-thymidine is generally considered as equivalent to net DNA synthesis, the second possibility is still a subject of debate, and no conclusive evidence has so far been produced (17–23, 8, 6, 9).

Clearly, in our case, we must confine the discussion to the first possibility because, in the

nuclei of the adrenal medulla cells, coincidental with the incorporation of the label an increase of DNA content takes place, which has been concurrently demonstrated with three independent methods (Feulgen photometry, interference microscopy with the use of DNase, and microchemical determinations) (1, 2).

An apparent discrepancy exists between histophotometric and radioautographic data, on quantitative grounds. It has been shown (1, 2) by histophotometric determinations that synthesis of DNA takes place in all nuclei during the recovery period, whereas incorporation of labeled thymidine occurs, in our experimental conditions, in only a limited number of nuclei. It has to be taken into account, however, that DNA synthesis does not take place to the same extent in all nuclei. On the other hand, the number of labeled nuclei closely depends on the time of availability of the precursor, which has been shown to be limited to 40 to 60 min (12–14). Increasing the time of availability of H^3-thymidine, such as that obtained in the experiments (see above) in which 4 doses of the precursor had been injected at intervals of 2 hr during the 8 hr of recovery, leads to an increase in the number of labeled nuclei (10%). On the other hand, in those nuclei which show a small synthesis of DNA on histophotometrical examination, H^3-thymidine would be incorporated in amounts too small to produce blackening of the photographic emulsion during the time the slides were exposed (10 days).

A prolongation of the time of exposure of the slides (30 days) leads to a considerable increase in the number of labeled nuclei (28.86%). Assuming an availability time of the precursor of 40 minutes after each injection, the total time the precursor is available to the cells in our experimental conditions amounts to 30% of the recovery time (8 hr). From histophotometric determinations, it appears that DNA synthesis occurs in almost all nuclei (90%) after 8 hr of recovery. The radioautographic results are, therefore, in agreement with the histophotometric measurements. The apparent discrepancy between histophotometric and radioautographic techniques reflects a fundamental difference between the two methods, since histophotometry gives the assembled result of a process which takes place asynchronously and with different intensity in a population of nuclei over a certain period of time, whereas radioautography gives a picture of the processes actually going on at a given moment.

It is possible to conclude, therefore, that the present experiments establish beyond doubt the fact that a synthesis of DNA takes place in the adrenal medulla nuclei in the recovery period after stimulation, in full agreement with the previous observations (1, 2).

The demonstration that during the recovery there is a real synthesis of DNA may help to clarify further the decrease in DNA content observed upon exposure to cold. The most likely explanations of this phenomenon, along the lines of the discussion by Mirsky and Osawa (24) of an apparently similar case, i.e., decrease in DNA content in liver nuclei induced by cortisone (Lowe et al., 25, 26) are: 1) that the diminution is real, i.e., the amount of the DNA per nucleus after exposure to cold is less in vivo; 2) that a part of the DNA is changed in such a way as to escape determination through loss during the analytical manipulations. In the first case, in the period of return to normal or supranormal DNA values, synthesis of new DNA should take place. In fact, this demonstration has been given in our case; the relevant conclusion is, therefore, that the decrease in the amount of DNA per nucleus induced by exposure to cold is real.

In view of the fact that DNA synthesis is generally considered to precede cellular division, it is necessary to examine whether this applies also to our case, inasmuch as in some nuclei the values of DNA reach, in the recovery period, levels corresponding to double the normal values. In all our observations, on both normal adrenal medullas and experimental ones, during exposure to cold and during the recovery period, no mitosis has ever been detected, in agreement with the observations of Mitchell (27). Moreover, the present data, in agreement with those of Diderholm and Hellman (7), demonstrate that, except during the recovery period, there is no significant incorporation of H^3-thymidine, and hence DNA synthesis, evident in these cells.

The possibility that the DNA synthesis observed in the recovery period might be due, to some extent, to a diurnal rhythm can be ruled out by the fact that no significant number of labeled nuclei is found in control animals injected at the same hour of the day as the experimental ones.

It is clear, therefore, that the DNA synthesis during the recovery period is not related to the

preparation for mitosis but exclusively to the DNA loss which had occurred previously upon exposure to cold. Incidentally, this shows that irreversible postmitotic cells, like those of the adrenal medulla, although they have lost the ability to divide, maintain the ability to synthesize DNA under suitable experimental conditions. The DNA synthesis is not limited to the restoration of the normal amount of DNA but goes well beyond it, constituting an example of an "overshoot" reaction.

Since no indication exists that the observed decrease of the DNA per nucleus is associated with death of the cells (and this, in view of the extent of the decrease, should have been easily detected), it is evident that we are dealing here with a peculiar reversible modification related to the functional activity of the cells. As shown by histological data reported by Cramer (28), upon intermittent exposure to cold the adrenal medulla is rhythmically stimulated (with consequent increase of functional activity) without reaching exhaustion.

It can be concluded that the long-debated question (see Brachet (29); Mirsky and Osawa (24); and Viola-Magni (2)) of whether changes of the content of DNA per nucleus may occur in relation to the functional activity of the cell, has a positive answer, at least so far as the adrenal medulla is concerned. A more precise correlation of the DNA changes with the functional activity of the cells (based upon evaluation of the secretory activity) will be attempted in future investigations. It is possible that this situation occurs only in this tissue and cannot be extended to other types of cells. In this connection it must be considered that (*a*) these cells are irreversibly postmitotic or perennial (30), and that (*b*) their content of DNA per nucleus is greater (from 16 to 39%), according to reference 2) than that of a typical diploid cell, such as that of the kidney tubules. The first circumstance does not allow the tissue to respond to a strong stimulation with an increase in the number of the cells, as is the case in other tissue (the adrenal cortex (31) and other endocrine glands (32)), in which hyperplasia brings about a reduction of the functional load onto the individual cells. The second circumstance suggests (as pointed out previously (2)) that the over-all variations of DNA content may involve mainly this extra fraction of DNA, and only partially the DNA associated with the chromosomal sets.

This consideration raises the question of the heterogeneity of DNA. Separation of various fractions of DNA with several analytical techniques (fractional extraction, chromotography, heat denaturation curve, ultracentrifugation) has been reported by various authors (see 24 for reference). Up to now, no relationship has been established between these fractions and any portion of DNA identifiable on the basis of structural or functional criteria. The experimental situation described here seems to offer a perspective in this direction.

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