THE EFFECT OF CORTISONE ON DNA CONTENT OF RAT HEPATOCYTES*

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Cell division in rapidly proliferating tissues is inhibited by many agents of widely different nature. Two agents in particular, x-radiation and cortisone, because of therapeutic usefulness have received special attention (1–4). Since DNA synthesis is a concomitant of cell division the effect of oncolytic agents on this aspect of cell division has been of especial interest.

A rapid rate of incorporation by DNA of P^{32} and other labelled precursors occurs in growing tissue but not at nearly the same rate in non-dividing tissue. X-radiation prevents this increase in incorporation (5) but in non-proliferating tissue it is difficult to demonstrate any effect by x-radiation on incorporation into DNA. It is believed that DNA synthesis occurs during interphase or prophase and is a process necessarily preceding cell division (6). Whether the effect of x-radiation on DNA synthesis is a primary effect or is secondary to other biochemical events within the cell at the time of division is unknown.

Because of the analogous effects of x-radiation and cortisone upon cell division under certain conditions (1, 2, 4), experiments were undertaken to explore the mechanisms whereby these effects are brought about. First, it seemed of interest to study the effects of cortisone upon DNA synthesis in both rapidly growing and non-proliferating tissue. Secondly, it seemed desirable to compare the effects of x-radiation, cortisone, and both agents combined upon the DNA content of nuclei.

With the use of P^{32}, the first type of experiment would reveal whether cortisone had an inhibitory effect on the incorporation of P into DNA in a manner similar to x-radiation. The second type or experiment would show whether x-radiation and cortisone exerted an effect upon the DNA content of adult cell nuclei as a mechanism which would indirectly affect cell division.

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Materials and Methods

Animals.—Male rats of the Wistar strain,[1] weighing from 100 to 120 gm., were fed *ad libitum* on a lablox R diet.[2] All animals were fasted for 24 hours before removal of the livers for study, and to avoid alterations due to diurnal variations in metabolism, were killed between 9:00 and 10:00 a.m.

Five groups of animals were studied:

1. Control.—Animals received no treatment.

2. Cortisone.—Animals received cortisone acetate[3] (25 mg./day, intramuscularly) for from 1 to 5 days.

3. Partial Hepatectomy.—Partial hepatectomy was performed according to the method of Higgins (7); the animals were fed 20 per cent sucrose water *ad libitum* and sacrificed 18 hours after operation. Studies were performed either on normal rats or on animals which had received injections of cortisone for 5 days prior to the operation. No sham operated controls were prepared.

4. X-radiation.—Total body x-radiation, 1300 roentgens air dose was administered with 250 kv. Westinghouse generator using 1 mm. aluminum and ¼ mm. copper filtration. The animals were radiated at a distance of 50 cm. in a plastic box. Survival studies indicated that 50 per cent of the animals were dead in 7 days and all were dead in 11 days. Approximately 10 per cent of the animals died during the 5 day experimental period. Animals were killed from 1 to 5 days after x-radiation.

5. Cortisone Plus X-radiation.—X-radiation was administered as indicated and cortisone was started within 1 hour after radiation for periods of from 1 to 5 days. In the 5 day experimental period, careful handling insured that the death rate did not exceed 10 per cent.

Cellular Disruption.—At the time of sacrifice, the animals were lightly anesthetized with ether, the hepatic vein was severed, and the liver excised. The livers were rinsed in the suspending medium, blotted dry, and placed in a chilled, steel meat press. The expressed liver was then rapidly weighed on a chilled watch glass and transferred to an iced Potter-Elvehjem type, plastic plug homogenizer. Slightly alkaline saline (containing 2 ml. of 0.1 N NaOH per liter of 0.85 per cent *w/v* NaCl) was used as a suspending medium and 20 per cent homogenates were prepared. The brei was maintained at 4°C. throughout the whole procedure.

In some experiments, "purified nuclei" were obtained by repeated centrifugation and multiple washes in 2 per cent *w/v* citric acid (8).

Enumeration of Nuclei.—Direct enumeration of nuclei in a brei was performed as follows: A suitable dilution, (usually 1:10) of the saline brei was made with 1 per cent acetic acid in a NBS white cell diluting pipette, and nuclei were counted by phase microscopy in a hemacytometer for phase optics.

In selected experiments, isolated "purified nuclei" were suspended in 2 per cent citric acid, diluted appropriately, and counted as described above. This procedure allowed a comparison of the values of DNA per nucleus obtained in this direct manner with values obtained from homogenates.

Errors of Counting Nuclei.—The nature of the errors inherent in any counting procedure have been discussed by Berksun (9). He lists three major sources of error: (*a*) pipette error, (*b*) chamber error, (*c*) field error or deviation from the Poisson distribution curve in the chamber because of the nature of the material.

[1] Carworth Farms, Inc., New City, New York.

[2] Allied Mills, Peoria, Illinois.

[3] Made available through the courtesy of Dr. Frederick K. Heath, Merck & Co., Inc., Rahway, New Jersey.

Pipette error and chamber error were minimized by using a small group of NBS-certified diluting pipettes and only two counting chambers. In a series of replicate nuclear counts from normal animals, using a single pipette, it was demonstrated that field error could be ignored.

It was assumed that there was no significant difference in the behavior of nuclei from the various experimental groups with respect to the counting procedure. This seemed valid since: (*a*) there was no gross clumping observed in the diluting pipettes from the various experimental groups; (*b*) there was no tendency towards clumping observed in the hemacytometer from any of the experimental groups; (*c*) there was no evidence of change in nuclear friability as a result of the experimental regimens. If nuclear disruption had occurred as a general phenomenon or selectively in certain groups, DNA would pass into the suspending menstruum and there would have been an increase in apparent DNA/nucleus. In a pilot experiment using 11 normal and 8 cortisone-treated animals, it was found that the results using isolated "purified nuclei" were similar in direction and significance to those obtained from homogenates.

In addition to the various factors mentioned, the size of sample influences the reliability of the results. In the present instance sufficient numbers of nuclei were counted to insure, in theory, an experimental error of 10 per cent or less, at least 90 per cent of the time (10).

Chemical Analyses.—The total nucleic acid content of a brei or suspension of "purified nuclei" was extracted by the method of Schneider using hot trichloracetic acid (TCA) (11). The TCA extract was analyzed for deoxyribose by the diphenylamine reaction (12). The accompanying tables use the term DNA to indicate deoxyribose since this was the material determined. This value can be converted to the appropriate value for nucleic acid by simple multiplication. Deoxyribose $\times$ 2.19 = DNA.

Calculations.—Deoxyribose was determined on a suitable aliquot of homogenate and expressed as micrograms of deoxyribose per gram of wet liver. Nuclei, in another aliquot, were enumerated and expressed as nuclei per gram of wet liver. Simple division provided a value "per nucleus."

Tracer Techniques.—P^{32} (0.4 μc./gm. body weight) was injected intraperitoneally. The rats were killed in the usual fashion 1 hour after injection. Inorganic P was precipitated as the Mg salt from a neutralized cold TCA extract (13). Any material to be analyzed for phosphorus was wet ashed with sulfuric acid on a sand bath. Oxidation was completed with concentrated nitric acid and phosphorus was estimated by the colorimetric method of Fiske and SubbaRow (14). DNA was isolated from nuclei by the method of Vermund *et al.* (15) and an aliquot of DNA nucleotides was wet ashed in sulfuric acid for phosphorus determination.

The radioactivity of the ashed material was measured[4] on the same sample

[4] A Model 163 tracer lab scaler was used with a radiation laboratory model 80-S-1 Geiger Muller dipping tube.

from which a portion was taken for P analysis. An aliquot was obtained for deoxyribose analysis prior to ashing, and unless the values for deoxyribose and P agreed within 5 per cent, assuming a statistical tetranucleotide, the data were discarded. Since this was an infrequent occurrence, it was thought that the procedure was reasonably reliable. Counts were recorded as relative specific activity, (RSA):

$$\frac{\text{Counts/min./}\mu\text{g. P}}{\text{Counts/min./}\mu\text{g. inorganic P}} \times 100$$

EXPERIMENTAL RESULTS

P^{32} Studies: The P^{32} data are summarized in Table I. DNA phosphorus isolated from livers of normal animals had a relative specific activity of 0.08;

TABLE I

Effect of X-radiation and Cortisone on Incorporation of P^{32} by DNA

Type of experiment	No. of animals	Relative specific activity
		mean S.E. *mean*
Normal	19	0.08 ± 0.01
Cortisone, (5 days)	9	0.03 ± 0.01*
Normal, partial hepatectomy, (18 hrs.)	15	0.67 ± 0.14
Cortisone, partial hepatectomy, (18 hrs.)	4	0.26 ± 0.13
X-ray, 4 days (1300 r)	7	0.09 ± 0.01
Cortisone, (5 days), 3 day recovery	6	0.12 ± 0.02‡

Comparison to normal.
* $p < 0.02$.
‡ $p < 0.05$.

DNA phosphorus from cortisone-treated animals showed a relative specific activity of 0.03. This suggested interference with DNA synthesis, and it is clear from examination of results in the partially hepatectomized animals that this is indeed the case.

P^{32} uptake at the 4th day after x-radiation and 3 days after the last administration of cortisone was not significantly different from that observed in the normal.

Normal and cortisone-treated animals subjected to partial hepatectomy yielded contrasting data. DNA-P obtained from normal operated animals had a RSA of 0.76; while in the cortisone-treated animals, this fraction had a RSA of 0.26. The high rate of P^{32} incorporation into the hepatic DNA phosphorus of normal, partially hepatectomized animals has been observed previously (5, 6). The reduced incorporation of P^{32} accompanying cortisone administration may be related to the interference with cell division reported by Roberts (1) and Einhorn (2).

DNA Content of Nuclei.—Tables II, III, IV show the effects of cortisone, x-radiation, and the combination of these agents upon deoxyribose per gram of liver, deoxyribose per nucleus, and nuclei per gram of pressed liver.

TABLE II

*Effect of Cortisone on DNA Content of Rat Liver**

Length of cortisone administration	Liver weight‡	DNA/gm.§	Nuclei × 10^{-6}/gm.	DNA/nucleus‖
days				
0	4.40	1090	237	4.68 ± 0.15
1	4.86	924	222	4.29 ± 0.19
2	6.39	749	168	4.49 ± 0.14
3	6.49	718	197	3.68 ± 0.12
4	6.37	698	208	3.46 ± 0.18
5	6.37	741	193	3.83 ± 0.11
5 + 3 day recovery	—	863	200	4:36

* Mean values of 10 animals except day 0 = 12 animals; day 5 = 11 animals; recovery = 4 animals.

‡ Grams of pressed liver.

§ Micrograms of deoxyribose per gram of wet liver.

‖ Picograms of deoxyribose per nucleus; mean values and standard error of mean.

TABLE III

*Effect of X-radiation on DNA Content of Rat Liver**

Days following radiation	Liver weight‡	DNA/gm.§	Nuclei × 10^{-6}/gm.	DNA/nucleus‖
0	4.40	1090	237	4.68 ± 0.15
1	5.27	867	186	4.75 ± 0.12
2	4.90	871	207	4.23 ± 0.01
3	4.49	886	240	3.73 ± 0.10
4	3.47	1124	262	4.27 ± 0.16
5	3.47	1226	267	4.71 ± 0.18

* Mean values of 10 animals except day 0 = 12 animals.

‡ Grams of pressed liver.

§ Micrograms of deoxyribose per gram of wet liver.

‖ Picograms of deoxyribose per nucleus; mean values and standard error of mean.

The value of DNA per nucleus is an average value for a mixed population of liver nuclei. The cells counted and analyzed for DNA include diploid and polyploid hepatic nuclei, leukocytes, bile duct cells, Kupffer cells, etc. The implications of measurements on this heterogenous cell population are considered below.

Table II summarizes the results in the cortisone-treated animals. Deoxyribose per nucleus showed a steady fall from 0 to 5 days; when day 5 was compared

to day 0 by the t test (16), the differences were highly significant ($P < 0.01$). It can be calculated that there was no significant change in the DNA per liver but a moderate increase in the cellularity of the liver.

Table III shows the effects of x-radiation on hepatic nuclei. Deoxyribose per nucleus fell during days 0 to 3 and then increased on days 4 and 5. The value per nucleus at day 3 was significantly different from day 0 ($P < 0.01$). The average value for day 5, however, was indistinguishable from that of day 0. The calculated value of DNA per liver fell and then rose much as did the value for DNA/nucleus. The number of cells per liver did not change significantly.

Table IV shows the combined effects of x-radiation and cortisone. The

TABLE IV

*Effect of X-radiation and Cortisone on DNA Content of Rat Liver**

Days of cortisone administration following radiation	Liver weight‡	DNA/gm.§	Nuclei × 10^{-6}/gm.	DNA/nucleus‖
0	4.40	1090	237	4.68 ± 0.15
1	5.95	800	170	4.76 ± 0.20
2	6.64	733	173	4.32 ± 0.14
3	6.43	770	186	4.22 ± 0.26
4	4.75	945	222	4.39 ± 0.13
5	4.06	1036	260	4.01 ± 0.04

* Mean values of 10 animals except day 0 = 12 animals; day 4 = 11 animals.

‡ Grams of pressed liver.

§ Micrograms of deoxyribose per gram of wet liver.

‖ Picograms of deoxyribose per nucleus; mean value and standard error of mean.

deoxyribose value per nucleus fell slowly but steadily until day 5. This value was significantly different than found for day 0 ($P = 0.01$). It is evident that the administration of cortisone prevented the rise at days 4 and 5 seen in animals receiving x-radiation alone.

The calculated values for deoxyribose per liver show in general the changes exhibited by the values per nucleus. In the cortisone experiments there seemed to be an apparent increase in cellularity of the liver because of increase in numbers of nuclei counted and analyzed. It is difficult to accept the fact that any of the experimental treatments increased the number of nuclei and cells in the liver; this observation, therefore, would seem to be due to an artifact and was probably the result of increased friability of the liver and hence expression of more cells from the meat press. Regardless of cause, varying numbers of cells were analyzed for deoxyribose. Any increase in the numbers of nuclei analyzed would certainly increase the apparent DNA per liver, but would have no predictable effect on the DNA/nucleus.

DISCUSSION

The data presented in Tables II, III, and IV indicate that under certain conditions, the apparent DNA content of nuclei will be found to change. It has been demonstrated (17, 18) that DNA is a stable component of cells, present in equal concentration in non-proliferating diploid cells of a variety of tissues in a given species and resistant to change by a number of agents. It, therefore, seems important to examine with care both the evidence available on the stability of DNA and possible sources of error in the present experiments.

Studies with P^{32} have shown that non-dividing cells incorporate only small amounts of P^{32} into DNA phosphorus (5, 6). Experiments with other precursors (C^{14} formate, C^{14} or N^{15} glycine, N^{15} or C^{14} adenine) (5) suggested from comparison of the ratios of activities of precursors incorporated into RNA with respect to DNA that these precursors were utilized at a faster rate than P^{32} in DNA synthesis. Tyner's data, however, obtained following simultaneous administration of P^{32} and C^{14} glycine, lead her to conclude that differences in the rate of incorporation existed in the pathways of RNA synthesis and not in the pathways of DNA synthesis (19). Assuming that DNA is a homogeneous compound, the experimental data indicate that the metabolic "turnover" of DNA is very slow; however, these data do not exclude the existence of more than one form of DNA. Bendich reported the existence in several tissues of at least two types of DNA. These could be separated by a special extraction method and were found to have individually different rates of synthesis for components of the purine and pyrimidine molecules (20–22). The present data suggest the possibility that part of the DNA may be labilized and lost to the cell as a result of cortisone administration or x-radiation. This conclusion is made easier to accept by assuming that nuclear DNA is heterogenous and that the turnover rate of the various DNAs may differ.

Before accepting changes in nuclear DNA as real, it is necessary to rule out the possibility that some unknown factor or artifact produced an apparent rather than real alteration. The results could have been influenced by the following artifacts: (*a*) selective death of hepatocytes (diploid and/or tetraploid), (*b*) change in the number of non-hepatic diploid cells,[5] (*c*) selective change in number of polyploid hepatocytes.

To obviate the first possibility, a histological survey was made of liver to determine the presence of necrosis. None was found. The second and third possibilities may be explored by a simple mathematical analysis. This is summarized in Table V. The cell population of the liver is considered to be made up of non-hepatic diploid, hepatic diploid, and hepatic tetraploid cells. In Davidson's survey of the DNA content of nuclei of rat tissue, the ratio of the DNA content of diploid nuclei to the DNA value per average hepatic cell was found

[5] The non-hepatic cells may be considered as all diploid.

to be 0.74. By the use of this value in conjunction with the data obtained in the present experiments, the normal diploid DNA deoxyribose value was calculated as 3.40 picograms. In terms of DNA phosphorus, this is equivalent to 0.85 picograms, a value which is somewhat higher than Davidson reported.

TABLE V

Mathematics of DNA

A. Cell population of liver	Number	DNA/nucleus
Non-hepatic diploid: T_{ND}	x	a
Hepatic diploid: T_{HD}	y	a
Hepatic tetraploid: T_{HP}	z	$2a$

B. Equations

Total nuclei per liver: $T_N = x + y + z$

Total DNA per liver: $T_D = ax + ay + 2az$

DNA per average nucleus: $\dfrac{T_D}{T_N} = \dfrac{ax + ay + 2az}{x + y + z}$

$$\frac{\text{True diploid DNA per nucleus}}{\text{Average DNA per liver nucleus}} : \frac{\dfrac{a}{\dfrac{T_D}{T_N}}}{} = \frac{a}{\dfrac{ax + ay + 2az}{x + y + z}}$$

C. By measurement

Total nuclei: $1042 \times 10^6 = T_N$

Total DNA: $4796\ \mu g. \times 10^6 = T_D$

Non-hepatic cells: 30 per cent of $1036 \times 10^6 = T_{ND}$

From Thompson *et al* (17): $\dfrac{a}{\dfrac{T_D}{T_N}} = 0.74$

By substitution: $\dfrac{T_D}{T_N} \times 0.74 = a$

$a = 3.40$ picograms of deoxyribose.

Nevertheless, for the purpose of the following discussion, it is entirely satisfactory.

If one assumes that the action of cortisone is solely on hepatocytes and that the number of these cells analyzed stays constant,[6] then one may calculate from the equations in Table V and the experimental data, deoxyribose values per diploid hepatocyte for any experimental period. These theoretical figures

[6] This should be approximately true since none of the experimental regimens is known to stimulate the growth of liver; in contrast, the number of non-hepatic cells in the liver is a function of many factors, such as vascularity, lympholysis, effective perfusion, and others.

are plotted in Fig. 1. The heavy black lines are the least square lines for the cortisone group and cortisone plus x-radiation group. The x-radiation data could obviously not be treated in the same manner. Comparison with a similar plot of the experimental data from Tables II, III, and IV was made and showed that the shapes of the experimentally determined curves were the same as the theoretical curves. Therefore, if one assumes that the number of hepatocytes remained constant, the calculated changes in DNA content are as

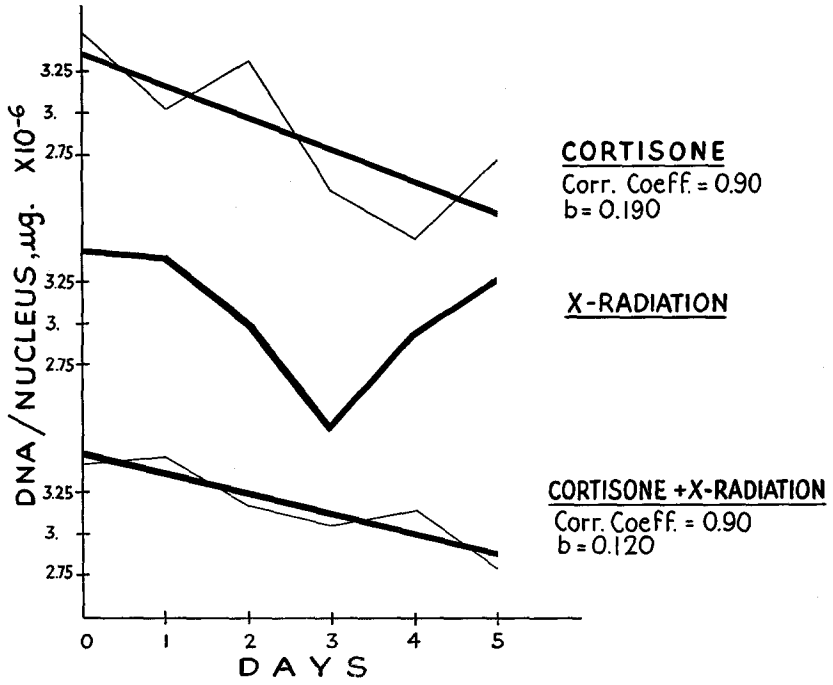

FIG. 1. DNA per diploid parenchymal nucleus.

great or greater than those found in the mixed cellular population and are in the same direction in each instance.

One may calculate in the same manner the per cent of increase in the diploid non-hepatic cells that would be necessary to produce the observed fall in average DNA/nucleus. This value was found to be 70 per cent. Since the histological evidence indicates no great change in number of non-hepatic cells,[7] it was concluded that any observed fall in DNA per average nucleus could not have

[7] For this calculation, it was necessary to determine by histological examination of fixed and stained tissue the per cent of non-hepatocytes analyzed in a given homogenate. This was found, in three experiments on normal and the same number on cortisone- and x-ray-treated animals, to be close to 30 per cent.

been caused by a major increase in the numbers of non-hepatic diploid nuclei analyzed.

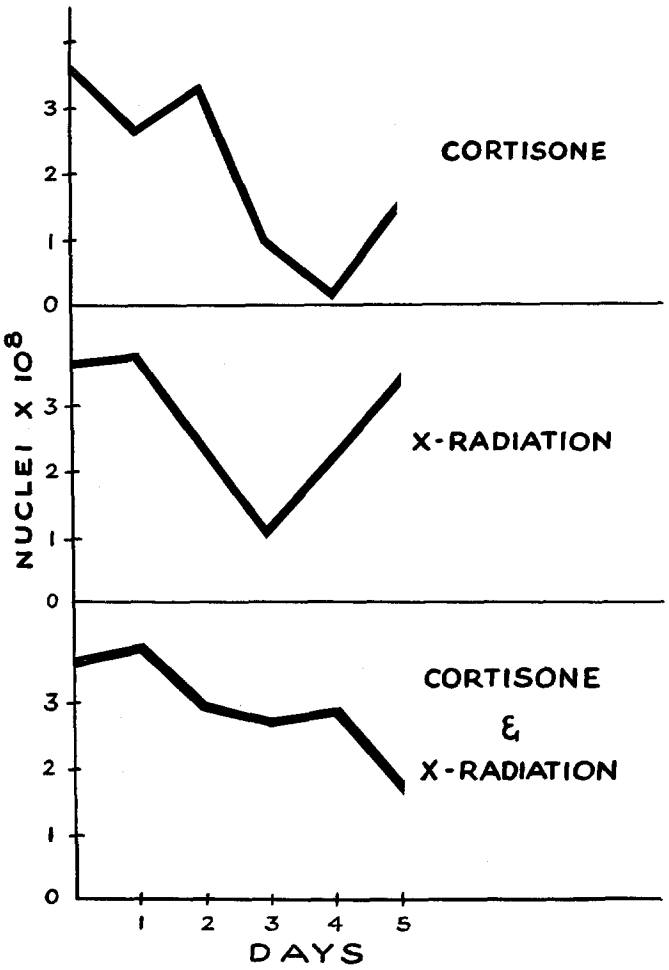

FIG. 2. Effect of cortisone and x-radiation on the number of tetraploid nuclei.

The experimentally determined value of DNA per nucleus is in part a function of the numbers of tetraploid nuclei present. One may assume for purpose of calculation that the diploid value of DNA per nucleus stayed constant for both hepatic and non-hepatic cells and that only the number of tetraploid nuclei changed. The equation in Table V may then be solved for the numbers of tetraploid nuclei necessary to give the experimentally determined value of deoxyribose per nucleus. These theoretical plots are found in Fig. 2. It is clear

that changes in frequency of polyploid cells could explain the experimental results, though the fall in the numbers of these cells would be extremely large. In the case of the cortisone-treated animals, only total destruction of polyploid cells could cause the observed fall in DNA per average nucleus. Since no direct determination was made of changes in the numbers of polyploid cells, it was not possible to eliminate this explanation for the observed results.[8]

In effect, then, there appears to be two possible explanations for the observed fall in nuclear DNA. Either the number of polyploid nuclei selectively changed or DNA per diploid hepatocyte nucleus changed. Both phenomena require a loss of DNA/nucleus, though in the first instance the loss was only in a select group of nuclei.

Several explanations for the loss of DNA are possible: (*a*) DNA synthesis was decreased; (*b*) the amounts of DNA determined by the Schneider-TCA procedure were changed.

The rate of incorporation of P^{32} by DNA and DNA synthesis in the resting liver is normally very low and hence changes induced in this rate by cortisone and/or x-radiation are difficult to evaluate. Even if DNA synthesis were completely prevented, it seems unlikely that a cessation of synthesis for only 5 days could account for a loss of 20 to 30 per cent in nuclear DNA. Of course, any effects on alternate pathways of DNA synthesis which would not be measured by incorporation of P^{32} cannot be excluded.

With respect to the second possibility, it may be recalled that when animals that had received cortisone for 5 days were allowed to recover for 3 days, the DNA per nucleus was found to increase toward normal. Likewise, on days 4 and 5 in the x-radiated series, DNA per nucleus was found to increase. The isotope data in Table I show that increases in the rate of DNA synthesis are of a small order of magnitude and probably cannot explain the increases in DNA/nucleus noted on the days under discussion.

Although multiple mechanisms may be at work, it is tempting to present a hypothesis which would partially account for the behavior of the data with respect to the cortisone and x-radiated series. The Schneider-TCA procedure differentiates between "cold acid-soluble" nucleotides and "cold acid-insoluble" nucleic acids. It is possible that some agent present in the livers of the cortisone-treated and x-radiated animals caused a depolymerization of DNA to small nucleotides, but only to the extent that the "missing" DNA became cold acid-soluble but not lost to the cell. One may assume thus a set of conditions prevailing which leads to the depolymerization of some DNA during periods of apparent loss and repolymerization on days showing apparent increase. A repolymerization of DNA in these animals with an ensuant increase in DNA/

[8] Changes in numbers of octaploid or higher forms would only change the position of the theoretical curve but not the conclusion that changes in the frequency of polyploid cells are consistent with the data.

nucleus, but no significant change in uptake of P^{32} would account for the experimental observations.

Finally, it should be noted as very significant that cortisone treatment prevented the rise in DNA per nucleus seen on days 4 and 5 of the x-radiated series. This effect could have been due to a simple delay in the rise normally seen. Alternately, if the recovery from x-radiation represents simple repolymerization, cortisone seems to have prevented this phenomenon.

SUMMARY AND CONCLUSIONS

Rats were treated with cortisone, x-radiation, and both agents in combination, and the effect noted on the DNA content of hepatocytes. Nuclei were enumerated both in whole liver homogenates and following isolation. The incorporation of P^{32} into DNA was also studied in relation to these agents. The following observations were made:—

1. The DNA content of nuclei fell both during cortisone administration and following x-radiation. In the former instance, the fall was progressive with continuing administration of hormone; in the latter instance, there was a return to normal 5 days after radiation.

2. Cortisone administration to x-radiated rats caused a fall in DNA/nucleus and prevented the return to normal at 5 days.

3. There was no evidence that the effects of cortisone and x-rays were additive in reducing DNA/nucleus.

4. These data indicate an alteration in DNA/nucleus, but simple changes in ploidy cannot be excluded. Either explanation requires that the agents used affect the DNA of non-regenerating nuclei.

5. Cortisone interfered with the incorporation of P^{32} into the DNA of regenerating liver. Only a small effect on DNA synthesis in resting liver was observed with cortisone or x-radiation.

6. DNA content of nuclei returned to normal 5 days after x-radiation and 3 days after discontinuance of cortisone. Slight increase in the incorporation of P^{32} by DNA was observed during recovery phases.

7. The hypothesis is proposed that the apparent losses and increases in DNA/nucleus were due to depolymerization and repolymerization of DNA. Following x-radiation and/or cortisone administration, it is proposed that some DNA is depolymerized and becomes cold acid-soluble and dissociated from organized chromatin. Later, conditions are such that this degraded DNA is repolymerized.

8. These data might be interpreted to indicate that a portion of the DNA is not essential to cell integrity; alternatively, there may be two or more species of DNA, one of which is more readily affected by the agents investigated in the present report.

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