maximal effect ascribed to ornithine in Tables 2 and 3. This change would far exceed the possible error in the control of flow rate in these experiments. It follows that the observed inducing effect of ornithine is a real one, whatever its mechanism.

§ Further support of this conclusion has been obtained in unpublished experiments of Dr. I. B. Weinstein. *E. coli* W160-37 type 1-3 was depleted of arginine, treated with chloramphenicol (200μg/ml) to prevent protein synthesis, and incubated for 5 min with C^{14}-arginine (10 μg/ml) in the absence and in the presence of an excess of unlabeled ornithine (500 μg/ml). The results showed that ornithine did not interfere with the uptake of arginine.

[1] Vogel, H. J., and B. D. Davis, *Federation Proc.*, **11**, 485 (1952).

[2] Wijesundera, S., and D. D. Woods, *Biochem. Jour.*, **55**, viii (1953).

[3] Novick, A., and L. Szilard, *Science*, **112**, 715 (1950); *Dynamics of Growth Processes* (Princeton: Princeton University Press, 1954), p. 21.

[4] Roberts, R. B., *et al.*, *Studies of Biosynthesis in Escherichia coli* (Carnegie Institution of Washington, D. C., 1955), p. 160.

[5] Gorini, L. (unpublished results).

[6] Gorini, L., and H. Kaufman, *Science*, **131**, 604 (1960).

[7] Davis, B. D., and E. S. Mingioli, *Jour. Bacteriol.*, **60**, 17 (1950).

[8] Gorini, L., *Bull. Soc. Chim. Biol.*, **40**, 1939 (1958).

[9] Gorini, L., and W. K. Maas, *Biochim. et Biophys. Acta*, **25**, 208 (1957).

[10] Pardee, A., F. Jacob, and J. Monod, *Jour. Mol. Biol.*, **1**, 165 (1959).

THE REPLICATION OF DESOXYRIBONUCLEIC ACID IN HEPATOCYTES

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The process of replication of DNA in hepatocytes in rats following partial hepatectomy has been extensively studied by biochemical methods;[1-9] however, one of the major limitations of the biochemical methods is that it has not been possible to determine the time sequence of replication of the DNA in individual hepatocytes because of the changing population of hepatocytes synthesizing DNA.

Individual hepatocytes have been followed throughout replication of the DNA content by labeling nuclei with tritiated thymidine and measuring the DNA content microspectrophotometrically at varying times after administration. Quantitative autoradiography and microspectrophotometry have given independent methods for the measurement of the relative changes of DNA during replication in the same nuclei.[10, 14, 20]

Materials and Methods.—Combined autoradiographic and cytochemical studies have been carried out on rats sacrificed between $18^1/_2$ and 26 hr after partial hepatectomy. The weights have been between 200-300 grams and the age between 4-5 months. All rats were given intravenously 50 μc of tritiated thymidine (either 1.9 or 3 curies per millimol) diluted to a volume of 0.5 cc in normal saline.

The regenerating livers were minced with scissors and mixed. Random specimens of these small pieces of liver were taken and gently pressed between cover slips, followed by immersion in liquid propane. The specimens were quickly transferred to ethyl alcohol (cooled to -78 degrees C by solid CO_2).[11] All liver speci-

mens were stained by the Feulgen technique prior to preparation of the autoradiographs.[12] Autoradiographs of the liver specimens following Feulgen staining were prepared by the stripping film technique devised by Pelc.[13]

The measurement of the DNA content of the Feulgen stained nuclei has been made by means of two wavelength microspectrophotometry.[15, 16] The Schwarzchild–Villiger Phenomenon was not considered to introduce a significant error in these microspectrophotometric measurements.[17] The measurements were expressed in relative units between the 4N and 8N amount of DNA. The ratios of either the 2N to 4N, 4N to 8N, or 2N to 8N amount of DNA were within the limits of experimental error of the DNA determinations of either the 2N, 4N, or 8N hepatic nuclei.

The procedure recommended by Mendelsohn[18, 19] has been used routinely in the measurement of the DNA content of hepatocytes measured microspectrophotometrically. The total quantity of chromophore in the nucleus was obtained by substitution of these results into the appropriate equations and tables given by Patau.[16]

The combined autoradiographic and cytochemical measurements on the same nucleus were made in the following manner. All nuclei, both labeled and unlabeled, were mapped within a field. Grains over the labeled nuclei were counted and consecutive fields were mapped until a total of approximately 25 labeled nuclei had been recorded. The silver bromide grains were removed chemically and the DNA content measured microspectrophotometrically.

Results.—The results of the experiment in which tritiated thymidine was injected shortly after the onset of synthesis ($15^1/_2$ hr) are shown in Figure 1. During the availability of tritiated thymidine from $15^1/_2$ to $16^1/_2$ hr, only 1.51 ± 1.02 per cent of the hepatocytes were found to be labeled. The animal was sacrificed at $18^1/_2$ hr and the DNA content of 25 nuclei determined. The mean DNA content of the labeled hepatocytes was 34 per cent above the 4N value for DNA at this time (Table 1).

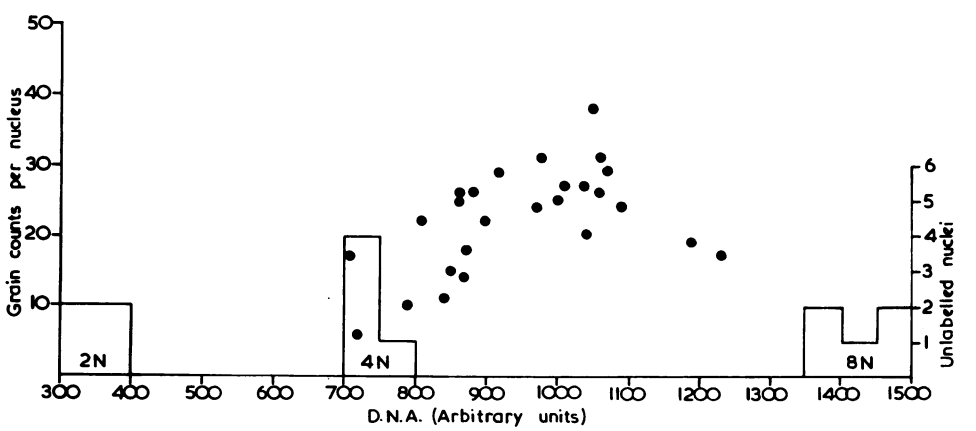

FIG. 1.—Plot of grain counts versus DNA content of the same hepatic nuclei of a rat given tritiated thymidine $15^1/_2$ hr after hepatectomy and sacrificed 3 hr later. ● labelled nuclei. □ unlabelled.

The hepatocytes in the experiment in which the tritiated thymidine was given at 17 hr, and the animal sacrificed at 23 hr, after hepatectomy is shown in Figure 1 (on page 700 of these PROCEEDINGS). Twenty-two of the 27 nuclei were

considered to be tetraploid nuclei which had doubled their DNA content to become octoploid nuclei. These results also demonstrated that 8 hr is an upper limit for the mean doubling for an hepatocyte.

Discussion.—A. Per cent of tritiated thymidine-labeled hepatocytes at different times after hepatectomy: It has been previously shown that the per cent of hepatocytes labeled with tritiated thymidine gradually increases from the onset of DNA synthesis at 15 hr until 20 hr after hepatectomy.[14] No labeled hepatocytes were found in either a normal rat or 2 rats injected at 14 hr after hepatectomy. The increase in the labeled hepatocytes between 15 and 20 hr after hepatectomy is approximately 4 per cent per hr. Between 20 hr and the onset of mitosis at 24 hr after hepatectomy, there is a decrease of labeled hepatocytes. This indicates that the initial group of hepatocytes which begin DNA synthesis at 15 hr after hepatectomy completes synthesis between 20 to 23 hr after hepatectomy. This also indicates that DNA synthesis and mitotic division occur in cycles, an observation first made by Ponfick in 1889.

B. *The mean time for replication of the DNA content of hepatocytes:* There is a large body of indirect information concerning the time for replication of the DNA in the hepatocytes of regenerating liver. It is well established that DNA synthesis begins approximately 15 hr after hepatectomy and mitosis begins at approximately 24 hr after hepatectomy and reaches a peak approximately

TABLE 1

CHANGE IN DNA CONTENT WITH TIME AFTER HEPATECTOMY MEASURED MICROSPECTROPHOTOMETRICALLY IN TRITIATED THYMIDINE LABELED-HEPATOCYTES

	Unlabeled Nuclei				Labeled Nuclei		
	Time after hepatectomy (hr)	2N	4N	8N	Mean Content 25 Nuclei	S.D.	D*
21.7.59 (A)	$15\frac{1}{2}$–$18\frac{1}{2}$ (C)	347	735	1447	975	±115	0.34
17.3.59 (B)	17 –20 (C)	265	636	1146	963	±229	0.64
21.7.59 (B)	17 –23 (C)	314	692	1223	1317	±82	1.20
24.4.59	20 –$20\frac{1}{2}$ (C)	184	389	725	641	+77	0.75

* Mean DNA content of labeled nuclei expressed as a fraction between the 4N and 8N amount of DNA.

4 hr later. Little precise information about the time of replication of an hepatocyte can be obtained from this indirect evidence because of the inability to separate the DNA synthesis in hepatocytes in different cycles.

It was not possible to determine if the labeled tetraploid nuclei were diploid nuclei which had completed synthesis or if they were tetraploid nuclei beginning synthesis However, it was shown by Naora[21] that 83.4 per cent of the hepatocytes were tetraploid in rats weighing 200 grams or more. The results of this study agree with the observations of Naora. Furthermore, the small nonparenchymal cells were not found to synthesize DNA during the period under consideration, and no octoploid hepatocytes were found to be labeled. It can therefore be assumed that synthesis of DNA is primarily confined to tetraploid hepatocytes in the rats used in this study.

The results of this and other experiments have shown that the DNA content is doubled prior to division. The following method has been discussed to express the mean DNA content of the synthesizing hepatocytes in terms of the fraction of the content between the 4N and 8N amount of DNA.

L = mean content of labeled hepatocytes
8N = DNA content of octoploid hepatocytes
4N = DNA content of tetraploid hepatocytes
D = DNA content of labeled hepatocytes expressed as fraction of the content between 4N and 8N

The following equation expresses the changes in mean DNA content of the labeled nuclei from 4N to 8N as a change from 0 to 1 in relative units:

$$D = \frac{L - 4N}{8N - 4N}.$$

The results of the combined autoradiographic and cytochemical studies, in which the DNA content is expressed in terms of the fraction of the DNA content between 4N and 8N, are given in Table 1.

Estimates have been made of the time required for the DNA content of an hepatocyte to be doubled from the results of these experiments. The estimates have been made in the following way:

D = DNA content of labeled nuclei expressed as a fraction between the 4N and 8N amount of DNA
S = time after the onset of DNA synthesis at 15 hr until the sacrifice of the animal
T = time in hr for doubling of the DNA content of an hepatocyte.

Therefore,

$$\frac{D}{S} = \frac{1}{T}; \quad T = \frac{S}{D}.$$

For an example, in the 17-to 20-hr experiment, see Table 2.

$$D = 0.64$$
$$S = 20 - 15 = 5 \text{ hr}$$
$$T = \frac{5}{0.64} = 7.4 \text{ hr}$$

TABLE 2

ESTIMATIONS FOR TIME FOR DOUBLING OF THE DNA CONTENT OF AN HEPATOCYTE

No.	Date	Time after hepatectomy (hr)	Time after onset of synthesis at 15 hr (hr)	D*	Estimated doubling time (hr)
1	21.7.59 (A)	$15^1/_2$–$18^1/_2$	3.5	0.34	10.3
2	17.3.59 (B)	17 –20	5	0.64	7.8
3	21.7.59 (B)	17 –23	8	1.20	8.0
4	24.4.59	20 –$20^1/_2$	5.5	0.75	7.4

* DNA content of labeled nuclei expressed as a fraction between the 4N and 8N content.

These estimates are based on the assumption that the mean change in DNA content for 25 nuclei is constant. This is specifically pointed out to differentiate it from the autoradiographic results of individual nuclei in which a changing rate of synthesis was found. It was shown from both the autoradiographic and the combined autoradiographic and cytological results that DNA synthesis in regenerating liver is not synchronized. The change in the mean DNA content of the hepa-

tocytes synthesizing DNA measured microspectrophotometrically is in essence the integration of the rate of synthesis over the period under consideration. By assuming that the change in the mean DNA content of the population is constant, a reasonably good approximation of the doubling time of DNA of an hepatocyte is obtained. This is borne out by the experimental results shown in Figure 3 of Part II. It should be noted that a straight line can almost be fitted to the experimentally determined value of the mean DNA content of labeled hepatocytes at the different times after hepatectomy. The estimates of the time for replication of the DNA of an hepatocyte from the data of different experiments is given in Table 2.

It would be expected that the estimate of the doubling time based on the results obtained at the beginning of synthesis would be high since the rate of synthesis is lower at this time than at later times when the rate of synthesis has increased. Estimates based on the $15^1/_2$ to $18^1/_2$ hr experiment gave a doubling time of 10.3 hr.

The hepatocytes in the 17 to 23 hr experiment have doubled their DNA content. This gives an upper limit of DNA doubling time of 8 hr which is based directly on experimental results. Estimates based on both the 17 to 20 hr and the 20 to $20^1/_2$ hr experiments are in close agreement with the doubling time based directly on the experimental results above. The estimated doubling time for the labeled hepatocytes of these two experiments was 7.8 and 7.4 hr respectively.

It was shown that by 20 hr in the combined autoradiographic and cytochemical study that some of the hepatocytes had completed synthesis. The histogram of the grain counts per nucleus in the 20 to $20^1/_2$ hr experiment show therefore nuclei in all stages of synthesis. The shape of the histogram should also reflect the duration of the changing rates of synthesis of an hepatocyte in terms of the fraction of time necessary for DNA doubling. Since the histogram of the log of the grain counts per nucleus can be fitted to a normal curve, a normal curve with the same mean and standard deviation as the mean and standard deviation for the log of the grain counts per nucleus has been used as the model for the changing rates of DNA synthesis.

The general equation for the normal distribution is as follows: The frequency in any infinitesimally small range, dx, can be expressed as

$$df = \frac{1}{\sigma\sqrt{2\pi}} e^{-1/2 \cdot \frac{(x-\mu)^2}{\sigma^2}} dx$$

μ = mean of the distribution
σ = standard deviation
x = distance of the observation from the mean.

The following information about the log of the grain counts per nuclei for the control of the 20 to $20^1/_2$ hr experiment

μ_1 = the mean of the log of the grain counts per nucleus = 1.767
σ_1 = ± 0.225.

Substitution of the above information into the general equation for the normal distribution gives the following differential equation:

$$df = \frac{1}{\pm 0.225\sqrt{2\pi}} e^{-1/2} \frac{(x - 1.767)^2}{(0.225)^2} dx.$$

Since the histogram of the log of grain counts per nucleus can be fitted to a normal curve, the same curve can be used as the model for the changing rates of DNA synthesis. The time axis is superimposed on that of log grain counts, the 8 hr for replication covering that part of the axis within which readings fall 95 per cent of the time.

Transforming these values to the time scale, t = time in hours of the observation from the midpoint of the time for replication (8 hr).

The new standard deviation σ_2 is obtained from the knowledge that $\pm 1.96 \times$ S.D. will give values in which readings will fall 95 per cent of the time.

Therefore,

$$1.96 \times \sigma_2 = 4$$
$$\sigma_2 = 2.041.$$

By substitution into the general equation for the normal curve, the rate of DNA synthesis becomes

$$dR = \frac{1}{2.041\sqrt{2\pi}} e^{-1/2} \left(\frac{t}{2.041}\right)^2 dt,$$

where

t = time in hr
R = rate of DNA synthesis
σ_2 = 2.041 = new standard deviation
μ_2 = mean = 0.

A model for the changing rate of synthesis during the doubling of the DNA content of an hepatocyte is shown in Figure 2. This model was constructed by

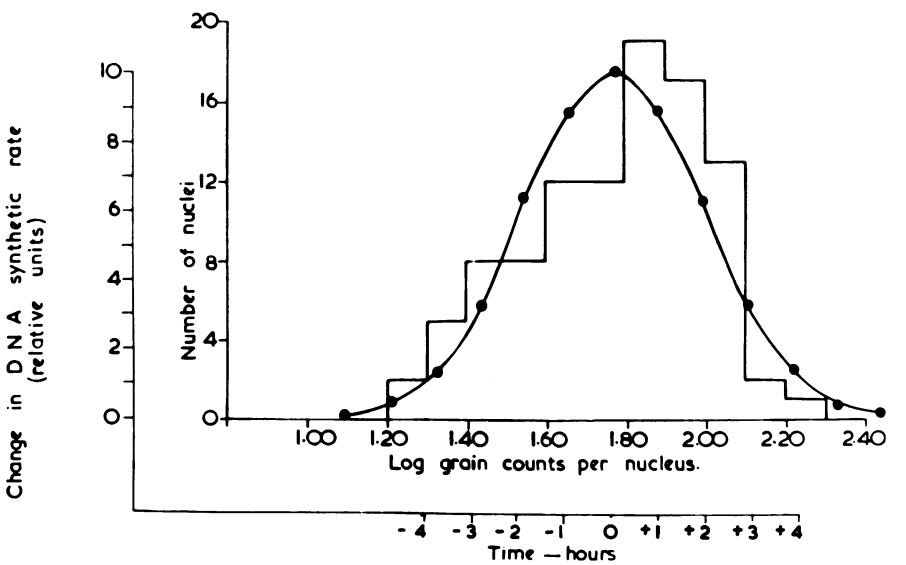

FIG. 2.—Model for the doubling of the DNA content of an hepatocyte (from histogram of log of grain count distribution and superimposed normal curve).

superimposing a normal curve with the same mean and standard deviation of the log of the grain counts per nucleus of the 20 to $20^1/_2$ hr experiment. Upon integration of the equation for the rate of DNA synthesis of an hepatocyte, the DNA content at time "t" is as follows:

$$\text{DNA (content)} = \int_{-4}^{t} \frac{1}{2.04\sqrt{2\pi}} e^{-1/2\left(\frac{t}{2.04}\right)^2} dt.$$

The values for the DNA content were obtained in the following manner. The results obtained experimentally from the autoradiographic studies have been used to determine the theoretical DNA content at time "t" during the period of replication:

$$t = \frac{x - \mu_1}{c},$$

where c is a conversion factor between the standard deviation for the log of the grain counts per nucleus σ_1 and the new standard deviation σ_2.

$$c = \frac{\sigma_1}{\sigma_2} = \frac{0.225}{2.041} = 0.110.$$

Therefore,

$$t = \frac{x - 1.767}{0.110}.$$

Upon substitution of these values into the preceding equation for DNA content and integrating, the following theoretical values of the DNA content have been obtained for different times during the 8-hr period of replication.

TABLE 3

THEORETICAL VALUES FOR DNA CONTENT

Time (hr)	Theoretical DNA Content*
−4	0.0250
−3	0.0683
−2	0.1610
−1	0.3096
0	0.4750
+1	0.6854
+2	0.8340
+3	0.9266
+4	0.9500

* Expressed as a fraction of the DNA content between 4N and 8N.

The theoretical curve for the change in DNA content during the period of replication is shown in Figure 3. Note the close proximity of the values for the DNA content determined experimentally to the theoretical curve. Thus, the predicted values for the DNA content obtained by integration of the curve for the changing rate of DNA synthesis from autoradiographic results agree with the experimental values for DNA content determined by the cytochemical measurement of the labeled hepatocytes at different times during replication.

The doubling time for the first 4 experiments was estimated based on the assump-

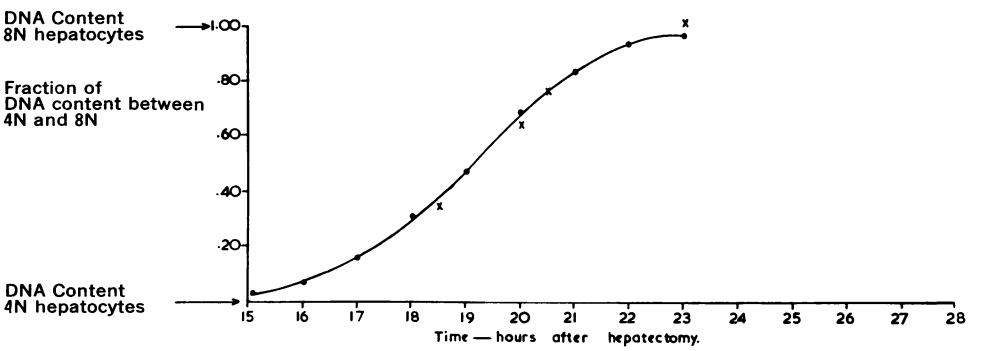

FIG. 3.—Change in DNA content with time after hepatectomy measured microspectrophotometrically in tritiated thymidine-labelled hepatocytes. ●—● theoretical curve for change in DNA content between 4N and 8N. × DNA content determined experimentally.

TABLE 4

ESTIMATIONS OF DNA DOUBLING BASED ON AN ASSUMED EXPONENTIAL CHANGE IN DNA CONTENT DURING REPLICATION

No.	Time after hepatectomy	Estimated DNA Content* Assuming 8 hr doubling time	Estimated DNA Content* Assuming 9 hr doubling time
1	$15^1/_2$–$18^1/_2$	1.12	0.91
2	17 –20	1.08	0.88
3	17 –23	1.00†	...
4	20 –$20^1/_2$	0.99	0.85

* DNA content of labeled nuclei expressed as a fraction between 4N and 8N amount.
† This value was determined experimentally.

tion that the change in DNA content during replication was exponential rather than linear.

The estimates of the DNA content at 8 hr obtained from substituting the values of the DNA content into the equation for the normal curve given previously and integrating are shown in Table 4. Note that all of the values are close to the anticipated value of one. In order to check further the reliability of these results it was assumed that the time for doubling was 9 hr rather than 8 hr. The values obtained by substitution into the equation for the normal curve are shown in the last column of Table 4. Since the values of the DNA content are all less than one it is evident that assuming an 8 hr doubling time more closely fits the experimental results than assuming a 9-hr doubling time.

Summary and Conclusions.—It has been found that the histogram for the log of the grain counts per hepatic nucleus could be fitted to a normal curve with the same mean and standard deviation. The changing rate of DNA synthesis of an hepatocyte during replication has therefore been expressed as an exponential function.

It has been possible to follow individual nuclei throughout the period of replication by labeling the nuclei with tritiated thymidine and measuring the DNA content of the labeled hepatocytes microspectrophotometrically at different times after tritiated thymidine administration. The experimental results from the cytochemical studies have been fitted to a curve from an integrated equation for changing rates of DNA synthesis. Thus, the theoretical curve predicted from the autoradiographic results agree with the experimentally determined cytochemical results. The mean time for replication of the DNA content of an hepatocyte was found to be 8 hr.

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[1] Higgins, G. M., and R. M. Anderson, *Arch. Path.*, **12**, 186 (1931).

[2] Davidson, J. N., and C. Waymouth, *Biochem. J.*, **38**, 375 (1944).

[3] Novikoff, A. B., and V. R. Potter, *J. Biol. Chem.*, **173**, 223 (1948).

[4] Price, J. M., and A. K. Laird, *Cancer Research*, **10**, 650 (1950).

[5] Holmes, B. E., and L. K. Mee, *Radiobiology Symposium*, 220, Leige (1954), ed. A. M. Bacq and P. Alexander (London: Butterworth Scientific Publ., 1955).

[6] Nygaard, O. F., and H. P. Rusch, *Cancer Research*, **15**, 240 (1955).

[7] Mirsky, A. E., in *Proc. III International Congress Biochemistry*, ed. C. Lie-Bacq (New York: Academic Press, 1956), p. 349.

[8] Beltz, R. E., J. Van Lancker, and V. R. Potter, *Cancer Research*, **17**, 688 (1957).

[9] Davidson, J. N., "Chemistry of the Liver Cell," *Brit. med. Bull.*, **13**, 77 (1957).

[10] Looney, W. B., Dissertation for Ph.D. Degree, University of Cambridge, England, November, 1959.

[11] Simpson, W. L., *Anat. Rec.*, **80**, 173 (1941).

[12] Lillie, R. D., *Histopathologic Technic and Practical Histochemistry* (New York: Blakiston, 1954).

[13] Pelc, S. R., "Autoradiographic Technique," *Nature*, **160**, 749 (1947).

[14] Looney, W. B., *Proceedings of the IXth International Radiological Congress* (Munich: Georg Thieme, Verlag (in press).

[15] Ornstein, L., *Lab. Invest.*, **1**, 250 (1952).

[16] Patau, K., *Chromosoma*, **5**, 341 (1952–53).

[17] Naora, H., *Science*, **114**, 279 (1951)

[18] Mendelsohn, M. L, Dissertation for Ph.D. Degree, University of Cambridge, Cambridge, England, 1957.

[19] Mendelsohn, M. L., *J. Biophys. Biochem. Cytol*, **4**, 407 (1958).

[20] Looney, W. B., in *Faraday Society Conference on the Cell Nucleus*, Ed. B. E. Holmes (London: Butterworth Scientific Publ., 1960).

[21] Naora, H., *J. Biophy. Biochem. Cytol.*, **3**, 949 (1957).

THE EFFECT OF IRRADIATION ON THE REPLICATION OF DESOXYRIBONUCLEIC ACID IN HEPATOCYTES

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By 1925 it was generally accepted that the cell nucleus was more sensitive to the effects of irradiation than the cytoplasm.[1] The discovery by Muller[2] in 1927 that X rays could produce gene mutations firmly established the importance of the effects of irradiation on the genetic material in the nucleus. Mitchell[3, 4] and Euler and Hevesy[5] were first to show that irradiation would produce disturbances in nucleic acid metabolism.

Additional results suggesting that irradiation directly affects DNA synthesis were reported by Holmes,[6] Hevesy,[7] Skipper and Mitchell,[8] Pelc and Howard,[9] Klein and Forssberg,[10] Vermund, Barnum, Huseby, and Stenstrom,[11] Bennett, Kelly, and Kreukel,[12] Lajtha, Oliver, and Ellis,[13] Looney,[14, 15] Lajtha, Kumatori, Oliver, and Ellis,[16] Holmes and Mee,[17] Beltz, Van Lancker, and Potter,[18] and