during the DNA synthetic phase of the generative cycle, but that a certain spacing in time exists (*15*). The duration of the synthesis of a single chromosome probably corresponds to about one-third or one-half of the total duration of duplication of the genome of the cell (*16*). An increased synchrony would, therefore, result in a reduction of the total time a cell spends in synthesizing DNA.

The ovarian hormones clearly influence the duration of the process of DNA synthesis. Therefore, the simplest interpretation of the variation in the length of this process in mammary epithelium of different intact animals taken at random in the experiment is that this is a consequence of differences in the endocrine titer among mice due to the variable rhythm of the ovarian secretion. As shown in Table 1, the variation in the duration of DNA synthesis is decreased in ovariectomized animals under steady hormonal treatment with 17-β-estradiol and progesterone. This result supports the previous hypothesis.

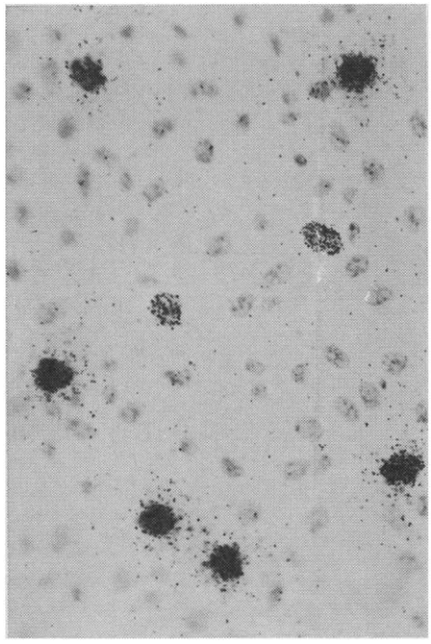

Fig. 1. Radioautography of a "squash" preparation of mammary alveolar cells from a C3H/HeJ female mouse injected first with thymidine-H^3 and then with thymidine-C^{14}. The nuclei containing C^{14} show a spray of β-tracks on several planes (mean energy of C^{14}-β-rays = 49 kev; mean distance traveled, 50 μ), while the nuclei containing only H^3 show photographic grains in their immediate vicinity and on one plane only (mean energy H^3-β-rays = 5.7 kev; mean distance traveled, 1 μ) and thus can be differentiated. Magnification, × 400.

Within the system of the mammary gland, the process of DNA synthesis has proved to be extremely variable in duration. Ovarian hormones are able not only to initiate the process of cell division, but also to accelerate at least a step of this process—that is, the duplication of DNA. The hypothesis of "constancy" of duration of DNA synthesis and of the "doubling sequence" in general, with variation in generation time dependent entirely upon variation of the G_1 phase, appears therefore to be undoubtedly disproved.

The fact remains that most of the cell systems so far studied have a duration of DNA synthesis close to a low of 7 to 8 hours. One might postulate that in these cell systems the duration of DNA synthesis is close to, or has reached, a "minimum" of fairly constant value for different cell types in a given species. This is suggested by the observations that, as shown in Table 1, steady treatment of animals with large doses of hormones decreases variation and clusters the values of duration of DNA synthesis around the low average time of 10.7 hours and that the lowest values of duration of DNA synthesis in cells of the mammary gland are close to those most commonly found in other cell systems of the mouse. A "minimum" duration of DNA synthesis is expected on a theoretical basis. As the quantity of DNA to be synthesized appears to be essentially the same in different cell types of the same species, duplication of DNA at maximum speed should essentially last the same "minimum" time. A certain variance in the duration of this process among different cell types could be expected as a result of difference in synchrony of chromosome duplication. On the other hand, influences or lack of influences on cell metabolism, as for example by hormones, can possibly lengthen the process of DNA duplication without any theoretical expected "maximum." The hypothesis of "minimum" duration appears to give a satisfactory interpretation of all facts known up to now. That is, many cell systems appear to have virtually the same duration of DNA synthesis, while this process is variable in length and can last significantly longer in other cell types in the same animal species.

FRANCESCO BRESCIANI

Biology Department, Brookhaven National Laboratory, Upton, New York, and *Institute of General Pathology, University of Naples, Naples, Italy*

References and Notes

1. A. Howard and S. R. Pelc, *J. Exptl. Cell. Res.* **2**, 178 (1951); in *Isotopes in Biochemistry*, CIBA Foundation Conf. (Blakiston, Philadelphia, 1951), p. 138; *Heredity Suppl.* **6**, 261 (1953).
2. Terminology after Howard and Pelc (*1*). A recent analysis of the cell generative cycle is given by H. Quastler in *Cell Proliferation*, L. F. Lamerton and R. J. Fry, Eds. (Davis, Philadelphia, 1963), p. 18. It should be mentioned that the abbreviation G in the nomenclature of the cell cycle stands for gap—"slanderous appellation, since gap exists only in the investigator's knowledge, not in the activity of the cell"—H. Quastler, in *Chemical and Biological Effects of Radiations*, M. Haissinsky, Ed. (Masson, Paris, 1962), p. 147.
3. P. Reichard and B. Estborn, *J. Biol. Chem.* **188**, 839 (1951); M. Freidkin, D. Tilson, D. Roberts, *ibid.* **220**, 627 (1956).
4. W. L. Hughes, V. P. Bond, G. Brecher, E. P. Cronkite, R. B. Painter, H. Quastler, F. G. Sherman, *Proc. Natl. Acad. Sci. U.S.* **44**, 476 (1958).
5. H. Quastler and F. G. Sherman, *Exptl. Cell Res.* **17**, 420 (1959); C. Pilgrim and W. Maurer, *Naturwissenschaften* **49**, 544 (1962).
6. D. E. Wimber and H. Quastler, *Exptl. Cell Res.* **30**, 8 (1963).
7. E. Koburg and W. Maurer, *Biochim. Biophys. Acta* **61**, 229 (1962).
8. V. Defendi and L. A. Manson, *Nature* **198**, 359 (1963).
9. S. M. Cattaneo, H. Quastler, F. G. Sherman, *Radiation Res.* **12**, 587 (1960); *Nature* **190**, 923 (1961); C. Pilgrim and W. Maurer, *Naturwissenschaften* **49**, 544 (1962).
10. W. S. Bullough, *Nature* **199**, 859 (1963).
11. ——— and E. B. Laurence, unpublished results, cited in (*10*).
12. F. G. Sherman, H. Quastler, D. R. Wimber, *Exptl. Cell Res.* **24**, 114 (1961).
13. H. Quastler and F. G. Sherman, *ibid.* **17**, 420 (1959); E. Koburg and W. Maurer, *Biochim. Biophys. Acta* **61**, 229 (1962); R. N. Staroscik, W. H. Jenkins, M. L. Mendelsohn, *Nature* **202**, 456 (1964); M. Wolfsberg, *Exptl. Cell Res.*, in press.
14. D. E. Wimber, H. Quastler, O. L. Stein, D. R. Wimber, *J. Biophys. Biochem. Cytol.* **8**, 327 (1960).
15. A. Lima de Faria, *ibid.* **6**, 457 (1959); J. H. Taylor, *ibid.* **7**, 455 (1960); D. E. Wimber, *Exptl. Cell Res.* **23**, 402 (1961); J. L. German, *Trans. N.Y. Acad. Sci.* **24**, 395 (1962); L. Atkins, P. D. Taft, P. K. Dalal, *J. Cell. Biol.* **15**, 390 (1962).
16. P. S. Moorhead and V. Defendi, *J. Cell. Biol.* **16**, 202 (1963).
17. Research carried out at Brookhaven National Laboratory under the auspices of the AEC.

4 September 1964

Antibody Synthesizing Cells: Appearance after Secondary Antigenic Stimulation in vitro

Abstract. *Suspensions of spleen cells from rabbits immunized with sheep erythrocytes can be stimulated to produce antibody-synthesizing cells in vitro. The cellular response is antigen specific.*

Spleen and lymph node suspensions and slices (*1*) taken from animals that had received in vivo a secondary stimulation by antigen have been shown to synthesize antibody in vitro. After secondary antigenic stimulation in vitro lymph node and spleen fragments synthesize antibody (*1, 2*), and antibody-containing cells appear in lymph node fragments (*3*). We report here on the

Table 1. Antibody-forming cells per 10^7 spleen cells after antigen stimulation in vitro. The results show the number of plaques (cells producing antibody) after the cells had been cultured for the number of days indicated.

Day 2	Day 4	Day 5	Day 6	Day 7	Day 8
		No antigen			
	14	12	27	7	
	4	9	32	6	
		20	50		
			59		
Av.	9	14	42	7	
	Antigen present throughout				
12	6	4240	4090	283	81
	4	4960	1750	336	316
	15	TNTC*	2300	690	
		TNTC	1620	757	
Av.	8	4600	2440	519	199
	Antigen not replaced on changing medium				
11	8	TNTC	1470	47	90
	9	TNTC	1490	676	87
	5		760	596	9
			1000	467	12
Av.	7		1180	449	50

* TNTC, too numerous to count, entire tube plated.

use of the Jerne plaque counting technique (*4*) to show that antibody-forming cells appear in suspensions of spleen cells from 4 to 6 days after in vitro stimulation with an antigen.

Rabbits were immunized by intravenous injections of 3 ml of a 1 percent suspension of washed erythrocytes on day 1, and four times a week from day 8 to 26. Six months after the last injection the rabbits were killed and spleen cell suspensions were prepared and incubated in a modified Eagle's tissue culture medium (*5*). Replicate cultures containing 1×10^7 spleen cells in 1 ml of medium were incubated in the presence or absence of 4×10^6 sheep erythrocytes, the stimulating antigen. One half of the medium was carefully replaced every 2nd day without disturbing the cells. Control and antigen-stimulated cultures were harvested at the times indicated, and the number of antibody-forming cells was determined (*4*). The spleen cells were washed once, and the entire culture, or a portion of it on days 4 and 6, was suspended in 0.7 percent agar prepared with Eagle's medium to which sheep erythrocytes had been added at 45°C. This mixture was poured over a supporting layer of 1.4 percent agar in a 9-cm petri dish. The plates were incubated for 3 hours in an atmosphere of 5 percent carbon dioxide in oxygen, before the plaques were developed with complement (a 1 to 5 dilution of guinea pig serum). The plates were incubated for 30 minutes more, and then they were stained with benzidine. That an antibody-forming cell was present was indicated by a clear zone of lysed sheep erythrocytes around the cell. The plaques were counted under magnification (25 ×) with a binocular microscope.

Table 2. Specificity of antigenic stimulus (erythrocyte antigen) to the development of antibody-forming cells (measured with sheep erythrocytes as indicator cell in agar, day 5). The results are given as the number of plaques arising from spleen cells stimulated on day 1 with erythrocytes from the species indicated.

None	Sheep	Cow	Man	Chicken
18	6080	39	8	262
9	4470	92	69	46
12	3080	84	32	181
	154	90	7	397
	289	167	16	437
		Average		
13	2800	94	26	265

The number of plaques in control cultures remained in the range 4 to 59 per 10^7 cells throughout the incubation period (Table 1). In contrast, up to 5000 plaques per 10^7 cells appeared on days 5 and 6 in cultures which were incubated with antigen. The number of plaques had fallen again by days 7 and 8. A somewhat smaller number of plaques appeared if the antigen was not replaced on changing the medium.

The specificity of the antibody in plaque-forming cells was examined with erythrocytes from several other species as indicator cells. Many more plaques appeared on plates containing sheep erythrocytes; with sheep, cow, human, and chicken erythrocytes there were 519, 77, 16, and 24 plaques, respectively. The cell suspensions always contained a few cells producing antibodies against either cow, human, or chicken red blood cells in spite of the absence of known previous antigenic stimulation. Possibly these cells are responsible for the presence of the antibodies against foreign red blood cells in normal serum. Or it may represent the cross reactivity of antibody to sheep erythrocytes with the erythrocytes of other species.

The specificity of the response is demonstrated in Table 2. Human and bovine erythrocytes are much less effective antigens than sheep erythrocytes for eliciting the secondary response as measured with sheep erythrocytes as indicator cells. Chicken erythrocytes stimulated the appearance of more cells producing antibody capable of lysing sheep erythrocytes than did human and bovine erythrocytes.

The results show that completely in vitro a secondary response can be obtained with spleen cell suspensions. It has been previously shown, under identical conditions, that the addition of soluble antigens to spleen cell suspensions from immunized rabbits stimulated DNA synthesis and cell division (*5*). In our experiments a similar response was obtained when sheep erythrocytes were added to spleen cell suspensions from rabbits immunized with this antigen. Two days after addition of antigen, 1 to 2 percent of the cell population were dividing. On days 4 to 6, 0.05 percent of the cells were synthesizing or already contained antibody. No conclusion can be drawn concerning the relation between the number of dividing cells at day 2 and the antibody-forming cells at days 4 to 6, for two reasons: (i) the efficiency of the plaque-counting technique for counting antibody-forming cells is not known; and (ii) the culture conditions may not provide an adequate environment for the survival of more than a fraction of dividing cells up until day 6.

The results lend additional support to the thesis that the antigen-dependent stimulation of DNA synthesis (*5*) represents the proliferative phase of the secondary immunological response.

The fact that antibody-forming, or antibody-containing, cells do not appear until a considerable time after many cells can be shown to be proliferating in response to antigen suggests that maturation of the dividing cells must occur before antibody is synthesized in any quantity.

MARVIS RICHARDSON
Department of Microbiology and Public Health, Michigan State University, East Lansing
RICHARD W. DUTTON
Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California

References and Notes

1. For references, see A. B. Stavitsky, *Advan. Immunol.* **1**, 211 (1961).
2. M. C. Michaelides and A. H. Coons, *J. Exptl. Med.* **117**, 1035 (1963).
3. T. F. O'Brien, M. C. Michaelides, A. H. Coons, *ibid.* p. 1053.
4. N. K. Jerne and A. A. Nordin, *Science,* **140**, 405 (1963); N. K. Jerne, A. A. Nordin, C. Henry, in *Cell-bound Antibodies,* B. Amas and H. Koprowski, Eds. (Wistar Institute Press, 1963), p. 109.
5. R. W. Dutton and J. D. Eady, *Immunology,* **7**, 40 (1964).
6. Publication No. 74 from the Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California, and No. 3381 from the Michigan Agricultural Experiment Station. Supported in part by a USPHS grant to R.W.D. and a NSF grant to M.R.; in part by a USPHS fellowship (ESP-223-R1) to M.R.

3 August 1964