not be present in the rat. In either case, however, the rat possesses the capacity for precise lunar-day and solar-day timing. Since the period of an overt rhythm which deviates from a natural one is known in the mammal (8) to be a function of constant illumination level, it is clear that such unnatural periods must be derived within the exogenously rhythmic organism in reaction with its environment. It has been pointed out(1,7) that all the evidence at hand is compatible with an hypothesis that the mechanism of their deviation involves the well-known phasing phenomenon, utilizing the demonstrated daily rhythm of sensitivity to light and temperature as phasing agents(9,10) to effect regular daily autophasing in constant conditions.

Summary. 1. A white rat displayed in constant low illumination a rhythm of spontaneous running of precise lunar-day frequency over 60-day study. This obviously resulted in accurate monthly recurrence of daily running patterns. 2. Activity was high during hours the moon was below the horizon, and low when above. Spontaneous running at first lunar hour was 6 times that at 11th hour. Correlation between average lunar-day cycles for 2 consecutive months was $+0.70 \pm 0.11$, and mean cycle for each month was quite similar to earlier published lunar-day cycle in the rat. 3. Superimposed upon the dominant lunar-day periodism were minor, transient, solar-day patterns of locomotor activity, terminating spontaneously or through apparent submergence in the larger amplitude, lunar-day cycles. 4. Since these 2 basic periodisms in rat activity, solar-day and lunar-day, are the same frequencies as the widely, and probably universally, distributed exogenous metabolic periodisms, they are considered simple behavioral expressions of a fundamental exogenous "clock-system" from which regular physiological rhythms of other than natural frequencies, often present, may be derived by the organism.

1. Brown, F. A., Jr., Shriner, J., Ralph, C. L., *Am. J. Physiol.*, 1956, v184, 491.
2. Park, O., Woods, L. P., PROC. SOC. EXP. BIOL. AND MED., 1944, v43, 366.
3. Brown, F. A., Jr., *Biol. Bull.*, 1958, v115, 81.
4. Brown, F. A., Jr., Bennett, M. F., Webb, H. M., *Proc. Nat. Acad. Sci.*, 1958, v44, 290.
5. Brown, F. A., Jr., Webb, H. M., Brett, W. J., *Gunma J. Med Sci.*, 1959.
6. Webb, H. M., Brown, F. A., Jr., *Biol. Bull.*, 1958, v115, 303.
7. ———, *Physiol. Rev.*, 1959, v39, 127.
8. Johnson, M. S., *J. Exp. Zool.*, 1939, v82, 315.
9. Webb, H. M., *Physiol. Zool.*, 1950, v23, 316.
10. Stephens, G. C., *ibid.*, 1957, v30, 55.

Received April 16, 1959. P.S.E.B.M., 1959, v101.

Autoradiographic Studies of Antimitotic Action of Maleuric Acid.* (24980)

JESSE SISKEN, TADASHI A. OKADA AND EUGENE ROBERTS
Depts. of Biochemistry and Exp. Pathology, Medical Research Inst., City of Hope Medical Center, Duarte, Calif.

Maleuric acid (monomaleylurea) produces mitotic abnormalities and depresses mitotic index both in plant and animal cells(1).† In our study an analysis was made of mode of action of this compound on Ehrlich ascites tumor cells. By observing distribution of incorporated tritiated thymidine (T-H^3) in mitotic and interphasic cell populations using high resolution autoradiographic technics, it has been possible to show that administration of maleuric acid (MA) results in marked inhibition of the incorporation of T-H^3 into deoxyribonucleic acid (DNA) and, in addition, prevents progression of interphase cells into mitosis.

Methods. A sample of MA was obtained

* This investigation supported by research grants from Nat. Cancer Inst., U.S.P.H.S.

† LD_{50} in normal mice for single intraperitoneal injection was approximately 775 mg/kg and single daily doses of 150 mg/kg were tolerated for 8 days with no loss in weight or sign of toxicity.

from Naugatuck Chem. Division, U.S. Rubber Co., Naugatuck, Conn. On 4th day after transplantation of approximately 5 x 10^6 Ehrlich ascites tumor cells into C57 black mice averaging 20 g in weight, mice were injected intraperitoneally with 33 μc of T-H^3 (Schwartz Laboratories specific activity 360 mc/mM) in 0.1 ml saline. Single doses of 5 mg of MA in 0.5 ml saline were administered at times indicated below for individual experiments. Samples of tumor cells removed by peritoneal puncture were squashed with acetic dahlia for cytological examination or smeared for preparation of autoradiographs. The dried smears were fixed in absolute ethanol 5 minutes, brought to water through graded alcohol series, hydrolyzed in 1N HCl at 60°C 8 minutes, and treated with Feulgen stain. After appropriate bleaching and washing, the autoradiographic film was applied according to Taylor and McMaster(2) modification of stripping film technic. After 14-16 days of exposure, the autoradiographs were processed according to schedule of Taylor(3) and mounted in immersion oil. A nucleus was considered to be labeled if there were 6 or more grains above background level in the photographic emulsion over it. Since background generally amounted to 1 to 2 grains in an area as large as that overlying a nucleus, this criterion of labeling might tend to make our estimate of percentages of labeled cells slightly low. On the other hand, the level of labeling was such that the number of borderline cells was small in samples studied.

Results. As shown previously(1), MA produces marked cytoplasmic damage in all cells and chromosomal abnormalities characterized by clumping and stickiness (Fig. 1, normal cells; Fig. 2, treated cells). Photographs of typical results of labeling of untreated interphase and mitotic cells are shown in Figs. 3 to 5 and of cells treated with MA in Figs. 6 and 7. Usually between 500 and 600 interphases were counted for each sample with exception of samples obtained at later time intervals following injection of MA, when few tumor cells could be found in the smears. The smallest number of interphases observed for any sample was 88 cells. About 100 mitotic cells generally were counted, but at the later time intervals after treatment only a small number of mitoses could be observed.

In control experiments rapid incorporation of T-H^3 took place into interphase cells, approximately 40% of the cells being labeled in one hour after injection of this isotope (Fig. 8), the earliest time at which a sample was taken. Thenceforth, there was a slight increase of doubtful statistical significance in percentage of labeled cells up to 24 hours. The minimal period between time that exogenously supplied thymidine was incorporated into DNA and identification of labeled mitotic cell was 3 hours. In the 3-hour sample only 4 out of 194 (2.6%) mitotic cells were labeled, while at 4 hours the number rose to 26 out of 216 (12%). Maximal labeling of mitotic cells was attained at 12 hours. Actually, in one case, while only 2 of 96 mitotic cells were labeled at 3 hours, 53% of prophases, 7% of metaphases, and 3% of anatelophases were labeled at 4 hours. These values increased to 70, 29, and 4%, respectively, at 4.5 hours and 71, 53, and 54% at 6 hours. At 8 hours all phases were estimated to be labeled to approximately the same extent, 70-90%, no significant decrease being noted to 24 hours, the last period observed. Data from these control experiments appear to be in reasonable agreement with previously published results on incorporation of adenine-C^{14} into Ehrlich ascites cells(4).

Pretreatment of animals with MA had a profound effect on incorporation of T-H^3. When 5 mg of MA was injected 30 minutes *prior* to T-H^3 there was no incorporation into ascites tumor cells (Table I). In experiments in which 3- and 24-hour intervals elapsed between injection of MA and T-H^3 there was

TABLE I. Effect of Pretreatment with Maleuric Acid on Thymidine-H^3 Incorporation.*

	% of interphases with label			
		⌐ MA before T-H^3 ¬		
Hr after T-H^3	Control	30 min.	3 hr	24 hr
1	44	0		0
3	45	0	2	0
6	51	0	15	7
10		0	20	
12	55			
24	47		0	

* 5 mg MA in 0.5 ml saline given intraper.

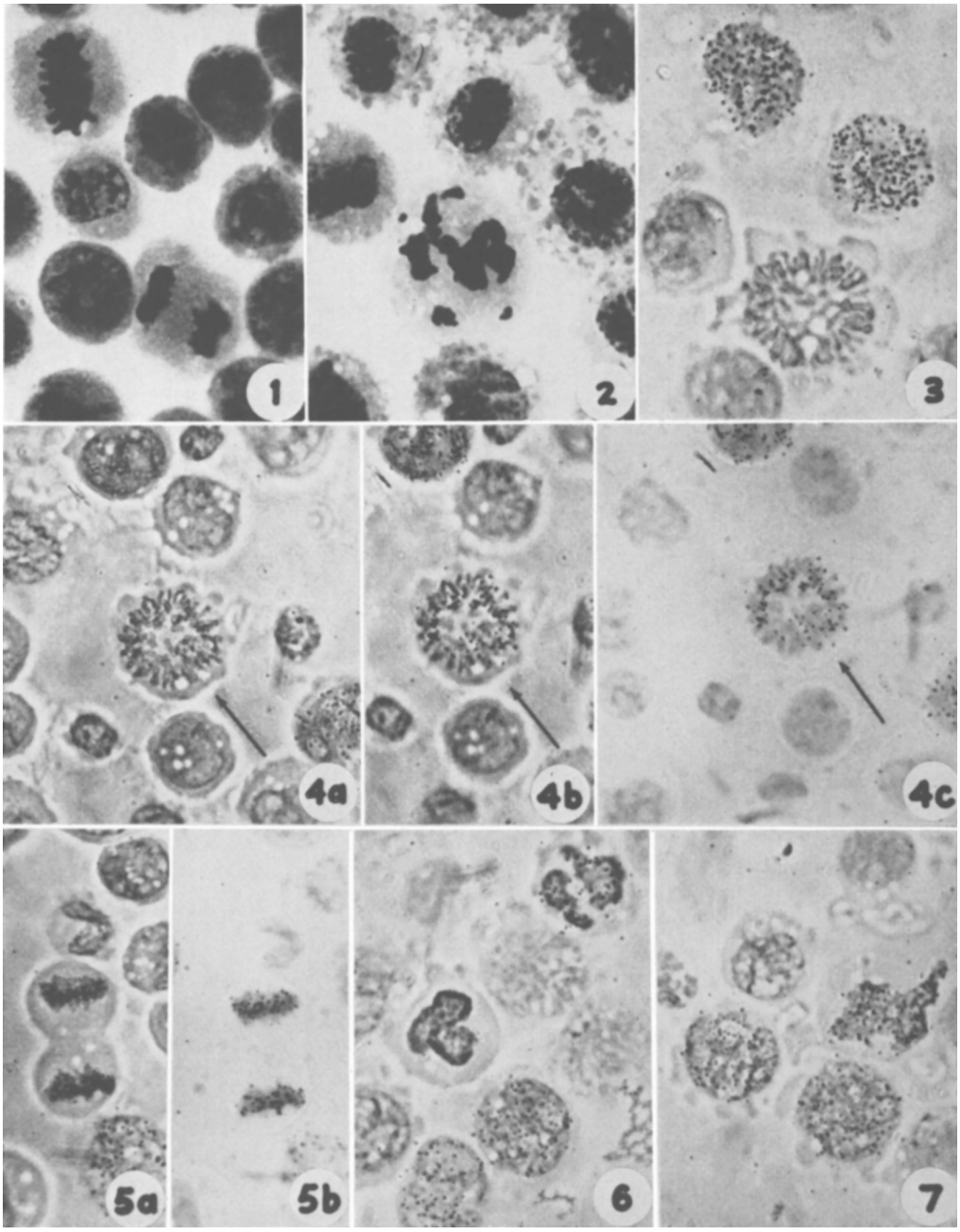

FIG. 1-7. Photomicrographs of Ehrlich ascites tumor. × 975.

FIG. 1. Untreated Ehrlich ascites tumor. Squash preparation stained with acetic dahlia.

FIG. 2. Tumor cells 3 hr after inj. of MA.

FIG. 3-7. Autoradiographs of Ehrlich ascites tumor stained by Feulgen reaction.

FIG. 3. Labeled interphase cells and unlabeled metaphase cell 3 hr after intraper. inj. of tritiated thymidine (autoradiograph; Feulgen stain; phase).

FIG. 4a-4c. Labeled metaphase cell (indicated by arrow) and both labeled and unlabeled interphase cells 10 hr after intraper. inj. of tritiated thymidine. (a) Phase contrast, showing

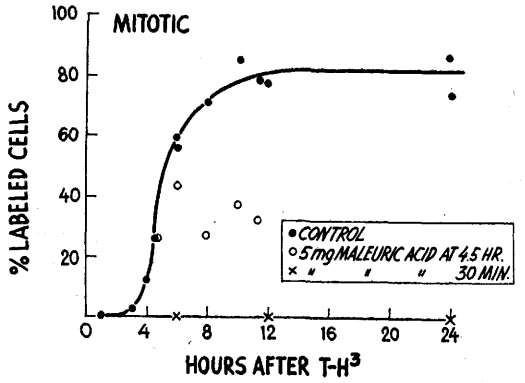

FIG. 8. Incorporation of T-H^3 into mitotic cells.

still a greatly reduced incorporation of label by comparison with controls.

When MA was injected 30 minutes or 4.5 hours *after* T-H^3, the percentage of labeled interphases decreased somewhat from control level during latter half of experiment. This decrease was slightly more pronounced when only 30 minutes elapsed between the 2 injections. Although number of cells in ascitic fluid was reduced greatly at 12- and 24-hour intervals, it is evident that MA did not cause greatly preferential destruction of labeled interphase cells; if lysis had occurred, it must have occurred at only slightly greater rates among labeled than unlabeled cells.

Observations on percentage of labeled mitotic cells clearly show that MA blocks entry of preprophase cells into mitosis. When injection of MA followed that of T-H^3 by 30 minutes, 47, 35, 26, and 27% of interphase cells were labeled at 3, 6, 12, and 24 hours, respectively, but not a single labeled mitotic figure was observed in samples studied. In the experiment in which MA was injected 4.5 hours after T-H^3, progression of labeled interphase cells into mitosis soon ceased even though labeled interphase cells were entering mitosis at a rapid rate (Fig. 8) at time of injection of MA. Although the accuracy of cell characterizations was reduced because of extensive damage to the mitotic figures, the impression was gained that a blocking of mitosis occurred at metaphase, probably related to observed stickiness and clumping. Thus, of 1511 untreated mitotic cells counted, the percentage distribution in prophase, metaphase and ana-telophase was 13, 55, and 32, respectively. In 326 cells counted after treatment with MA the distribution was 5, 82, and 13%.

Experiments to be published elsewhere showed that treatment with MA in doses comparable to those employed in present experiment in combination with other antitumor agents, while producing maximal cytological effects, caused no inhibition in ability of tumor cells to incorporate radioactive glutamine, glycine or lysine into cellular proteins at 3 hours after treatment.

Summary. The above results show that maleuric acid, at dosage level used, has at least 3 effects on cell cycle in Ehrlich ascites tumor cells. It prevents incorporation of tritiated thymidine into deoxyribonucleic acid, inhibits progression of premitotic cells into mitosis, and causes severe mitotic abnormalities which seem to result in a metaphase block.

1. Okada, T., Roberts, E., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 329.

2. Taylor, J. H., McMaster, R. D., *Chromosoma,* 1954, v6, 489.

3. ———, *Physical Techniques in Biological Research,* 1956, v3, 545.

4. Hornsey, S., Howard, A., *Ann. N. Y. Acad. Sci.,* 1956, v63, 915.

Received April 17, 1959. P.S.E.B.M., 1959, v101.

cells; (b) phase contrast in same field, showing developed photographic grains in field above cells; (c) same field, phase plate removed, showing developed photographic grains in film above cells.

FIG. 5a-5b. Labeled late anaphase cell 10 hr after inj. of tritiated thymidine. (a) Phase contrast, showing cell; (b) same field, phase plate removed, showing developed photographic grains in film above cell.

FIG. 6. Labeled and unlabeled abnormal mitotic cells at 8 hr after inj. of tritiated thymidine and 3.5 hr after inj. of MA.

FIG. 7. Labeled abnormal mitotic cell and labeled interphase cells at 8 hr after inj. of tritiated thymidine and 3.5 hr after inj. of MA.