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IN VITRO EPIDERMAL CELL PROLIFERATION IN RAT SKIN PLUGS

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Full-thickness skin plugs from immature and adult rats have been shown to incorporate ^{3}H-thymidine in vitro in a semisynchronous fashion for up to 54 hr. DNA synthesis is minimal at 16 hr post sacrifice but increases again at 24 and 48 hr, and cells in S phase at 48 hr have passed through at least one mitosis in vitro (between 32 and 39 hr). Advantage can be taken of this semisynchronous burst of DNA synthetic activity to test the effects of potential inhibitors of skin cell proliferation, and to determine at which phase of the cell cycle these inhibitors act. High concentrations of epinephrine or isoproterenol ($\sim 10^{-5}$ M) are required to cause inhibition but the effect is specific for the G_2 phase of the cell cycle. Propranolol does not demonstrate β-antagonism in this system, since it is a very potent inhibitor acting in G_1 phase. Dibutyryl cyclic AMP and theophylline have two effects: one is a specific inhibitory action on cells in the G_2 phase and the second is a short-term action limiting thymidine uptake by cells in S phase without affecting the transition of cells from G_1 to S.

With the increasing interest in tissue-specific growth factors [1] and chalones [2] in skin, and the search for specific agents for the control of hyperproliferative diseases such as psoriasis [3], it has become necessary to develop adequate model systems for the assay of potential stimulators or inhibitors of skin cell proliferation. Time-honored techniques [4-6] dependent upon counting of mitoses or labeled cells are laborious and do not lend themselves to the assay of large numbers of compounds. Recently more attention has been paid to direct scintillation counting of the thymidine incorporated either in vivo [7] or in vitro [8,9] into the DNA of proliferating epidermal cells, and in this paper we describe the applications of these latter techniques to the investigation of an in vitro wound response model [10,11].

MATERIALS AND METHODS

Tissue preparation. Male Sprague-Dawley rats were received at 17 days and maintained at least 4 days on a constant (12-hr) light-dark cycle prior to use. Animals were sacrificed at the same time each day (7:00-8:00 AM) in order to reduce any diurnal variation effects. The rats (21-27 days old) were killed in a CO_2 atmosphere, the backs were shaved with electric clippers, swabbed with a Nystatin suspension in 70% ethanol (left to evaporate over 15 min), and the dorsal skin was excised and immersed in medium. During all subsequent procedures, precautions were taken to keep the skin moistened with medium. Subcutaneous fat and muscle were removed from the underside of the dermis by vigorous scraping with a dull scalpel. After rinsing, the skin was uniformly flattened (dermis down) on a Teflon sheet, and 6-mm circles were cut with a biopsy punch; up to 50 pieces could be obtained from each rat.

Incubations. Two circles of skin (10-20 mg total) from the same rat were placed in loosely capped vials with 3 ml of Dulbecco's Modified Eagle Medium* (Grand Island Biological) and the vials were incubated in a gyrotary shaker (150-200 rpm) at 37°C, under an atmosphere of 10% CO_2 in air. When required, test substances were added as freshly prepared solutions in 100 μl of medium or phosphate-buffered saline. For assessment of DNA synthetic activity, thymidine (methyl-^{3}H) (New England Nuclear, NET 027X, 20 Ci/mmole) was added to each vial to a final concentration of 10 μCi/ml, the incubation was allowed to proceed a further 2 hr, and the uptake was terminated by freezing the vials in dry ice. The vials were stored at -20°C until workup either by scintillation counting or autoradiography.

Thymidine incorporation. The vial contents were thawed quickly on a steam bath, the two tissue pieces were removed, blotted dry, and weighed together. Homogenization was carried out with 2 ml of ice-cold 5% perchloric acid in a motor-driven Ten Broeck glass-on-glass tissue grinder. The homogenate was transferred to centrifuge tubes with three washings (10 ml total), and spun at 3000 × *g* for 5 min. The precipitate was washed twice with 4 ml of ice-cold 5% perchloric acid and twice with 2 ml of absolute ethanol. The precipitate was digested in 0.5 ml of NCS tissue solubilizer (Amersham/Searle) with 1 drop of water at 60°C for 30 min, and the digest was transferrred to scintillation vials with 10 ml of Liquifluor toluene-based scintillation fluid. Radioactivity was determined and corrected for background and quenching (^{3}H-toluene internal standard).

Means and standard deviations for controls and treated tissues were calculated and the significance of differences was determined by application of Student's *t*-test for unpaired data. The amount of ^{3}H-thymidine incorporated into DNA was expressed as disintegrations per minute (dpm) per milligram of wet tissue.

Autoradiography. After thawing and blotting, the tissues were fixed overnight in Hollande-Bouin's solu-

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*This medium differs from the formula published by the Tissue Culture Standards Committee (In Vitro 6:93, 1970); it contains 4.6 gm/liter glucose and no sodium pyruvate. Penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) were added to the medium.

tion, dehydrated, embedded in paraffin, and sectioned at 7 μ. After deparaffinizing, the slides were coated with Kodak NTB-3 liquid emulsion, and exposed in the dark at room temperature for 20 hr. The slides were developed and stained lightly with Harris' hematoxylin–eosin through the emulsion. For each section, the length in millimeters and the number of labeled cells were determined.

RESULTS

Short-term Incubations

When ^{3}H-thymidine was present from the beginning of the incubation, the incorporation of radioactivity was found to be linear up to 4 hr (Fig. 1); by the 5th to 6th hr the rate began to fall off as described by others [8]. Tissue which had been heated to 65°C for 15 min prior to the incubation incorporated less than 1% of the radioactivity of the unheated tissues, confirming that there was very little physical binding of ^{3}H-thymidine to the precipitates, and that uptake was dependent upon metabolic competence of the cells. Autoradiography of tissue sections showed very little background, and the label was confined to the nuclei of cells in the proliferative layer. Consequently, tissues were incubated for a total of 4 hr in the presence of the appropriate agents and ^{3}H-thymidine was added for the last 2 hr.

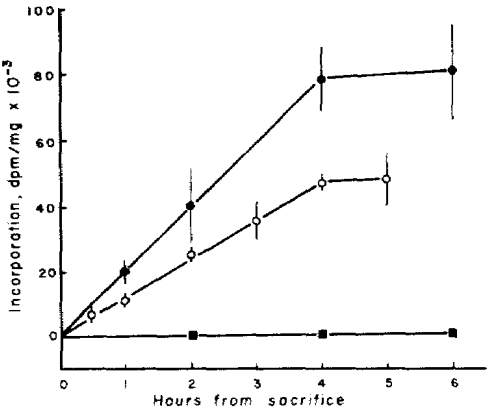

FIG. 1. Cumulative incorporation of ^{3}H-thymidine immediately postsacrifice in two experiments (●, ○) and tissues heated at 60°C for 10 min (■). Each point is the average of four vials (± standard deviation).

Experiments with hydroxyurea, which is known to inhibit DNA synthesis in skin in vivo [12], showed that 4-hr in vitro incubations were sufficient to demonstrate inhibition of DNA synthesis (Tab. I). Under these conditions cyclic AMP had no significant effect presumably because the cell membranes were impermeable to this compound. The dibutyryl derivative of cyclic AMP and/or theophylline, however, caused significant inhibition of the uptake of labeled thymidine within the 4-hr incubation period. This was of interest because it had been shown that cyclic AMP or its dibutyryl derivative inhibits cells in the G_2 phase of the cell cycle [13,14] whereas the effects seen here must be limited to cells in S phase. The possibility that cells could pass from G_2 into S during a 4-hr incubation is eliminated by the fact that neither Colcemid nor colchicine caused any inhibition at mitosis-blocking concentrations (Tab. II). Epinephrine, isoproterenol, and propranolol were also without effect under these conditions (data not shown). In order to demonstrate the effect of these latter compounds it was necessary to extend the length of incubation so that cells which incorporated tritiated thymidine had sufficient time to pass through an entire cell cycle in vitro before entering S phase.

The differences in control values from experiment to experiment (Tabs. I, II) reflect the differences in age and weight to the rats used. Twenty-one-day-old rats weighing 50 to 55 gm gain in size rapidly and weigh approximately 90 gm by 27 days.

TABLE I. *Inhibition of ^{3}H-thymidine uptake in short-term incubations*

Tissues were incubated in the presence of compounds for 4 hr total immediately post sacrifice, and ^{3}H-thymidine was added for the final 2-hr period. The number of vials for each determination is indicated in parentheses.

Compound	Concentration	dpm/mg ± S.D. (× 10^{-3})	p Value	Percent control
Control		6.4 ± 2.4 (6)		100
Hydroxyurea	10^{-5} M	7.4 ± 2.9 (8)	N.S.	114
	10^{-4} M	2.8 ± 1.1 (8)	<0.01	44
Control		13.9 ± 4.1 (4)		100
cAMP	10^{-4} M	16.3 ± 3.3 (4)	N.S.	117
	10^{-3} M	16.8 ± 5.6 (4)	N.S.	120
Control		15.5 ± 3.6 (4)		100
DBcAMP	5×10^{-3} M	8.4 ± 1.6 (4)	<0.02	54
Aminophylline	5×10^{-3} M	7.9 ± 2.0 (4)	<0.02	51
DBcAMP + aminophylline	5×10^{-3} M	5.0 ± 0.6 (4)	<0.001	32

During this period the dermis thickens and the follicles lengthen, giving rise to a concomitant increase in tissue weight of the 6-mm plugs. For this reason, animals were matched with respect to age and weight when more than one was required for an experiment, and each rat provided both control and experimental plugs.

Semisynchronous DNA Synthesis during Long-term Incubations

When skin plugs were incubated for periods up to 50 hr prior to the addition of ^{3}H-thymidine, a burst of DNA synthetic activity centered around 46 hr became apparent (Fig. 2). As before (see Fig. 1) there was an initial drop in rate of incorporation with DNA synthesis reaching a minimum 15 to 16 hr after sacrifice. Between 20 and 36 hr, ^{3}H-thymidine incorporation increased erratically, but by 40 hr incorporation had reached or surpassed that observed immediately post sacrifice. In a parallel experiment, the skin plugs were prepared for autoradiography (Fig. 2, Tab. III). Again the tissue

TABLE II. *Effect of mitotic inhibitors in short-term incubations*

Each value represents the average of six vials, and the differences between treatments and their respective controls are not statistically significant.

Compound	Concentration	dpm/mg ± S.D. ($\times 10^{-3}$)
Control		15.7 ± 6.8
Colcemid	0.33 μg/ml	14.2 ± 5.9
Control[a]		28.1 ± 6.2
Colchicine	10 μg/ml	22.6 ± 7.4
Control[b]		8.4 ± 2.4
Colchicine	10 μg/ml	9.5 ± 1.9

[a] 21-day-old animal
[b] 27-day-old animal

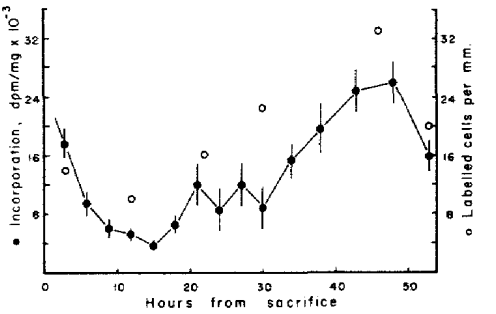

FIG. 2. Variation of ^{3}H-thymidine incorporation with length of incubation. The solid circles (●) are the averages (± standard error) obtained by liquid scintillation counting of one vial from each of 8 rats. The open circles (○) are the averages of number of labeled cells per millimeter of four 6-mm sections from each of 4 rats. In each case ^{3}H-thymidine (10 μCi/ml) was added to the appropriate vials 2 hr prior to the time indicated.

TABLE III. *Effect of incubation time on DNA synthesis by tissue section autoradiography*

^{3}H-thymidine was added for the final 2 hr of each indicated incubation period. Two remote sections from each plug were counted and averaged, and each value in the table is the mean of 16 independent determinations, 4 plugs from each of 4 rats.

Hours post sacrifice	Number of labeled cells per millimeter ± S.D.							
	Surface[a]	p[b] Value	Follicle[c]	p[b] Value	Sebaceous[c] gland	p[b] Value	Total	p[b] Value
3	2.9 ± 1.1		9.5 ± 4.4		0.6 ± 0.6		12.8 ± 5.5	
		<0.001		N.S.		N.S.		<0.1
12	1.2 ± 0.7		8.1 ± 2.3		0.6 ± 0.5		9.8 ± 3.2	
		<0.001		<0.001		<0.01		<0.001
22	0.3 ± 0.2		14.7 ± 5.3		1.4 ± 0.9		16.4 ± 6.0	
		<0.005		<0.01		N.S.		<0.02
30	1.0 ± 0.8		20.7 ± 5.7		1.1 ± 0.9		22.6 ± 7.4	
		<0.001		<0.1		<0.01		<0.02
46	9.1 ± 7.4		25.8 ± 10.4		0.4 ± 0.4		35.3 ± 17.6	
		<0.02		<0.001		<0.1		<0.002
54	3.7 ± 2.9		14.1 ± 5.0		0.2 ± 0.1		18.1 ± 8.4	
		N.S.		N.S.		<0.1		N.S.
70	4.7 ± 2.4		13.9 ± 8.5		0.1 ± 0.2		18.5 ± 11.1	
		<0.01		N.S.		<0.1		N.S.
77	2.2 ± 2.1		11.6 ± 7.4		0.0 ± 0.0		13.9 ± 9.5	

[a] All labeled surface cells were clearly restricted to the epidermal basal layer and not associated with follicular openings.
[b] Significance of differences between successive time periods.
[c] Labeled follicular cells were restricted to the upper two-thirds of a layer clearly contiguous with the surface basal layer. Sebaceous cells were counted as such only when they could be distinguished from follicular epithelium.

pieces appear to reach a minimum in DNA synthetic activity at 12 to 16 hr and a maximum between 40 and 48 hr. The tissue slices were cut parallel with the slant direction of the hair follicles so that an entire follicle together with its sebaceous gland and surface opening were apparent in the section. In this way a reasonable distinction could be made between sebocytes and follicular keratinocytes on the basis of structural position. More arbitrarily, all cells below the shoulders of the follicular surface opening were distinguished as follicular as opposed to surface keratinocytes. It is clear from Table III that activity occurs in all parts of the tissue although more than 70% of the labeled cells are associated with the upper two-thirds of the follicle and less than 9% of the labeled cells could be considered to be sebocytes rather than keratinocytes. Overall, the number of cells labeled during a 2-hr period in the presence of ^{3}H-thymidine closely parallels the curve determined by scintillation counting of total incorporated radioactivity.

Inhibition of Mitoses by Colcemid

Since it was our aim in this study to investigate the effects of various agents on epidermal cell proliferation as measured by DNA synthesis, it was important to determine whether the cells in S phase between 40 and 50 hr had been stimulated directly from G_0 or G_1 [6,7,10,11,15,16] or had passed through a preliminary mitosis in vitro.

Treatment of tissues from the beginning of the incubation with Colcemid resulted in no reduction in the amount of ^{3}H-thymidine incorporation after 25 hr in culture, although the amount of incorporation was reduced 75% by 10^{-4} M propranolol (Tab. IV). When the incubation was extended to 48 hr with Colcemid addition occurring at various times following sacrifice, it was clear that the majority of cells synthesizing DNA at this time underwent at least one postsacrifice mitosis. The data from several experiments, accumulated in Figure 3, show that Colcemid must be added at least 7 hr prior to the ^{3}H-thymidine addition for any inhibition of cells 46 to 48 hr following culture, and it is inferred from this that the minimum transit time from M to the beginning of S is approximately 7 hr. Furthermore, at least 85% of the cells which are labeled at 46 to 48 hr pass through mitosis between 8 and 16 hr earlier. Addition of Colcemid at 22 hr (7986 ± 1788 dpm/mg, 39% of control) instead of 32 hr (2871 ± 1484 dpm/mg, 14% of control) results in significantly ($p < 0.01$) less inhibition, and thus the exact time of addition appears to be quite critical.

TABLE IV. *Effect of agents added at beginning of incubation upon ^{3}H-thymidine incorporation at 24 to 26 hr*

Compounds were added to the medium to give the indicated concentrations at the start of the incubation. ^{3}H-thymidine was added at 24 hr and incorporation was measured at 26 hr. Each value is the calculated mean and standard deviation of five vials.

Compound	Concentration	dpm/mg ± S.D. ($\times 10^{-3}$)	p Value
Control		14.0 ± 1.7	
Colcemid	0.33 μg/ml	13.6 ± 1.3	N.S.
	0.17 μg/ml	13.9 ± 2.4	N.S.
Control		14.3 ± 1.9	
Isoproterenol	10^{-4} M	11.8 ± 2.3	N.S.
	10^{-6} M	14.0 ± 2.8	N.S.
Control		18.0 ± 1.3	
Propranolol	10^{-4} M	4.2 ± 1.3	<0.001
	10^{-6} M	12.1 ± 2.8	<0.01

Inhibition in G_2 Phase by β-Adrenergics

Isoproterenol and epinephrine also inhibit ^{3}H-thymidine incorporation at 46 to 48 hr if added at the appropriate time (Fig. 3). The time inhibition curves for these two compounds closely parallel, but slightly precede, that for Colcemid in any given experiment, although the time difference is not significant. Addition of the β-adrenergic compounds up to 9 hr before the tritium label causes no inhibition, thus eliminating possible effects on cells in G_1 or S phase. Dose-response curves for isoproterenol and epinephrine (Fig. 4) are superimposable and show that relatively high concentrations of these compounds are required to produce inhibition of DNA synthesis.

In a series of experiments (data not shown), tissues were treated for varying periods with 10^{-4} M isoproterenol or epinephrine, and then various concentrations of propranolol were added in an attempt to overcome the inhibition. No stimulation or initiation of DNA synthesis was observed up to 30 hr after propranolol addition; in fact, even greater inhibition was achieved. Propranolol itself was a very potent inhibitor of ^{3}H-thymidine incorporation when added as little as 2 hr before the isotope at 46 to 48 hr (Figs. 3,5).

From the time-response curve (Fig. 3) it is

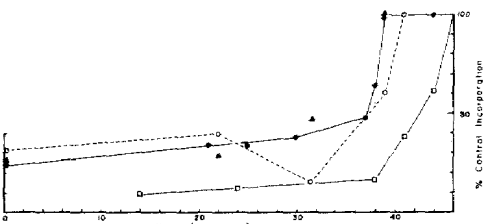

FIG. 3. Effect of time of addition of various compounds on ^{3}H-thymidine incorporated between 46 and 48 hr. 0.33 μg/ml Colcemid (O); 10^{-4} M epinephrine (●); 10^{-4} M isoproterenol (▲); and 10^{-4} M propranolol (□) were added at the indicated times. ^{3}H-thymidine was added to all vials at 46 hr, and the amount of incorporation was determined at 48 hr.

apparent that isoproterenol and epinephrine inhibit cells in late G_2 phase some 9 to 16 hr before these same cells would otherwise enter S phase. Since propranolol inhibits cells in late G_1 or S phase, it is not possible in this system to establish a β-adrenergic mode of action for these compounds.

Short- and Long-term Effects of Dibutyryl Cyclic AMP (DBcAMP)

The short-term inhibition of ^{3}H-thymidine incorporation caused by DBcAMP and/or theophylline (Tab. I) was investigated further. In a 48-hr incubation, addition of 5×10^{-3} M DBcAMP simultaneously with, or up to 7 hr before, the addition of ^{3}H-thymidine caused 50% inhibition (Tab. V). This inhibition was not paralleled by a corresponding decrease in the number of labeled cells observed in autoradiographs of tissue sections (Tab. VI), although the labeling was lighter in the treated tissues than in the controls. When

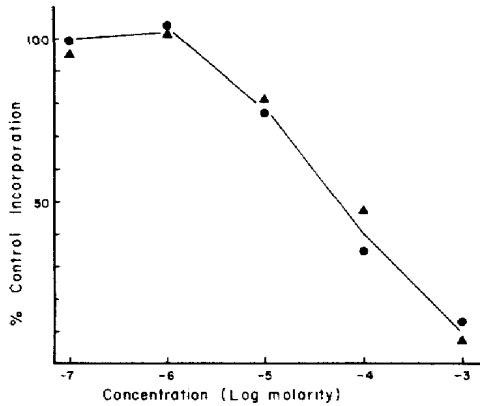

FIG. 4. Inhibition of ^{3}H-thymidine incorporation by isoproterenol (▲) and epinephrine (●). Each point is the average of four vials from each of 2 rats. Compounds were added at the beginning of the incubation and ^{3}H-thymidine was present between 46 and 48 hr.

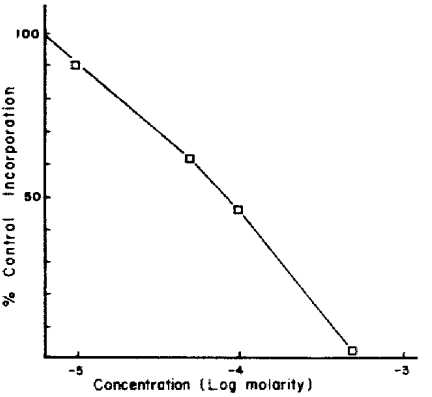

FIG. 5. Inhibition by propranolol. Tissues were incubated for 48 hr, propranolol was added at 39 hr and ^{3}H-thymidine at 46 hr.

TABLE V. *Effect of dibutyryl cyclic AMP (4 mM) added at varying times prior to the 46- to 48-hour ^{3}H-thymidine window*

All tissues were incubated in vitro for 48 hr post sacrifice. ^{3}H-thymidine was added at 46 hr and appropriate additions of DBcAMP were made as indicated. Each value is the average of two vials from each of 4 rats. Treatment values are not significantly different from each other, but all are significantly different from control ($p < 0.001$)

Time of addition prior to ^{3}H-thymidine	dpm/mg ± S.D. ($\times 10^{-3}$)	Percent control
Control-No addition	21.4 ± 4.7	100
0 hr	11.6 ± 2.5	54
2 "	11.4 ± 3.9	53
3 "	10.3 ± 1.4	48
4 "	9.3 ± 2.8	43
5 "	11.2 ± 2.9	52
6 "	11.7 ± 1.5	54
7 "	12.2 ± 2.7	57

TABLE VI. *Comparison of liquid scintillation counting and autoradiographic techniques: effect of elevated intracellular cAMP levels in short- and long-term incubations*

Time of addition prior to ^{3}H-thymidine	Addition 5 mM	Scintillation counting			Autoradiography		
		dpm/mg ± S.D.[a] ($\times 10^{-3}$)	p Value	Percent control	Labeled cells/mm ± S.D.[b]	p Value	Percent control
2 hr	None	16.1 ± 2.3		100	16.8 ± 5.8		100
	DBcAMP	10.2 ± 1.4	<0.01	63	17.4 ± 10.6	N.S.	104
	Theophylline	7.5 ± 4.0	<0.01	47	20.8 ± 9.1	N.S.	124
26 hr	None	13.2 ± 2.6		100	19.3 ± 6.4		100
	DBcAMP	1.1 ± 0.3	<0.001	8.2	2.6 ± 1.3	<0.001	13.5
	Theophylline	1.0 ± 0.6	<0.001	7.8	2.1 ± 1.3	<0.001	10.9

[a] Average of four vials from 1 rat

[b] Average of counting of two sections from each of 8 skin plugs

DBcAMP or theophylline pretreatment was extended to 26 hr, the results of scintillation counting and autoradiography were identical and an inhibition amounting to 90% was observed. Comparison of these data with the curves in Figure 3 shows that elevated levels of intracellular cAMP prevents cells from entering mitosis from the G_2 phase of the cell cycle. The short-term results are those expected for an inhibition of ^{3}H-thymidine phosphorylation [17] and even entry into the cells [18] rather than for an actual inhibition of cell cycle in late G_1 or S phase.

DISCUSSION

The studies reported here confirm the utility of direct scintillation counting of incorporated ^{3}H-thymidine in the investigation of skin cell proliferation in vitro, and the extension of the incubations to 48 hr greatly increases the scope and flexibility of the method. As Marks et al [8] have shown previously, tissues from freshly killed animals rapidly decline in DNA synthesizing ability in vitro, and consequently their studies were limited to 4 to 6 hr post sacrifice. Such short-term incubations limit observations to S phase alone; in fact, from the shape of the curve shown in Figure 2 it would appear that no cells undergo a G_1-S transition during the first 10 to 14 hr of incubation. This temporary inhibition is a response to wounding rather than diurnal viariation, since the minimum invariably occurs at 14 to 16 hr post excision regardless of the time of day (data not shown).

The amount of resurgence in DNA synthesis between 20 and 30 hr varied from experiment to experiment but was always greater than that seen at 16 hr and less than that observed at 48 hr. Since the incorporation at 24 hr could not be blocked by isoproterenol, epinephrine, or Colcemid, this population does not pass through mitosis in vitro and must arise from cells which were in G_1 at the time of sacrifice. In contrast, the majority of cells synthesizing DNA at 48 hr pass through mitosis sometime after 32 hr but before 40 hr (Fig. 3). Since the system is only semisynchronous and a spread in the timing of individual cell mitoses corresponding to the length of S phase is to be expected, we have not attempted to determine the timing of mitoses more exactly. Nevertheless, if these cells arise from those which were in S phase at the moment of sacrifice, and mitosis does not occur until after 32 hr, then these cells have spent between 20 and 30 hr in G_2 and would appear to have been blocked in this phase [6]. Preliminary experiments indicate that this blocking may be due to some factor(s) released by the tissues and degraded over time since the resurgence in DNA synthesis can be postponed for 24 hr by doubling the amount of tissue incubated in each vial. This effect is consistent with the release of phase-specific chalones [19], and is being investigated.

Since the DNA synthesizing cells appearing at 48 hr have passed through a full cell cycle in vitro, this system affords an opportunity for investigating the effects of agents at all phases of the cell cycle. Isoproterenol and epinephrine have been reported to block entry of epidermal cells into mitosis from G_2 phase [14,20–22]. Our results confirm this G_2 inhibition and show that the effects of these compounds are limited to this phase of the cell cycle. There would appear to be little doubt that these two compounds act through the adenyl cyclase–cAMP system as suggested by the work of Voorhees et al [14,20–22] and that the system is of the β-adrenergic type. Propranolol has been shown to inhibit the isproterenol stimulation of adenyl cyclase [21] and to antagonize the epidermal cell division inhibition of epinephrine [22]. That propranolol does not appear to deblock or antagonize β-adrenergic inhibition in our system is not inconsistent with the above since propranolol exerts an inhibitory action in the G_1 phase of the cell cycle. It is possible that propranolol does release cells from the G_2 block only to catch them again in G_1 before they reach S. Experiments to clarify this point are in progress.

The experiments reported here further demonstrate that in vitro ^{3}H-thymidine incorporation provides an easy and reliable method for measuring epidermal cell proliferation and provides support for previously established control mechanisms. With this method, not only can potential inhibitors be detected, but the phase of the cell cycle in which they act can also be determined.

REFERENCES

1. Cohen S: Epidermal growth factor. J Invest Dermatol 59:13–16, 1972
2. Elgjo K: Chalone inhibition of cellular proliferation. J Invest Dermatol 59:81–83, 1972
3. Weinstein GD, Velasco J: Selective action of methotrexate on psoriatic epidermal cells. J Invest Dermatol 59:121–127, 1972
4. Bullough WS, Laurence EB: Mitotic control by internal secretion. The role of the chalone–adrenaline complex. Exp Cell Res 33:176–194. 1964
5. Olvey SK, McDonald CJ: The *in vitro* cell cycle of adult mammalian epidermis: a preliminary study. J Invest Dermatol 58:175–179, 1972
6. Gelfant S, Candelas GC: Regulation of epidermal mitosis. J Invest Dermatol 59:7–12, 1972
7. Hamilton E, Potten CS: Influence of hair plucking on the turnover time of epidermal basal layer. Cell Tissue Kinet 5:505–517, 1972
8. Marks R, Fukui K, Halprin K: The application of an *in vitro* technique to the study of epidermal replication and metabolism. Br J Deramtol 84:453–460, 1971
9. Hell EA, Maibach H: A comparison of *in vivo* and *in vitro* methods of identifying human epidermal cells in DNA synthesis. Br J Dermatol 86:506–507, 1972
10. Hell EA, Cruickshank CND: The effect of injury upon the uptake of ^{3}H-thymidine by guinea pig epidermis. Exp Cell Res 31:128–139, 1963
11. Gelfant S: Patterns of cell division: the demonstration of discrete cell populations, Methods in Cell Physiology, vol II. Edited by DM Prescott. New York, Academic Press, 1966, pp 359–395
12. Singer RM, Gelfant S: Continuous inhibition of DNA synthesis in mouse ear epidermis using hydroxyurea. Exp Cell Res 73:270–271, 1972

13. Marks F, Reiben W: Cyclic 3′,5′-AMP and theophylline inhibit epidermal mitosis in G_2-phase. Naturwissenschaften 59:41–42, 1972
14. Voorhees JJ, Duell EA, Kelsey WH: Dibutyryl cyclic AMP inhibition of epidermal cell division. Arch Dermatol 105:384–386, 1972
15. Potten CS: Early and late incorporation of tritiated thymidine into skin cells and the presence of a long-lived G_0-specific precursor pool. J Cell Biol 51:855–861, 1971
16. Potten CS: Tritiated thymidine incorporation into hair follicle matrix and epidermal basal cells after stimulation. J Invest Dermatol 56:311–317, 1971
17. Hauschka PV, Everhart LP, Rubin RW: Alteration of nucleoside transport of Chinese hamster cells by dibutyryl adenosine 3′:5′-cyclic monophosphate. Proc Natl Acad Sci USA 69:3542–3546, 1972
18. Van Wijk R, Wicks WD, Bevers MM, and Van Rijn J: Rapid arrest of DNA synthesis by N^6,$O^{2'}$-dibutyryl cyclic adenosine 3′,5′-monophosphate in cultured hepatoma cells. Cancer Res 33:1331–1338, 1973
19. Levine M: The growth of adult human skin *in vitro*. Br J Dermatol 86:481–490, 1972
20. Powell JA, Duell EA, Voorhees JJ: Beta adrenergic stimulation of endogenous epidermal cyclic AMP formation. Arch Dermatol 104:359–365, 1971
21. Duell EA, Voorhees JJ, Kelsey WH, Hayes E: Isoproterenol sensitive adenyl cyclase in a particulate fraction of epidermis. Arch Dermatol 104:601–610, 1971
22. Voorhees JJ, Duell EA, Kelsey MH, Hayes E: Regulation of epidermal proliferation *in vitro* by epinephrine, adenyl cyclase and cyclic AMP. Fed Proc 30:671, 1971