to include selective depression of all transmitter-coupled sodium conductances with preservation of all transmitter-coupled chloride and potassium conductances. Furthermore, the close correspondence between the concentration of depressant producing a half-maximal depression of the acetylcholine depolarization and the plasma concentration present under conditions of general anesthesia or anticonvulsive therapy (*9, 20*) suggests that a similar, selective postsynaptic depression of excitatory postsynaptic potentials may be involved in these forms of central nervous system depression. The mechanism underlying this selective depression of the transmitter-coupled sodium ionophore requires investigation.

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12. *Aplysia* saline consisted (in millimoles) of 500 NaCl, 10 KCl, 50 $MgCl_2$, 10 $CaCl_2$, and 10 tris-Cl buffered to *p*H 7.8. Snail saline (in millimoles) was 100 NaCl, 10 $CaCl_2$, 10 tris-Cl buffered to *p*H 7.8, 4 KCl, and 5 $MgCl_2$. Crayfish saline (in millimoles) was 160 NaCl, 15 $CaCl_2$, 10 $MgCl_2$, 5 KCl, and 5 tris-Cl buffered to *p*H 7.8. Lobster saline (in millimoles) was 450 NaCl, 20 $CaCl_2$, 10 KCl, and 10 tris-Cl buffered to *p*H 7.8. Potassium was omitted from the lobster saline in the experiments where bath perfusion of putative transmitters was done in order to increase the size of the depolarization. Potassium was also omitted from the snail saline to enlarge both depolarizing and hyperpolarizing drug responses.
13. The following drugs were obtained from the following laboratories: acetylcholine and γ-aminobutyric acid (both from Sigma, St. Louis), L-glutamic acid (Calbiochem, Los Angeles), dopamine (Nutritional Biochemicals, Cleveland), sodium pentobarbital (Abbott Laboratories, North Chicago), sodium 5,5-diphenylhydantoin (K & K Laboratories, Plainview, New York), α-chloralose, chloroform, ethanol, and urethane (all from Fisher Scientific, Fair Lawn, New Jersey).
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18. The low concentrations of pentobarbital used in the present study did not alter the current-voltage relations of the cell over the range of membrane potentials used to observe postsynaptic potential behavior. Previously, Chalazonitis has reported that volatile anesthetics markedly increase membrane potential and conductance, abolishing both excitatory and inhibitory postsynaptic potentials at anesthetic vapor pressures twice those necessary to induce surgical anesthesia in man [N. Chalazonitis, *Anesthesiology* **28**, 111 (1967)]. He concluded that these agents were acting primarily postsynaptically to depress membrane excitability, but a systematic investigation of synaptic transmission at lower vapor pressures was not performed.
19. Recently, biochemical evidence has been presented using a neuromuscular junction preparation to demonstrate that, although three different general anesthetics depress the contractile response to nerve stimulation, they do not alter the release of the transmitter acetylcholine [S. O. Gergis, K. L. Dretchen, M. D. Sokoll, J. P. Long, *Proc. Soc. Exp. Biol. Med.* **141**, 629 (1972)]. These authors conclude from these results that general anesthetics are acting postsynaptically.
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Retinyl Acetate: Effect on Cellular Content of RNA in Epidermis in Cell Culture in Chemically Defined Medium

Abstract. *Cell cultures of epidermis from newborn mice were established in chemically defined medium. Additions of retinyl acetate to these cultures caused a significant increase in cellular RNA content. Addition of insulin and hydrocortisone to the cultures potentiated the effect of retinyl acetate on cellular RNA content.*

Although it was established over 50 years ago that vitamin A is required for normal differentiation of epithelial cells in many organs (*1*), the molecular mechanisms whereby vitamin A controls this process are still unknown. The structures of the metabolite or metabolites of vitamin A which control cell differentiation are also unknown; there is a great deal of evidence which suggests that structures other than vitamin A alcohol (retinol) may be involved in this process (*2*). Although there have been numerous reports of the ability of vitamin A to control epithelial cell differentiation in organ culture, particularly of its ability to suppress keratinization and enhance secretory activity (*3*), the molecular mechanisms involved have not been elucidated by organ culture studies. The use of chemically defined media for epithelial cell cultures

Table 1. Changes in levels of RNA, DNA, and protein in mouse epidermal cell cultures 3 days after culture in chemically defined medium supplemented with hormones or retinyl acetate or both. Hormone treatment was crystalline bovine insulin, 5 μg/ml, and hydrocortisone hemisuccinate, 5 μg/ml. Retinyl acetate was the *all-trans* isomer, at either 1.56 or 3.12 μg/ml, dissolved in dimethyl sulfoxide to give a final dimethyl sulfoxide concentration of 1.25 percent. Dimethyl sulfoxide was included in all cultures (final concentration, 1.25 percent). Values for total RNA, DNA, and protein have been normalized with respect to the cultures which received no treatment. Standard deviations from the mean are given for all values.

Supplements to medium	Total RNA per dish	Total DNA per dish	Total protein per dish	RNA/DNA	Protein/DNA
None	1.00*	1.00*	1.00*	0.61 ± .06	17.3 ± 1.9
Hormone	0.97 ± .08	1.00 ± .15	1.10 ± .19	.59 ± .07	19.1 ± 2.1
Retinyl acetate	1.40 ± .19†	1.18 ± .18	1.13 ± .14	.73 ± .03†	16.9 ± 2.1
Hormone and retinyl acetate	2.08 ± .23†§	1.40 ± .21†	1.54 ± .18†§	.92 ± .12†§	19.4 ± 3.2

* The mean values for this treatment were derived from five separate sets of experiments and were as follows: total RNA per dish, 10.1 μg; total DNA per dish, 16.6 μg; and total protein per dish, 291 μg. At the start of the experiment ("zero-time" controls), these mean values were: RNA, 7.3 μg per dish; DNA, 11.2 μg per dish; and protein, 149 μg per dish. † These values differ significantly from the corresponding values from untreated cultures ($P < .001$ by *t*-test) or from cultures treated with hormone alone ($P < .01$ for values for total DNA). § These values differ significantly ($P < .001$) from the corresponding values from cultures treated with retinyl acetate alone.

may prove valuable for studying molecular mechanisms, but such conditions have not been used previously for this purpose. We report here that newborn mouse epidermal cells, when grown in cell culture in medium without serum, respond rapidly to the administration of vitamin A acetate (retinyl acetate) with a significant increase in their total RNA content, and that this response is greatly affected by the inclusion of insulin and hydrocortisone in the culture medium.

Full details of the method for epidermal cell culture and of the alteration of differentiation in these cultures caused by retinyl acetate are in preparation (*4*). In brief, cultures of epidermal cells from 1-day-old Balb/c mice were prepared as follows: the mice were skinned and the total skin thickness (dermis and epidermis) was stretched out on a cork board and then floated (dermis down) overnight at 2° to 4°C in Hanks balanced saline solution containing 0.25 percent crude trypsin (*5*). After the skin floated for 20 to 24 hours in this solution, the epidermis and dermis were easily and totally separated from each other by gentle manipulation with a pair of forceps. The dermis was discarded, and the epidermis was minced with iris scissors and then stirred for 30 minutes at 37°C in a trypsinizing flask in the following medium: CMRL-1066 (*6*) with Earle's salts; 10 percent fetal bovine serum; 2 m*M* glutamine; penicillin, 100 unit/ml; and streptomycin, 100 μg/ml. The resulting epithelial cell suspension was filtered through nylon monofilament screening cloth (425 mesh, 25-μm openings) to remove keratin and any clumps of undissociated cells.

From the homogeneous suspension of epithelial cells obtained above, aliquots of 5 ml, containing approximately 2 million cells, were plated into plastic petri dishes (Falcon) 100 mm in diameter. After 24 hours at 37°C in CO_2-air (5 : 95), the medium was removed, and the cells, which had formed colonies adherent to the dish, were washed twice with Hanks saline. Fresh medium (10 ml) containing no serum (CMRL-1066; 0.5 percent crystalline bovine albumin; 2 m*M* glutamine; penicillin, 100 unit/ml; and streptomycin, 100 μg/ml) was then added; at this point hormones or retinyl acetate (*7*) or both were also added to some cultures, as noted below. The dishes were incubated for 3 days at 37°C in CO_2-air (5 : 95). The medium was then discarded, and the cells were washed with ice-cold Hanks saline. The cells were transferred quantitatively to centrifuge tubes, and sequential determinations for the contents of each individual dish were made for total protein (*8*) and total RNA and DNA (*9*). In each set of experiments, all individual treatments were performed in no less than triplicate.

Five separate sets of experiments, involving a total of over 200 dishes, have been performed, and the results are reported in Table 1. Since there was some variation from one experiment to another in terms of the total number of cells at the start of the experiment, the values for total RNA, DNA, and protein per dish have been calculated with respect to the values obtained in that particular experiment for the set of cells which received treatment with neither hormone nor retinyl acetate. Treatment of the cultures with insulin and hydrocortisone (5 μg of each hormone per milliliter) had little effect on their total content of RNA, DNA, or protein. Treatment of the cultures with retinyl acetate alone (1.56 μg/ml or 3.12 μg/ml) increased the total amount of RNA per dish by 40 percent; if the cultures were treated simultaneously with both the hormones and retinyl acetate, the increase in the total amount of RNA was much greater (108 percent). Treatment with either greater amounts (6.25 μg/ml) or lesser amounts (0.78 μg/ml) of retinyl acetate caused a less marked increase in cellular RNA. Retinyl acetate, insulin, and hydrocortisone had a less striking effect on the total amount of DNA and protein per dish. When used alone, neither the hormones nor retinyl acetate markedly increased the total amount of DNA or protein, although the combination of insulin, hydrocortisone, and retinyl acetate did cause a significant increase in the total amount of both DNA and protein. Data are also presented in Table 1 for ratios of cellular RNA/DNA and protein/DNA under the various conditions of treatment. These values are not merely a recalculation of the values for the total amounts of RNA, DNA, and protein per dish; since the ratio values are internally normalized, they are independent of any experimental error in seeding of the identical number of cells in each culture or recovery of cells from cultures. These values add further confirmation to the finding that retinyl acetate alone caused a significant increase in cellular RNA concentration (20 percent over untreated cultures), and that this increase was potentiated by insulin and hydrocortisone (51 percent over untreated cultures). Statistical analysis shows that the increase in cellular RNA concentration caused by retinyl acetate alone is highly significant, and, furthermore, that the potentiation of this effect by the addition of insulin and hydrocortisone is also highly significant.

The mechanisms whereby retinyl acetate causes the increase in both the amount and concentration of RNA in the epidermal cell cultures are not known, nor is the mechanism of interaction of retinyl acetate with insulin and hydrocortisone. Separate experiments in this epidermal cell culture system, as well as with organ cultures of mouse mammary gland (*10*), suggest that there are important interactions between insulin and hydrocortisone in control of RNA metabolism. It has previously been demonstrated that vitamin A controls the pattern of metabolism of newly synthesized, high molecular weight RNA in tracheal epithelium (*11*). The development of the present epithelial cell culture system, in which RNA metabolism responds so markedly to the addition of minute amounts of vitamin A, may be of help in elucidating some of the molecular mechanisms (as well as in identifying active metabolites) by which vitamin A controls the process of cell differentiation.

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