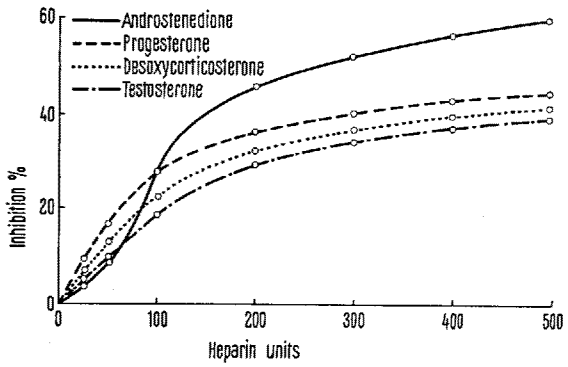
substrates. Upon purification, however, there was a disproportionate loss of correlation of the activities of the enzyme preparation with respect to various steroid substrates employed. Certain procedures inhibited the reductase activities but to different degrees for each steroid employed. These data were interpreted to mean that a group of steroid reductases are present in liver homogenates which are specific for each given steroid substrate.

In view of this specificity, it seems unlikely that heparin would have appreciable affinity for the steroid binding site of each reductase involved in this study and thus inhibit each in a somewhat similar manner. A more likely explanation for heparin inhibition is interference with a cofactor common to the reductases. NADPH is the specific cofactor for the $\Delta^4$ reductases and heparin may compete with this coenzyme for attachment to the specific protein. We have previously shown[1] that excess NADPH will diminish the inhibitory effect of heparin on cortisone reduction. Reduction of the C-20 ketone[2] and C-11 ketone (unpublished data) groups of cortisone by rat liver homogenates is not inhibited by heparin and the NADPH requirements of these functions are probably less rigorous than for the $\Delta^4$ reductases[5,6].




Heparin inhibition of steroid reduction by liver homogenates. %-inhibition refers to a comparison between steroid reduction in an experimental flask and in the corresponding control flask, both prepared with the same homogenate. Each point represents the mean of 5 experiments.

*Zusammenfassung.* Heparin hemmt die Reduktion der Doppelbindung in 4-Stellung des Rings A einer Reihe von C-11-desoxy, $\Delta^4$-4-keto-Steroiden in der Rattenleber. Es scheint, dass Heparin mit NADPH um das Steroid-reduktase-Apoenzym konkurriert.

PATRICIA T. KRZANOWSKI and R. C. TROOP

*Division of Clinical Pharmacology, University of Tennessee, Medical Units, Memphis (Tennessee 38103, USA), 25 September 1967.*

[5] H. J. HUBENER, D. K. FUKUSHIMA and T. F. GALLAGHER, J. biol. Chem. *220*, 499 (1956).

[6] This work was supported by grant No. AM-09151 from The National Institutes of Health, U.S.P.H.S.

## Cation Inhibition of DNA Synthesis in Mammary Epithelial Cells in vitro

The onset of DNA synthesis and subsequent cell division are precisely controlled events in mammalian cells. Although the intracellular mechanisms important in regulating the DNA cycle are as yet undefined, various chemical and physical changes in the external environment of the cell may initiate DNA synthesis or prevent its onset. The ability of these environmental factors to vary the duration of the post-mitotic $G_1$ period, but not the duration of the $S$ period suggests that mechanisms important in regulating cell proliferation are operative in $G_1$. Such factors as temperature[1], nutrient deprivation[2,3], hormones[4,5], tissue injury[6], and others have been shown to alter the duration of the $G_1$ period. This report describes the effect of various cations upon the initiation of DNA synthesis by mammary epithelial cells maintained in organ culture.

Both abdominal mammary glands of mid-pregnant (10–12 day) nulliparous C3H/HeN mice were removed aseptically, and explants were prepared and cultured in sterile Medium 199 (Microbiological Associates)[7]. Appropriate hormones were added to a final concentration of 5 $\mu$g/ml, and analytical grade crystalline salts were dissolved in Medium 199 to the desired final concentration. Each experiment was performed with tissue from a single animal, and replicate incubations were used for each determination. DNA synthesis was measured after exposing the explants to (Me-$^3$H)-thymidine (Schwarz, specific activity 8.0 C/m$M$) at a concentration of 0.5 $\mu$C/ml for 4 or 12 h labeling periods. The tissue was then weighed, de-fatted in acetone, and assayed for tritium-labeled DNA as previously described[5], except that dissolution of the explants in hydroxide of hyamine was accelerated by heating at 70 °C for 10 min. Explants taken for autoradiography were fixed in Bouin's solution and sectioned at 5 $\mu$. The slides were dipped in Kodak photographic emulsion, developed 3 weeks later, and stained with Delafield's hematoxylin. Casein synthesis was measured by exposing explants to medium containing $^{32}$P (30 $\mu$C/ml) for 4 h pulse-labeling periods. After the explants were weighed and homogenized, radioactive casein was isolated from the 105,000 $g$ supernatant by precipitation with rennin and calcium ions in the presence of bovine casein carrier, as previously described[7].

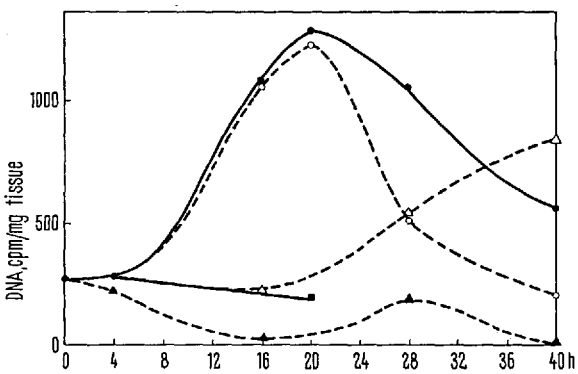
As shown in the Figure, mouse mammary explants cultured in the presence of insulin show a marked augmentation in rate of DNA synthesis. Previous studies[4] indicated that insulin acts upon these epithelial cells in the $G_1$

[1] C. L. SMITH, A. A. NEWTON and P. WILDY, Nature *184*, 107 (1959).
[2] J. F. WHITFIELD and R. H. RIXON, Expl Cell Res. *18*, 126 (1959).
[3] R. R. RUECKERT and G. C. MUELLER, Cancer Res. *20*, 1584 (1960).
[4] R. W. TURKINGTON, Endocrinology, in press.
[5] R. W. TURKINGTON and Y. J. TOPPER, Endocrinology *79*, 175 (1966).
[6] S. GELFANT, Expl Cell Res. *26*, 395 (1962).
[7] W. G. JUERGENS, F. E. STOCKDALE, Y. J. TOPPER and J. J. ELIAS, Proc. natn. Acad. Sci. USA *54*, 629 (1965).

phase of the cell cycle, and that this rise in DNA synthesis represents an increase in the number of cells synthesizing DNA rather than an acceleration of the rate of DNA replication/cell. Addition of lithium sulfate to the incubation medium in increasing concentrations resulted in progressively decreasing rates of DNA synthesis. At 2 m*M* lithium this decrease was apparent only after 28 h of incubation. At 20 m*M* lithium the rise in DNA synthesis was markedly delayed, and at 50 m*M* lithium the rate of DNA synthesis fell below that observed in the absence of insulin. Transfer of explants to lithium-free medium resulted in active resumption of DNA synthesis within 4 h. Since lithium ion inhibited DNA synthesis in the absence of insulin and reduced insulin-mediated DNA synthesis below the rate observed in insulin-free medium, its effect appears to be an inhibition affecting the induction of DNA synthesis per se rather than a direct interaction with insulin. As shown in Table I, inhibition of DNA synthesis by lithium ion or by ammonium ion involves a decrease in the number of epithelial cells undertaking DNA synthesis. The observation that the average number of grains overlying labeled cells is unchanged indicates that the rate of DNA replication/cell is not markedly altered by the presence of these ions. These results and the time course in the Figure indicate that lithium and ammonium ions delay the entry of cells into the *S* period from the $G_1$ period.

Table II lists the rates of DNA synthesis by mammary explants incubated in the presence of insulin and various other cations. Among those ions tested, only ammonium and lithium ions altered DNA synthesis at the concentrations listed. No inhibition was exerted by the sulfate anion. The effects observed could not be explained by the increased osmotic pressure, since equivalent osmolar concentrations of sucrose or other salts did not produce this effect. Lithium and ammonium ions caused corresponding decreases in mitotic indices, indicating that their effects on DNA synthesis reflected diminished cell proliferation rather than diminished uptake of radioactive precursor.

Alveolar differentiation and a concomitant augmentation in the rate of casein synthesis by mouse mammary explants requires the addition of insulin, hydrocortisone, and prolactin to the synthetic medium[7,8]. As shown in Table III, the synergistic action of these 3 hormones effects a threefold increase in the rate of casein synthesis. In the presence of lithium ion this increase was not observed. Addition of prolactin to post-mitotic daughter




Time course of the synthesis of DNA by mid-pregnant mouse mammary explants incubated in the presence of insulin and various concentrations of lithium sulfate: ■——■, no additions; ●——●, insulin; o-----o, insulin and 2 m*M* lithium; △-----△, insulin and 20 m*M* lithium; ▲----▲, insulin and 50 m*M* lithium. Each value represents incorporation of tritiated thymidine into DNA during the preceding 4 h period, and is plotted as the mean of closely-agreeing duplicate determinations. The results are representative of 3 such experiments.

Table I. Effect of cations upon the number of mammary epithelial cells undertaking DNA synthesis and mitosis

| System | DNA, cpm/mg tissue | % of cells labeled | Average grain count | Mitotic index |
|---|---|---|---|---|
| No additions | 298 | 8 ± 3 | 40 ± 6 | 7 |
| Insulin | 1320 | 40 ± 2 | 40 ± 5 | 22 |
| Insulin + $Li^+$, 20 m*M* | 302 | 10 ± 2 | 40 ± 5 | 8 |
| Insulin + $NH_4^+$, 20 m*M* | 651 | 24 ± 3 | 39 ± 7 | 13 |
| $Li^+$, 50 m*M* | 38 | 1.5 ± 1 | 38 ± 8 | 1 |
| $NH_4^+$, 20 m*M* | 290 | 6 ± 3 | 40 ± 6 | 6 |

Explants incubated in the indicated systems were exposed to tritiated thymidine during 20–24 h of incubation. Labeled cells and grain counts were determined after counting at least 500 epithelial cells, and mitotic indices are based on 1000 epithelial cells counted.

Table II. Effect of various cations on DNA synthesis by mouse mammary explants in vitro

| Cation | Added as | Cation concentration, m*M* Control | Final | DNA, cpm/mg tissue | % inhibition |
|---|---|---|---|---|---|
| – | None | – | – | 3300 | 0 |
| $Na^+$ | $Na_2SO_4$ | 140 | 160 | 3190 | 0 |
| $K^+$ | $K_2SO_4$ | 6.7 | 26.7 | 3170 | 0 |
| $Li^+$ | $Li_2SO_4$ | 0 | 20 | 358 | 90 |
| $NH_4^+$ | $(NH_4)_2SO_4$ | 0 | 20 | 1560 | 53 |
| $Mg^{++}$ | $MgSO_4$ | 1.3 | 21.3 | 3100 | 0 |
| $Ca^{++}$ | $CaSO_4$ | 1.26 | 21.26 | 3390 | 0 |
| – | Sucrose | – | 30 | 3200 | 0 |

Mid-pregnancy mouse mammary explants incubated in Medium 199 containing insulin and the indicated salt additions were allowed to incorporate tritiated thymidine into DNA during 12–24 h of incubation. The values obtained represent the mean of closely-agreeing duplicate determinations, and are representative of 2 such experiments.

Table III. Effect of lithium ion upon hormone-dependent synthesis of casein

| System | Casein, cpm/mg tissue |
|---|---|
| Initial period | 271 |
| IFP | 1081 |
| IFP + $Li^+$, 20 m*M* | 262 |
| IF | 263 |
| IF + $Li^+$, 20 m*M* | 278 |

Rates of casein synthesis were determined by exposing explants incubated in the indicated systems to $^{32}P$ during 32–36 h of incubation. I, insulin; F, hydrocortisone; P, prolactin.

[8] R. W. Turkington, W. G. Juergens and Y. J. Topper, Biochim. biophys. Acta *111*, 573 (1965).

cells formed in the presence of insulin and hydrocortisone induces casein synthesis[9,10], and this action of prolactin was not abolished by the presence of lithium at concentrations which prevent DNA synthesis. The 'baseline' level of casein synthesis, as measured in the initial period and maintained by medium containing insulin and hydrocortisone, appears to represent the biosynthetic function of differentiated (and presumably non-dividing) cells formed in vivo, and this function was not diminished by the presence of lithium ions. This inhibition of cell differentiation by the inhibition of DNA synthesis and subsequent cell proliferation confirms previous results indicating that new cell formation is necessary for the expression of new differentiated function in this system[5,9,10]. By these criteria, the effect of lithium ion is not a non-specific toxic effect, but rather appears to represent inhibition of the induction of DNA synthesis in cells preparing for proliferation.

These studies demonstrate that lithium and ammonium ions can act in $G_1$ to delay the onset of DNA synthesis in mouse mammary epithelial cells in vitro. Their action emphasizes the importance of mechanisms which may be operative in $G_1$ to regulate the rate of cell proliferation and thus govern the expression of new differentiated function in progeny cells. Grain counts overlying labeled cells indicated that the rate of DNA replication, once replication was initiated, was not detectably altered, suggesting that similar control mechanisms may not be operative during the $S$ phase[11].

*Zusammenfassung.* Lithium- und Ammoniumionen verhindern den Anfang der DNA-Synthese im Milchdrüsenepithel in vitro. Die Replikation von DNA, nach dem Anfang der $S$-Periode, wird jedoch nicht verhindert. Diese spezifische Verhinderung der Dauer der $G_1$-Periode durch die ionale Umgebung zeigt, dass Mechanismen, die für die Regulierung der Zellproduktion wichtig sind, während der $G_1$-Periode intervenieren.

R. W. TURKINGTON

*Department of Medicine, Duke University Medical Center and Veterans Administration Hospital, Durham (North Carolina 27705, USA), 23 October 1967.*

[9] R. W. TURKINGTON, D. H. LOCKWOOD and Y. J. TOPPER, Biochim. biophys. Acta *148*, 475 (1967).
[10] R. W. TURKINGTON, Endocrinology, in press.
[11] This work was supported in part by grant No. CA 10268 from the U.S. Public Health Service.

## The Specificity of Histones in Nucleated Erythrocytes

Due to their localization in the cell nucleus and due to their close association with DNA, histones are regarded as potential repressors of the genetic loci on DNA. Findings that histones function as potent inhibitors of the enzymatic synthesis of RNA in vitro or of the in vitro DNA replication support this possibility. Since histones have been shown to lack the tissue and species specificity once thought to be essential for their gene-regulatory function, other mechanisms such as interaction with acidic proteins, RNA, hormones, or direct chemical modification of the individual histone fractions by acetylation, phosphorylation, methylation, or thiolation were suggested to modify the histone-DNA interaction at sites involved in genetic transcription[1-5].

If histones function as gene repressors, a substantial deviation from the rather constant composition of somatic histones in vertebrata can be expected in cells with permanently arrested DNA and RNA synthesis. One of such changes can be seen during the spermatogenesis in fish and other animals. The transition of histone to protamine first observed by MIESCHER[6] and confirmed by many investigators is a natural example of a major involvement of histones in cellular differentiation.

Another example of a profound change in the composition of histones associated with a specialization of cellular functions appears again in cells with permanently arrested RNA and DNA synthesis – in chicken erythrocytes. Occurrence of a histone fraction specific for nucleated erythrocytes in domestic fowl has been reported by several authors[7-9]. The histone isolated by NEELIN et al.[10] as histone fraction V and by HNILICA[11] as histone fraction F2c, differs in its amino acid composition from the other histones known to occur in the vertebratae nuclei. It is relatively rich in lysine (21%), alanine (15%), serine (12%), and arginine (11%). In starch gel electrophoresis it migrates as a homogeneous band of a slightly slower mobility than the very lysine-rich histone F1. Because of its absence in other tissues of the chick, it appears that the F2c histone may serve as a permanent genetic repressor.

To investigate this possibility, histones from the erythrocytes of chicken, bull frog (*Rana catesbeiana*) and of the common jack (*Caranx hippos*) were isolated, fractionated, and analyzed. Blood from the sacrificed animals freshly collected with anti-coagulant (sodium citrate) was quickly frozen over pulverized solid $CO_2$. After thawing in an ice bath in the laboratory, the hemolyzed blood cells were washed several times in 0.14 $M$ NaCl containing 0.01 $M$ trisodium citrate. Nuclei, collected by differential centrifugation, were washed with isotonic saline, 0.1 $M$ *Tris* buffer pH 7.6, and 95% ethanol[11]. The

[1] E. STEDMAN and E. STEDMAN, Nature *166*, 780 (1959).
[2] L. S. HNILICA, E. W. JOHNS and J. A. V. BUTLER, Biochem. J. *82*, 123 (1962).
[3] L. S. HNILICA, Biochim. biophys. Acta *117*, 163 (1966).
[4] V. G. ALLFREY, B. G. T. POGO, A. O. POGO, L. J. KLEINSMITH and A. E. MIRSKY in *Histones* (Eds A. V. S. DEREUCK and J. KNIGHT; Churchill, London 1966), p. 42.
[5] L. S. HNILICA, Progr. Nucleic Acid Res. and molec. Biol. *7*, 25 (1967).
[6] F. MIESCHER, in *Die histochemischen und physiologischen Arbeiten* (Vogel, Leipzig 1897).
[7] E. STEDMAN and E. STEDMAN, Phil. Trans. R. Soc. [*B*] *235*, 565 (1951).
[8] J. M. NEELIN and G. C. BUTLER, Can. J. Biochem. Physiol. *37*, 843 (1959).
[9] J. M. NEELIN and G. C. BUTLER, Can. J. Biochem. Physiol. *39*, 845 (1961).
[10] J. M. NEELIN, P. X. CALLAHAN, D. C. LAMB and K. MURRAY, Can. J. Biochem. Physiol. *42*, 1743 (1964).
[11] L. S. HNILICA, Experientia *20*, 13 (1964).