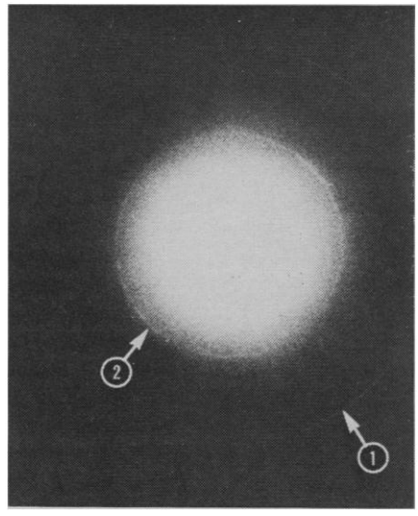

Fig. 2. Electron diffraction pattern of wet, unfixed, and unstained human erythrocyte membrane. The outer ring ① is from the aluminum-coated substrate; the inner ring ②, at a spacing of 4.18 Å, is from the membrane.

The two misoriented patterns in Fig. 1B show either the observation area straddling two neighboring domains, each with imperfect α-phase packing, or the packing of the two monolayers with different orientations. Yet the frequent simultaneous observation of three or more patterns indicates that domain boundaries must exist. The bilayer is made up of a mosaic of these domains, each with a dimension of several micrometers. This structure persists under any degree of hydration, as long as the temperature is below the transition temperature. When the temperature of the hydration stage was raised, an increase in unit cell dimensions was observed for various degrees of hydration, and a sharp transition to a less ordered state was indicated by the loss of sharp diffraction rings. Above the transition temperature, a diffuse ring with a spacing of 4.4 Å is observed, as shown in Fig. 1C. The transition temperature observed for small areas (< 1 μm) of single bilayers was identical with that obtained by other methods for larger volumes of multilayers (*7, 8*). The variation of the transition temperature with the degree of hydration agrees with the temperature-hydration phase diagram obtained from x-ray diffraction measurements of a bulk lipid-water system (*8*). A complete analysis of this work will be reported elsewhere (*9*).

The diffraction pattern from a wet human erythrocyte ghost membrane is shown in Fig. 2. The spacing for the discontinuous ring in Fig. 2 is 4.18 Å. The sharp ring on the outside is from aluminum deposited on the grids for calibration. The sharpness and unevenness of the inner ring in Fig. 2 suggest that the membrane may also consist of semicrystalline domains, as in the case of phospholipid bilayers. The diffused ring at 4.7 Å observed by x-ray diffraction was not seen here (*10*).

The advantage of electron diffraction for studying the structure of biological membranes can be readily seen from this report. By restricting the observed area to a size comparable to that of the domain, the mosaic structure of the membrane was directly observed for the first time. The ability to obtain data from a single sheet of membrane is important since, in general, biological membranes do not exist in nature in the multilayer forms required for most x-ray diffraction work. To meet the challenge of observing the structure of biological membranes in their natural states, electron diffraction seems to be a very useful technique among direct methods.

It is possible to use this technique to observe small angle diffraction from biological membranes, with an electron energy filter to discriminate the inelastically scattered electrons in the small angle region (*11*). It should be useful for studying large protein structures, such as intramembrane particles (*12, 13*), in biological membranes, and the structure of gap junctions (*13*).

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Spermidine in Hormone-Dependent Differentiation of Mammary Gland in vitro

Abstract. *Stimulation of milk-protein synthesis in mouse mammary epithelium in vitro requires insulin, hydrocortisone, and prolactin. The requirement for hydrocortisone can be replaced by spermidine. The possibility that spermidine mediates the effect of glucocorticoid is also supported by the observation that the cellular spermidine concentration increases before the accelerated synthesis of casein and α-lactalbumin.*

Spermidine is ubiquitous in living tissues, and relatively high concentrations of this polyamine are often found in mammalian tissues which exhibit relatively high rates of RNA and protein synthesis (*1, 2*). Furthermore, the spermidine concentration frequently undergoes marked changes during growth and differentiation (*3, 4*). In mammary gland, the spermidine concentration has been reported to increase dramatically during lactation (*2*). This prompted us to investigate the possible role of spermidine in lactogenesis. The results reported here demonstrate the involvement of spermidine in the synthesis of milk proteins during the hormone-dependent differentiation of mouse mammary epithelial cells in vitro and represent the first demonstration that spermidine can mediate the effect of glucocorticoid.

To determine the effects of spermidine on milk protein synthesis, mammary explants prepared from pregnant mice were cultured for 2 days on media containing several combinations of the polyamine, insulin, hydrocortisone, and prolactin. Maximal increases in casein synthesis (*5*) and α-lactalbumin

accumulation (*6*, *7*) occur after 48 hours of incubation with insulin, hydrocortisone, and prolactin. Results shown in Table 1 demonstrate that spermidine, in combination with both insulin and prolactin, produced an increase in casein synthesis and α-lactalbumin accumulation similar to that ordinarily produced by the presence of the triple hormone combination. Addition of spermidine to the triple hormone combination caused no further stimulation of casein and α-lactalbumin synthesis as produced by the combination of insulin, spermidine, and prolactin. Spermidine alone, or in combination with any one of the three hormones (not shown), or spermidine in combination with both insulin and hydrocortisone were all ineffective in augmenting milk protein synthesis. It should be noted that spermidine did not always completely replace the requirement for hydrocortisone because the stimulation, particularly in casein synthesis, was always lower in the spermidine-insulin-prolactin system than in the triple hormone combination. In similar experiments, spermidine was found to be effective at a concentration as low as $4 \times 10^{-4} M$. Related polyamines, spermine, and putrescine, and the cation, Mg^{2+}, were inactive.

The effects of various combinations of hormones on the concentration of spermidine in cultured mammary epithelium is shown in Table 2. After 15 hours of culture, the initial spermidine concentration was maintained in the presence of insulin, whereas the concentration decreased by one-half in the absence of any added hormones. Hydrocortisone alone or prolactin alone produced no increase in the spermidine concentration (not shown). When the hormones were tested in pairs, insulin and prolactin enhanced the polyamine accumulation by 50 percent, while hydrocortisone plus insulin produced only a slight increase over that observed with insulin alone. In contrast, the triple hormone combination produced a doubling of the spermidine concentration. This indicates that the enhancement of the polyamine accumulation in the presence of the three hormones occurs prior to the increase in milk protein synthesis, since milk protein synthesis is still quite low at this time (*5*, *6*). After the 48-hour incubation period, a further increase in spermidine concentration occurs in the triple hormone combination system. The level of spermidine was still elevated in the insulin-prolactin system, whereas in all the other systems the polyamine concentration declined. The concentration of spermine, in contrast to that of spermidine, increased slightly in the presence of the three hormones throughout the culture period, but the increase was not appreciably different from that observed in the presence of various combinations of hormones in pairs. Putrescine was present only in a trace amount, and hence its concentration could not be accurately determined in these experiments.

The data presented above strongly suggest the involvement of spermidine

Table 1. Casein synthesis and α-lactalbumin accumulation in mammary gland explants cultured in various combinations of insulin, hydrocortisone, prolactin, and spermidine. Mammary gland explants from C3H/HeN mice in the middle of their first pregnancy were cultured for 48 hours in medium 199 (Grand Island Biological Co.) containing combinations of insulin, hydrocortisone, prolactin, and spermidine. Final concentrations of insulin (crystalline pork zinc insulin, Eli Lilly Co.), hydrocortisone alcohol (Nutritional Biochemicals Corp.), and prolactin (ovine prolactin, NIH) were 5 μg/ml each, and spermidine (Sigma) was used at a final concentration of 4 m*M*. Details of mammary gland culture techniques were described previously (*5*). The extent of casein synthesis was estimated by labeling the explants with carrier-free $^{32}P_i$ (15 μc/ml of the medium, Schwarz-Mann) for 4 hours immediately before the termination of the culture. Isotopically labeled casein was isolated from the 105,000*g* supernatant of the tissue homogenates by precipitation with rennin and calcium ions in the presence of bovine casein carrier (*5*). Casein precipitates were dissolved in 1 ml of NCS solubilizer (Amersham/Searle) for the radioactivity measurement. Accumulation of α-lactalbumin, the B protein component of the lactose synthetase system, in the mammary explants was determined by measuring its ability to catalyze the transfer of galactose from [^{14}C]uridine-diphosphogalactose (297 mc/mmole, New England Nuclear) to glucose as described by Vonderhaar *et al.* (*7*). The reaction was run for 30 minutes at 37°C, with a blank containing no galactose acceptor to correct for endogenous hydrolysis of UDP-[^{14}C]galactose. The data in this table are representative of several experiments.

Culture conditions	α-Lactalbumin* (mean ± S.E.M.)	Casein † (mean ± S.E.M.)
Uncultured control	15 ± 3	80 ± 5
Insulin + hydrocortisone	5 ± 1	110 ± 10
Insulin + prolactin	25 ± 4	190 ± 10
Insulin + hydrocortisone + prolactin	75 ± 8	950 ± 50
Insulin + spermidine	3 ± 1	70 ± 5
Insulin + hydrocortisone + spermidine	10 ± 2	85 ± 7
Insulin + prolactin + spermidine	55 ± 5	550 ± 24
Insulin + hydrocortisone + prolactin + spermidine	52 ± 6	560 ± 35

* Picomoles of lactose formed per milligram wet weight of tissue per 30 minutes. † Counts per minute per milligram wet weight of tissue per 4 hours.

Table 2. Effect of various combinations of insulin, hydrocortisone, and prolactin on concentrations of spermidine and spermine in cultured mammary epithelium. Mammary gland explants pooled from five to ten pregnant mice were cultured for the designated period in the presence of the hormones shown. At the end of the culture period, the explants were weighed and treated with collagenase to remove fat cells as described previously (*7*). The isolated epithelial cell fractions were assayed for the concentrations of the polyamine as described by Pegg *et al.* (*4*). In brief, the procedure includes extraction into alkaline butan-1-ol, separation by high-voltage electrophoresis, at 30 volts per centimeter of Whatman No. 3 MM chromatography paper for 2 hours in a 0.1*M* citric acid–NaOH buffer, *p*H 4.3, and staining with acid ninhydrin. Recovery factors were determined by the addition of the appropriate [^{14}C]amine (0.15 μc) prior to homogenization. They varied from 77 to 87 percent. Each value represents the mean of at least two separate determinations. The data in this table are typical of those obtained in several experiments.

Culture conditions	Concentration of polyamine (nmole/g wet weight of tissue)	
	Spermidine	Spermine
Uncultured control	165	141
15 hours		
No hormone	108	115
Insulin	198	155
Insulin + hydrocortisone	230	165
Insulin + prolactin	260	195
Insulin + hydrocortisone + prolactin	330	189
Uncultured control	147	82
48 hours		
No hormone	31	30
Insulin	64	87
Insulin + hydrocortisone	123	136
Insulin + prolactin	220	125
Insulin + hydrocortisone + prolactin	400	110

in the hormone-dependent milk protein synthesis in mouse mammary epithelium in vitro. It is noteworthy that the largest accumulation of spermidine occurs in the presence of insulin, hydrocortisone, and prolactin, the combination of hormones that is necessary for the maximal stimulation of casein and α-lactalbumin in vitro (*5*, *6*). Correspondingly, in the presence of insulin and prolactin, small increases in milk protein synthesis were accompanied by small increases in the spermidine. Recently, Russell and McVicker (*2*) reported that the spermidine concentration increased to 5 μmole per gram of tissue during lactation in the rat mammary gland. The consistency between their observation and the data presented here affords confidence that enhancement of spermidine accumulation by insulin, hydrocortisone, and prolactin in vitro may reflect physiological changes in mammary epithelium during lactogenesis.

The ability of exogenously added spermidine to stimulate milk protein synthesis in cultured mammary cells provides more direct evidence for its involvement in lactogenesis. Spermidine is effective at a concentration as low as $4 \times 10^{-4} M$, which is in the range found in the cells that are actively synthesizing milk proteins under the influence of insulin, hydrocortisone, and prolactin. This effect of spermidine is specific for hydrocortisone, since spermidine did not replace the requirement for insulin or prolactin. At present, however, little is known about the mechanism of spermidine action on milk protein synthesis. Moreover, it is yet to be determined whether spermidine itself is the active agent or whether its effect is mediated through its metabolites.

Finally, it should be mentioned that spermidine is not quite as effective as hydrocortisone with respect to milk protein synthesis. This difference may simply be due to some unknown side effects of the polyamine or due to its limited entry into the cells. However, more interesting is an alternative possibility that the steroid hormone may exert some critical effects which cannot be fulfilled by the exogenous addition of the polyamine in the culture system.

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5-Methylcytidylic Acid: Absence from Mitochondrial DNA of Frogs and HeLa Cells

Abstract. *The content of 5-methylcytidylic acid in nuclear DNA and mitochondrial DNA of* Xenopus laevis *and HeLa cells has been determined. Both nuclear DNA's contain 5-methylcytidylic acid. The 5-methylcytidylic acid content of* X. laevis *DNA is 1.7 mole percent of total nucleotides, and that of HeLa cell DNA is 0.7 mole percent. In neither mitochondrial DNA could any 5-methylcytidylic acid be detected; the limit of sensitivity was judged at below 0.1 mole percent for* X. laevis *DNA and below 0.05 mole percent for HeLa cell DNA.*

Nuclear DNA from many animals contains 5-methylcytidylic acid (Me-C) in addition to the four major nucleotides (*1*). The presence of this minor constituent has been suggested in the mitochondrial DNA (mtDNA) of *Physarum* (*2*), but only recently has a report appeared on the methylation of animal mtDNA (*3*).

In the course of studies on the Me-C content of ribosomal DNA my colleagues and I have adapted a highly sensitive method for its detection (*4*). The method utilizes ^{32}P-labeled DNA, which is digested to either 3′ or 5′ mononucleotides by a suitable combination of nuclease and phosphodiesterase. The nucleotides are separated by two-dimensional thin-layer chromatography and visualized by radioautography. By increasing the exposure times, small amounts of ^{32}P-labeled

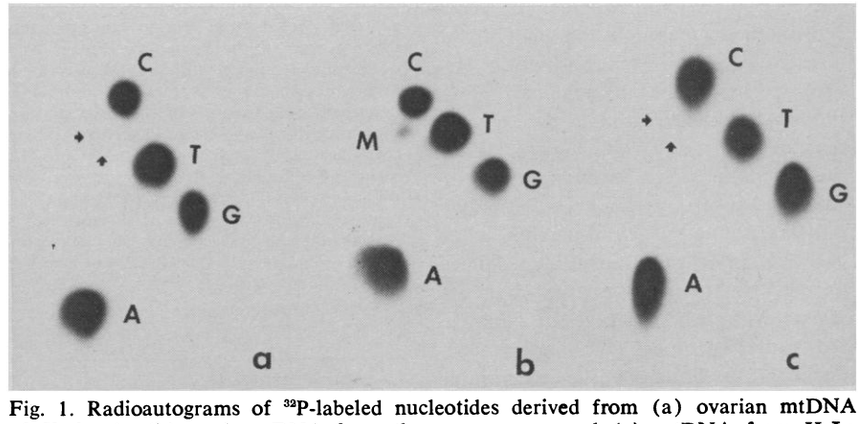

Fig. 1. Radioautograms of ^{32}P-labeled nucleotides derived from (a) ovarian mtDNA of *X. laevis*, (b) nuclear DNA from the same ovary, and (c) mtDNA from HeLa cells. A female *X. laevis* was injected with 1 mc of [^{32}P]phosphate; 3 days later the ovary was removed, and mtDNA was prepared as described (*8*); nuclear DNA was prepared from the connective tissue of the same ovary (*4*, *8*). HeLa cells were cultured for 3 days in the presence of [^{32}P]phosphate (10 μc/ml). The closed-circular component of mtDNA was prepared with the use of ethidium bromide–CsCl gradients (*9*). All DNA's were digested with micrococcal nuclease and spleen phosphodiesterase under the conditions described previously and separated by two-dimensional thin-layer chromatography (*4*). The individual nucleotides are indicated, with *M* standing for Me-C; *A*, adenylic acid; *C*, cytidylic acid; *G*, guanylic acid; and *T*, thymidylic acid. In (a) and (c) the position of carrier unlabeled Me-C in the chromatogram is indicated by two arrows.