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Ontogeny of Embryonic Chicken Lung: Effects of Pituitary Gland, Corticosterone, and Other Hormones upon Pulmonary Growth and Synthesis of Surfactant Phospholipids

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The actions of hormones on growth, cellular proliferation, and on synthetic rates of the major surfactant phospholipids, phosphatidylcholine (PC) and disaturated PC (DSPC), were studied in the lung of the chick embryo. Particular emphasis was placed on the effects of hypophysectomy, pituitary transplantation, and treatment with corticosterone (CORT). One study was concerned with hydrocortisone (HYCORT), estrogen (E_2), thyroxine (T_4), ovine prolactin (oPRL), and insulin. Hypophysectomy interfered with the normal gain in protein, the progressive dehydration of the embryonic lung, and also caused a reduction in the number of pulmonary cells on Days 16 and 18 of incubation. Absence of the pituitary gland diminished pulmonary PC by Day 16. Transplantation of one pituitary gland or exogenous CORT partially restored pulmonary phospholipid and PC (normalized per wet weight) in hypophysectomized (hypox) embryos. Transplantation also restored relative protein content in lungs of hypox individuals. Beyond this, transplantation was generally ineffective in reversing deficits of hypox individuals. All concentrations of CORT administered (30—100—300 μg) reduced the rate of pulmonary cell division. The highest dose was toxic as judged by its capacity to cause cellular death. Treatment of intact chicken embryos with CORT or E_2 for two days stimulated incorporation of [^{14}C]choline into PC and DSPC (the most surface-active component of PC) in the lungs of Day 17 embryos. CORT, but not E_2, stimulated DSPC synthesis when treatment was increased to 3 days. Other hormones tested (T_4, oPRL, insulin, and HYCORT) had no effect upon the rate of incorporation of [^{14}C]choline into PC or DSPC. These results indicate that during ontogeny the avian lung becomes sensitive to CORT, and possibly E_2, prior to 16 days of incubation. CORT, in particular, acts both to trigger the prehatching stimulation of surfactant phospholipid synthesis, especially the vital DSPC fraction, and to slow the rate of pulmonary cellular division coincident with biochemical differentiation of the surfactant system.

The air spaces of the avian and mammalian lung are lined with surfactant. This phospholipid-enriched secretion reduces surface tension, thus promoting pulmonary stability during breathing (Pattle, 1978). As in mammals, the major class of phospholipid in avian surfactant is phosphatidylcholine (PC). Quantitatively, the most important PC subcomponent is disaturated phosphatidylcholine (DSPC) (Fujiwara *et al.*, 1970; Hylka and Doneen, 1982). The spatial characteristics of the saturated 16-carbon fatty acid side-chains of DSPC are believed to be responsible for the potent surface-active properties of surfactant (Massoro, 1981). In the avian embryo both content and synthesis of pulmonary PC and DSPC increase prior to pipping which signals the onset of breathing (Tordet and Marin, 1976; Hylka and Doneen, 1982). A marked accumulation of surfactant also occurs in the mammalian fetal lung in the days preceding parturition (Van Golde, 1976).

Blackburn and coworkers (1972; 1973) showed in the fetal rat that absence of the

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pituitary gland, or deficits in hormones released in response to trophic pituitary agents, inhibited normal pulmonary growth. Many subsequent studies have identified specific hormones which promote growth and differentiation of the mammalian fetal lung. Most work has focused on the ability of glucocorticoids to accelerate both morphological and biochemical aspects of pulmonary development, particularly the ability of cortisol to stimulate surfactant synthesis and release (Ballard, 1981). Other hormones, such as estrogen, thyroid hormones, and, less certainly, prolactin, have also been implicated in maturation of the fetal pulmonary surfactant system (Smith and Bogues, 1980; Ballard, 1981).

The chick embryo possesses certain experimental advantages not possessed by the fetus; these include the possibility of long-term pituitary ablation, ease of access for administration of hormones, and isolation from immediate maternal endocrine influences. Nonetheless, identification of hormones which regulate pulmonary growth, maturation, and synthesis of surfactant in the avian embryo has not been achieved. Some experiments have suggested important roles for pituitary and adrenal hormones in ontogeny of the avian lung. For example, hypophysectomy of the chicken embryo by partial decapitation delayed the appearance of surfactant-secreting type II cells (Marin and Dameron, 1974; Marin *et al.*, 1978) and reduced content of PC in the lungs of embryos (Hylka and Doneen, 1980). Furthermore, organelles specialized for surfactant synthesis and storage (lamellar bodies) in type II cells could be stimulated to appear in lungs of hypophysectomized embryos when cocultured with either pituitary or adrenal glands (Dameron and Marin, 1978). Other evidence that hormones may be important in the development of avian surfactant is less direct. We have shown that content and synthesis of PC and DSPC increased most rapidly in the lung of the chicken embryo after Day 18 of incubation (Hylka and Doneen, 1980; 1982), which coincides with peak titers of plasma corticoids (Kalliecharan and Hall, 1974; Marie-Schlienger and Idelman, 1981), thyroid hormones (Thommes and Hylka, 1977; Bacou *et al.*, 1979), and prolactin (Harvey *et al.*, 1979). Though these findings are compatible with several hormones having roles in growth regulation of the embryonic lung, or in differentiation of its capacity to produce surfactant, no studies have investigated the effects of specific agents.

This paper reports the influences of hypophysectomy, pituitary replacement, and administration of corticosterone acetate upon growth, cellular proliferation, and differentiation of surfactant synthesis in the lung of the chick embryo. In addition, the actions of insulin, thyroxine, prolactin, and estrogen on the synthesis of the major surfactant phospholipids, PC and DSPC, were observed.

MATERIALS AND METHODS

Operations. Fertilized eggs (Rhode Island Red × White Leghorn) were obtained from Dave's Eggs and Poultry (Ann Arbor, Mich.). All eggs were incubated at 37.7 ± 0.7° in a forced-draft, rocking incubator. In experiments involving hypophysectomy or administration of hormones, a small hole (window) was made in the egg shell directly above the embryo on Days 1–2 of incubation. The window provided access for partial decapitation of the embryo, or for the later addition of hormones to the chorioallantoic membrane (CAM). The hole was taped and eggs were transferred to a stationary incubator (37.7°) for the duration of the experiment. At the conclusion of the incubation period, lungs were quickly dissected, blotted, weighed, wrapped in aluminum foil, and quick-frozen in a dry-ice ethanol bath. Tissues were stored at −25° until analyzed.

Embryos were hypophysectomized at 33–38 hr of incubation by the method of partial decapitation (Fugo, 1940). Incompletely decapitated embryos (those with remnants of eyes or upper beak) were not included in any of the results. Control embryos received a windowed shell, but were otherwise not operated upon. In transplantation experiments, single pituitary glands from 10-day-old donor embryos were transplanted onto the CAM of 9-day-old hypophysectomized (hypox) recipients (Betz, 1967). At sacrifice, transplanted pituitaries were examined with a dissecting microscope to determine viability of the gland (as judged by exten-

sive vascularization of transplant). Eggs containing nonviable transplants were discarded.

Hormone administration. The first set of experiments compared various markers of pulmonary growth in windowed controls and in hypophysectomized (hypox) embryos subjected to pituitary or to corticosterone (CORT) replacement. Animals received 100 μl of hormone solution on Days 13, 14, and 15 of incubation by direct addition to the CAM. Corticosterone-21-acetate (Sigma Chemical Co.) was dissolved in a small volume of warm 95% ethanol and diluted with 0.9% sterile saline to form a fine suspension (final ethanol = 3%). Three concentrations of CORT (30, 100, and 300 μg) were administered daily. Control treatment consisted of 0.9% sterile saline with 3% ethanol.

In experiments which measured *in vitro* incorporation of [^{14}C]choline into phospholipids of lung slices, hormones were administered to embryos as described above. Two incorporation experiments were performed. In the first (Table 5), embryos were treated on Days 15 and 16 of incubation and lungs were dissected for *in vitro* exposure to labeled precursor on Day 17. The following hormones and control substances were administered daily: 20 μg corticosterone-21-acetate (CORT: Sigma), 20 μg 17-β-estradiol (E_2, Sigma), 20 μg cholesterol (Supelco), 20 μg bovine insulin (Sigma), 20 μg ovine prolactin (oPRL; 35 IU/mg; NIH-P-S12), 100 ng L-thyroxine (T_4; Sigma), 0.9% NaCl. CORT and T_4 were also given in combination. As shown previously by induction of specific physiological responses, protein hormones, like the more lipophilic agents, enter the embryonic circulation after topical application onto the CAM, though the efficiency of transfer is not known (insulin: Thommes and Tamborino, 1962; prolactin: Doneen and Smith, 1982b). In the second experiment (Table 6), embryos were treated with hormones on Days 14, 15, and 16, and lungs from these were also removed on Day 17 of incubation. In this experiment, 20 μg CORT, 20 μg hydrocortisone (HYCORT; Sigma), 20 μg E_2, and 20 μg cholesterol were administered daily. In both experiments, control embryos received 100 μl of either 0.9% NaCl (3% ethanol) or a suspension of 20 μg cholesterol in 0.9% NaCl (3% ethanol). Cholesterol served as the steroid control. Since results from administration of saline and cholesterol did not differ (*t* test, $P < 0.05$), these values were pooled to form a single control group.

Lipid isolation and analysis. Lipids were extracted by homogenization in 20 vol of chloroform:methanol (2:1) according to Folch *et al.* (1957). Total extracted fat was measured gravimetrically, following evaporation of solvent and overnight desiccation *in vacuo* over KOH. Phospholipid content was estimated by measuring phosphorous (Bartlett, 1959) in lipid digests as described previously (Hylka and Doneen, 1982).

Individual phospholipid were separated by thin-layer chromatography using chloroform:methanol:0.25% KCl:2-propanol:triethylamine (30:9:6:25:18; Touchstone *et al.*, 1980). Visualization and elution of phospholipids has been previously described (Hylka and Doneen, 1982). Disaturated phosphatidylcholine (DSPC) was isolated by silica gel chromatography after reacting phospholipids with osmium tetroxide (Mason *et al.*, 1976). The use of this method to isolate DSPC from avian lungs with >95% recovery has been verified (Hylka and Doneen, 1982).

Methods for measuring both [^{14}C]choline incorporation into lung PC and DSPC *in vitro* and tissue choline specific activity (choline acetyl transferase assay) have been previously described in detail (Hylka and Doneen, 1982).

Protein, dry weight, water content. Protein was estimated in lung homogenates according to Lowry *et al.* (1951) using bovine serum albumin (Sigma) as standard. Tissue dry weight was determined by adding total lipid weight and lean dry weight (Marin *et al.*, 1978). Pulmonary water content was calculated by subtracting dry weight from wet weight.

DNA analysis. Lungs were homogenized in 10 vol of water and DNA content was measured in a 50-μl aliquot using diphenylamine (Burton, 1956) with calf thymus DNA (Sigma) as standard. The number of cells was estimated using the value of 2.573×10^{-6} μg DNA/chicken cell (Fasman, 1976).

Measurement of cell death. Cell death after treatment with CORT was measured from the loss of radioactivity in the DNA fraction isolated from lung cells previously labeled with [^{3}H]thymidine. Methyl-[^{3}H]-thymidine (New England Nuclear; 4.6 Ci/mmol) in 70% ethanol was evaporated to a small volume and diluted with sterile isotonic saline (final concentration: 5% ethanol). One hundred microliters of [^{3}H]thymidine (6.7 μCi) were added to the CAM on Day 10 of incubation. CORT or saline–ETOH was subsequently administered on Days 13, 14, and 15 as described above. On Day 13, lungs from several embryos not treated with hormones were collected for measurement of label incorporated. Cell death occurring by Day 16 was estimated from the percent label remaining compared with pretreatment (Day 13) values (i.e., no loss of cells = 100% of the [^{3}H]thymidine incorporated on Day 13 remained on Day 16). DNA was measured in lungs of 16-day embryos as described above. A second aliquot (100 μl) was pipetted onto a 2.3-cm Whatman No. 3 filter paper disk for measurement of ^{3}H incorporated into DNA (Fong and Bockrath, 1977). DNA was precipitated by immersing filters overnight in a beaker of ice-cold 5% trichloroacetic acid (TCA). These were then washed twice with 5% TCA and once each in 100% ethanol and acetone. Disks were dried and radioactivity was counted by placing disks in 10 ml of OCS (Amersham) scintillation fluid.

Statistics. Significance of differences between mean values was determined using either Students' *t* test or, in cases in which multiple comparisons were made,

from analysis of variance followed by Duncan's new multiple-range test, Dunnett's test, or the least-significant-difference (lsd) method (Steel and Torrie, 1980). The acceptance level for significance was set at 0.05.

RESULTS

The effects of hypophysectomy, and of hypophysectomy with CORT treatment or pituitary replacement, upon wet weight, hydration, and protein content in lungs of chicken embryos on Day 16 of development are summarized in Table 1. Pulmonary wet weight in hypox embryos was not different from windowed embryos. Administration of CORT, however, reduced pulmonary wet weight in a dose-dependent manner in both hypox (Table 1) and also in intact embryos (data not shown). Lung water as a percentage of the wet weight was increased in hypox embryos compared with the nonoperated (W + V) group ($P < 0.05$). Treatment with CORT significantly reduced pulmonary hydration in hypox animals, ($P < 0.05$), but hypophyseal replacement was ineffective. Hypox embryos also exhibited lowered pulmonary protein compared with W + V individuals. Pituitary transplantation restored protein to normal levels (when normalized to dry weight) in lungs of hypox embryos, whereas treatment with CORT had no similar effect.

The numbers of cells in lungs of intact and hypox embryos between Days 12–20 of incubation are illustrated in Fig. 1. Normal embryos exhibited three phases of pulmonary growth in this interval. The most rapid phase (Days 14–18) was bracketed by periods of relatively slower growth. On Day 12, lungs from hypox individuals contained significantly more cells compared with lungs of W embryos ($p < 0.05$). After Day 12, however, pulmonary growth rate in hypox animals was slowed compared with intact individuals, but only until Day 16 of incubation. Between Days 16 and 18, the rate of increase in the number of cells was only modestly reduced in hypox embryos compared with W individuals. Transplantation of a single pituitary gland into hypox embryos beginning on Day 9 of incubation did not restore numbers of pulmonary cells to W values on the one day of incubation examined (Day 16).

Tables 2 and 3 illustrate the effects of hypophysectomy and treatment with three doses of CORT upon cell number (Table 2)

TABLE 1

PULMONARY WET WEIGHT AND CONTENTS OF WATER AND PROTEIN IN THE 16-DAY NORMAL AND IN HYPOPHYSECTOMIZED CHICKEN EMBRYOS AFTER TREATMENT WITH CORTICOSTERONE-21-ACETATE OR PITUITARY REPLACEMENT[a]

Treatment[b,c]	(N)	Lung wet weight (mg)	(N)	Lung H_2O: % of wet weight	(N)	Protein per dry weight (mg/mg)
Hypox + V	(13)	151 ± 7	(6)	92.7 ± 0.8	(5)	0.70 ± 0.03
Hypox + 30 μg CORT	(12)	116 ± 9*	(6)	90.0 ± 0.06*	(6)	0.75 ± 0.01^{ns}
Hypox + 100 μg CORT	(8)	75 ± 6*	(6)	90.5 ± 0.4*	(6)	0.67 ± 0.03^{ns}
Hypox + 300 μg CORT	(15)	57 ± 3*	(6)	88.4 ± 0.2*	(6)	0.69 ± 0.02^{ns}
Hypox + PIT	(8)	139 ± 10^{ns}	(6)	91.4 ± 0.4^{ns}	(6)	0.91 ± 0.03*
W + V	(11)	145 ± 7^{ns}	(5)	87.0 ± 0.3*	(5)	0.86 ± 0.03*
		(F = 34.43)		(F = 17.13)		(F = 15.98)

[a] Values are means ± standard error of N individual samples.

[b] Abbreviations, CORT: corticosterone-21-acetate, Hypox: hypophysectomized, N: number of samples, PIT: pituitary transplantation, V: vehicle, and W: windowed controls.

[c] In each column, statistical comparisons were made between all treatment groups and Hypox + V (control). Also shown are values of the W + V group to indicate the state of unoperated embryos. Duncan's multiple range test was used, and F value for analysis of variance is given at the bottom of each column.

* $P < 0.05$; ns, not significantly different from controls.

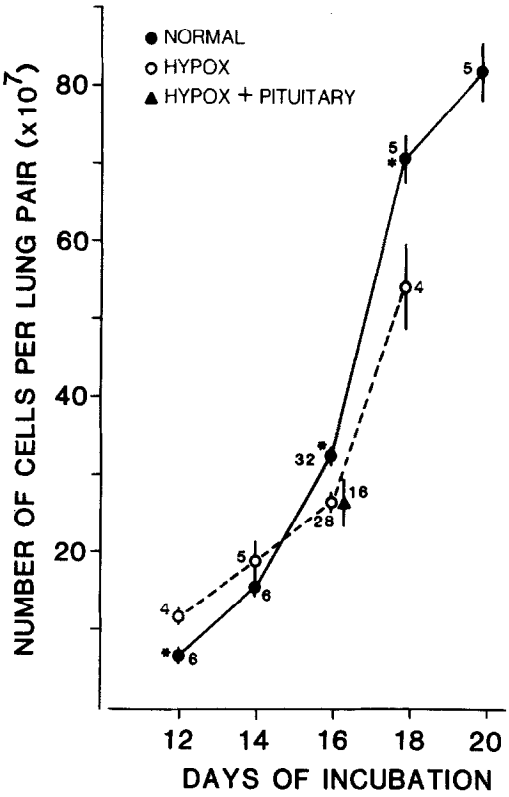

FIG. 1. Ontogeny of cell number in lungs of normal, hypophysectomized, and hypophysectomized chicken embryos receiving pituitary transplants. Values are means ± standard error (vertical bars). Sample size is indicated next to the symbols. Asterisks indicate that control values were significantly different ($P < 0.05$) from values for Hypox lungs on the same day of incubation (t test). Hypox = hypophysectomized.

and cell death (Table 3) in lungs. On Day 13 of incubation, no significant difference in cell number was observed between intact and hypox embryos. When measured after 3 days of treatment with CORT (Day 13–15), the two lowest doses (30 and 100 μg) permitted pulmonary growth in intact and hypox embryos as estimated from cell number, which exceeded Day 13 pretreatment values ($p < 0.05$, Dunnett's Test). However, CORT in amounts of 100 (W) or 300 μg (W and HYPOX), when administered for 3 days, decreased the number of pulmonary cells on Day 16 compared with saline-treated embryos in both groups. In contrast, the lowest dose of CORT (30 μg) permitted the normal increase in cell number to occur between Days 13–16 in both normal and hypox animals. It should be noted that CORT or hypophysectomy did not produce tissue-specific responses in lung growth. That is, absence of the pituitary and hormone treatment also reduced general body growth of the embryo (data not shown).

Estimation of cellular death measured from the disappearance of thymidine-labeled DNA in the lungs of intact embryos after treatment with different doses of CORT is presented in Table 3. Three hundred micrograms CORT produced a significant loss (22.1%; $P < 0.05$) of prelabeled cells. The reduction in [^{3}H]thymidine activity can be associated with the actual death of cells, rather than with cell turnover, since the total number of pulmonary cells was sharply reduced in response to the highest concentration of CORT (Table 2). The intermediate dose of CORT (100 μg) did not elicit cell death, but it was effective in attenuation of total cell number compared with untreated embryos in W and hypox groups (Table 2). Therefore, this level of CORT acted specifically to reduce the rate of cellular proliferation in the lung. In contrast, the lowest dose of CORT did not produce significant cellular death, and also permitted the normal increase in cell number (Table 2).

The effects of hypophysectomy, and of hypophysectomy with CORT treatment or pituitary transplantation, upon pulmonary lipid content are shown in Table 4. In lungs of hypox embryos, content of total lipid, total lipid phosphorous, and PC were significantly below levels observed in intact embryos ($P < 0.05$). However, treatment with CORT restored, or partially restored, these values in hypox groups. Transplantation of one pituitary gland onto the CAM of hypox embryos also produced significant elevations of total lipid, phospholipid, and PC after correction for differences in pulmonary wet weight.

TABLE 2
THE EFFECT OF VARIOUS DOSES OF CORTICOSTERONE ACETATE ON NUMBER OF CELLS IN THE LUNG OF THE NORMAL AND HYPOPHYSECTOMIZED CHICKEN EMBRYO

Day of incubation	Treatment[a]	N^b	Number of cells per lung pair ($\times 10^7$)[c]	
13	W (controls)	17	9.99 ± 0.51	
13	Hypox	6	10.80 ± 0.47[ns]	
16	W + V (controls)	17	29.93 ± 1.09	
16	W + 30 μg CORT	11	24.33 ± 1.63	
16	W + 100 μg CORT	11	19.51 ± 1.67*	$F = 52.2$
16	W + 300 μg CORT	20	12.40 ± 0.54*	
16	Hypox + V (controls)	12	22.70 ± 1.79	
16	Hypox + 30 μg CORT	6	23.51 ± 2.29	
16	Hypox + 100 μg CORT	6	15.12 ± 1.20	$F = 16.4$
16	Hypox + 300 μg CORT	13	12.48 ± 0.51*	

[a] CORT: corticosterone-21-acetate, Hypox: hypophysectomized, V: vehicle, W: windowed. Hormone was given on Days 13, 14, and 15 of incubation.
[b] N = number of individual lung pairs.
[c] Values are means ± standard error. [ns] Not significantly different from controls (T test).
* Significantly different ($P < 0.05$) from control values. (Dunnett's Test).

The rates of incorporation of [^{14}C]choline *in vitro* into pulmonary PC and DSPC following 2 days of treatment with various hormones are summarized in Table 5. The synthetic rates of both PC and DSPC were stimulated by treatment with CORT or E_2 ($P < 0.05$). Prolactin, T_4, and insulin had no statistically significant effect upon the synthetic rate of either phospholipid. The addition of T_4 in combination with CORT reduced the synthesis of both PC and DSPC to a rate intermediate between that of the control and CORT groups. The fraction of newly synthesized PC contributed by the DSPC subcomponent was similar in all treatment groups (19.5–21.9%).

Incorporation of [^{14}C]choline into PC and DSPC by lung slices from 17-day embryonic chickens after 3 days treatment with CORT, HYCORT, and E_2 is presented in Table 6. In this experiment, the two adrenal corticoids and E_2 did not alter the synthetic rate of pulmonary PC on Day 17. However, synthesis of the DSPC component was specifically stimulated by administration of CORT ($P < 0.05$); HYCORT or E_2 had no

TABLE 3
MEASUREMENT OF CELLULAR DEATH IN THE EMBRYONIC AVIAN LUNG AFTER ADMINISTRATION OF CORTICOSTERONE-21-ACETATE

Day of incubation	Treatment[a]	(N)	% of [^{3}H]Thymidine remaining in the lung[b]
13	W (control)	(10)	100.0
16	W + V	(7)	100.3[ns]
16	W + 30 μg CORT	(5)	102.5[ns]
16	W + 100 μg CORT	(5)	93.4[ns]
16	W + 300 μg CORT	(7)	77.9*

[a] Hormone was administered on Days 13, 14, and 15 of incubation. Abbreviations as in Table 1.
[b] Mean values shown. Standard error of the mean was less than 6% of the group mean in each treatment group. [ns] Not significant, Dunnett's test.
* $P < 0.05$ ($F = 3.41$), Dunnett's test.

TABLE 4

TOTAL PULMONARY LIPID, PHOSPHOLIPID, AND PHOSPHATIDYLCHOLINE IN THE 16-DAY NORMAL AND IN THE 16-DAY HYPOPHYSECTOMIZED CHICKEN EMBRYO AFTER TREATMENT WITH CORTICOSTERONE-21-ACETATE OR PITUITARY REPLACEMENT[a]

Treatment[b,c]	(N)	Total lipid per g wet weight (mg)	(N)	lipid P per g wet weight (μmol)	(N)	PC per g wet weight (μmol)
Hypox + V	(6)	14.0 ± 0.9	(6)	10.10 ± 1.03	(6)	5.07 ± 0.10
Hypox + 30 μg CORT	(6)	17.7 ± 0.8*	(6)	16.39 ± 0.42*	(6)	6.67 ± 0.23*
Hypox + 100 μg CORT	(6)	17.6 ± 1.9*	(6)	18.68 ± 0.58*	(6)	7.15 ± 0.48*
Hypox + PIT	(6)	16.3 ± 0.9*	(5)	14.65 ± 0.26*	(6)	6.70 ± 0.18*
W + V	(5)	22.0 ± 0.8*	(5)	17.58 ± 0.19*	(5)	7.46 ± 0.12*
		(F = 5.72)		(F = 29.23)		(F = 11.60)

[a] Values are means ± standard error of (N) individual samples. Corticosterone acetate or vehicle were given on Days 13, 14, and 15 of incubation.

[b] Abbreviations same as in Table 1.

[c] In each column, statistical comparisons were made between all treatment groups and Hypox + V (control). Also shown are values in the W + V group to indicate lipid content in unoperated embryos. Duncan's multiple-range test was used and F value for analysis of variance is given at the bottom of each column.

* $P < 0.05$.

stimulatory effect on DSPC synthesis. Furthermore, newly synthesized DSPC, as a percentage of newly synthesized PC, increased to 35% following CORT treatment for 3 days. In contrast, only 21% of the PC synthesized *in vitro* was contributed by DSPC in control and in HYCORT and E_2 groups ($P < 0.05$). In E_2-treated embryos the specific activity of choline in lung slices was significantly reduced compared with other groups ($P < 0.05$).

TABLE 5

INCORPORATION OF [^{14}C]CHOLINE *in Vitro* INTO PC AND DSPC BY LUNG SLICES FROM THE DAY-17 EMBRYONIC CHICKEN AFTER 2 DAYS OF HORMONE TREATMENT[a]

Treatment[c]	(N)	Nanomoles synthesized per g protein · hr^{-1}[b] PC	DSPC
W + V (controls)	(8)	16.5 ± 1.0	3.5 ± 0.4
CORT (20 μg)	(5)	21.0 ± 1.6*	4.6 ± 0.4*
E_2 (20 μg)	(4)	22.7 ± 2.4*	4.9 ± 0.5*
PRL (20 μg)	(5)	15.1 ± 0.8	3.2 ± 0.3
T_4 (100 ng)	(4)	15.3 ± 1.8	3.0 ± 0.5
CORT (20 μg) + T_4 (100 ng)	(4)	18.3 ± 1.6	3.9 ± 0.5
Insulin (20 μg)	(4)	20.5 ± 2.3	4.0 ± 0.3
		(F = 3.27)	(F = 2.70)

[a] Values are means ± standard error for N individual lung pairs after 4 hr incubation *in vitro*.

[b] Calculated from the choline specific activity determined in tissue slices. The specific activity of the choline pool was similar for lungs of all treatment groups, and averaged 9.18 ± 0.06 × 10^3 dpm/nmol (no significant differences among groups). Significance of differences between means was made using the lsd test.

[c] Abbreviations: PC = phosphatidylcholine, DSPC = disaturated phosphatidylcholine, CORT = corticosterone-21-acetate, E_2 = 17-β-estradiol, PRL = prolactin, T_4 = thyroxine.

* $p < 0.05$.

TABLE 6

RATES OF INCORPORATION OF [^{14}C]CHOLINE *in Vitro* INTO PC AND DSPC BY LUNG SLICES FROM THE DAY-17 EMBRYONIC CHICKEN AFTER 3 DAYS OF TREATMENT WITH VARIOUS STEROIDS[a]

Treatment[c]	(N)	Nanomoles synthesized per g protein · hr^{-1}[b] PC	DSPC
W + V (controls)	(15)	16.6 ± 0.6	3.5 ± 0.2
CORT (20 μg)	(7)	16.0 ± 1.8	5.6 ± 0.6*
HYCORT (20 μg)	(7)	16.9 ± 1.4	4.1 ± 0.4
E_2 (20 μg)	(8)	16.4 ± 1.2	3.5 ± 0.4
		(F = 0.035)	(F = 6.56)

[a] Values are means ± standard error for N lung pairs after 4 hr incubation *in vitro*.

[b] Calculated from choline specific activity determined in tissue slices. Specific activities (expressed as dpm/nmol choline × 10^3) were: controls—11.5 ± 0.70; corticosterone—11.42 ± 0.56; hydrocortisone—11.23 ± 0.68; 17-β-estradiol—9.63 ± 0.58.

[c] Abbreviations: CORT = corticosterone-21-acetate, HYCORT = hydrocortisone, E_2 = 17-β-estradiol.

* Significantly different from control values ($P < 0.05$) (lsd test).

DISCUSSION

These results show that pituitary hormones and corticosterone can influence growth, hydration, and lipid content of the lung of the chicken embryo. Of the specific hormones examined, CORT had the most prominent effects, both on pulmonary growth and on surfactant synthesis. CORT given alone, however, did not correct all biochemical and growth impairments observed in the lung of the hypox embryo, and was toxic when given in sufficiently high amounts.

Hormones can act on pulmonary growth in many ways. Since increased content of surfactant lipid in ontogeny has usually been normalized to protein, wet weight, dry weight, or DNA, possible independent actions of hormones on these aspects of lung growth must be considered.

Pulmonary growth in the chicken embryo in response to pituitary ablation and to corticosteroid or pituitary replacement has not been previously characterized. Whereas protein and wet weight did not attain normal levels in the lungs of Day 16 hypox embryos, pulmonary water as a percentage of the wet weight was greater than in lungs of intact controls. Pulmonary hydration in hypox embryos might have been associated with changed water permeability of lung cells, or to increased water in extracellular spaces (Marin *et al.*, 1978). Inasmuch as hydration of pulmonary cells is normally greater early in embryogenesis than later, absence of pituitary hormones or deficiencies of other hormones in hypox embryos might simply have retarded the time course of development (Hylka, 1982; Doneen and Smith, 1982a). Administration of CORT affected pulmonary weight in intact and hypox individuals (a) by reduction of water content in lungs of hypox embryos (Table 1), (b) by decreasing total number of cells when given in concentrated doses (Tables 2 and 3), and (c) by also reducing dry weight (data not shown) in the lungs of both normal and hypox individuals. CORT thus acts on several aspects of pulmonary growth. Depressed pulmonary weight after corticoid administration has also been observed in lungs of the fetal rabbit (Kotas and Avery, 1971; Carson *et al.*, 1973; Barrada *et al.*, 1980).

Most actions of CORT in the hypox embryo were not duplicated by transplantation of the embryonic pituitary gland. Pituitary transplantation did not reverse the high pulmonary hydration seen in hypox embryos. The inability of the transplant to duplicate these actions of CORT seems to indicate a lack of stimulation of the pituitary–adrenal axis. Although the transplant remained on the CAM for 7 days, during which time it became vascularized, the loss of hypothalamic connections apparently prevented release of pituitary hormones. It has been shown by Thommes and Jameson (1980) that although a transplanted pituitary on the CAM of a hypox chicken embryo could release TSH in response to exogenous TRH, the unstimulated pituitary could only partially restore thyroid function. Thus, the ectopic pituitary on the CAM probably produced suboptimal secretion of the pituitary hormones required for normal pulmonary growth and hydration. Another possibility is that the transplanted pituitary of the 10-day-old embryo had inadequate amounts of stored hormone, or failed to synthesize the amount required, to restore normal functions in the 13–16-day embryo. On the other hand, hypophyseal secretion seemed sufficient to increase pulmonary protein in hypox embryos. Thus, separate hormones responsible for various aspects of pulmonary growth and maturation may have different magnitudes of secretion from the transplant.

Hypophysectomy produced different effects on pulmonary cell number depending on the period of development. Early in incubation (Day 12), lungs from hypox embryos contained more cells than those in unoperated animals. Similar observations

have been made in lungs of rat fetuses decapitated 10 days prior to birth (Blackburn *et al.*, 1972; 1973). However, by Day 16 of incubation, lungs of hypox embryos contained fewer cells than controls. Moreover, the normal rapid phase of cellular division was delayed by 2 days in the lungs of hypox embryos (Fig. 1). Thus, the pituitary apparently restrained pulmonary cell division in the chick embryo early in organogenesis, whereas the rapid phase of cellular division occurred (albeit delayed) even in the absence of the hypophysis. Alternatively, autonomous secretion of hormones by target glands of the pituitary (i.e., adrenal, thyroid, gonads) could have been adequate for the rapid phase of pulmonary cell division.

Administration of CORT not only caused a reduction in lung size and weight in the chick embryo, but also reduced the number of cells. Although a reduced mitotic rate could account for the diminished number of cells in response to the lower doses of CORT (30 and 100 μg), the drastic reduction in cell number in response to the 300-μg dose was also accompanied by an actual loss of cells from the lung (Table 3). This proof of toxicity is important because doses as high as 1 mg/chicken embryo (Moog and Richardson, 1955; Thommes and Shulman, 1967) and 2 mg/rabbit fetus (Carson *et al.*, 1973) have been used in previous studies. One hundred micrograms of CORT reduced the mitotic rate of the lung as inferred from the diminished number of cells compared with untreated controls, and from the absence of cellular death (Tables 2 and 3). As 100 μg CORT simultaneously elevated total pulmonary phospholipid and PC content, action of the corticoid on the lung included parallel inhibition of cellular proliferation and stimulation of surfactant phospholipid production. The dual nature of corticosteroid action in the lung of the avian embryo is similar to that seen in the fetal mammal, in which cortisol inhibited increased pulmonary cell number while simultaneously promoting DSPC synthesis (Smith *et al.*, 1974). As in the chick embryo, physiological concentrations of glucocorticoid specifically attenuated the mitotic rate of lung cells in the fetus (Liggins and Kitterman, 1981).

Since hypophyseal and/or adrenal hormones directly stimulate the appearance of lamellar bodies in the avian embryonic lung (Dameron and Marin, 1978), it was important to correlate the effects of hormones on surfactant lipids (especially phospholipids and PC, which are abundant in lamellar bodies) with the previous morphological findings. The reduction of pulmonary PC content in the hypox avian embryo (Table 4) can be related to the delayed appearance of lamellar bodies in operated individuals (Marin and Dameron, 1974). Administration of CORT, as well as transplantation of one pituitary gland, elevated all aspects of pulmonary lipid content measured (per gram wet weight) compared with hypox embryos. Elevated PC in response to hypophyseal replacement represented a genuine increase in lung content, since the transplant did not significantly alter pulmonary hydration. In hypox embryos receiving CORT, some of the increased lipid content (per unit wet weight), but not all, could be attributed to the parallel reduction in lung hydration. These results suggest that hormones of the pituitary and CORT can increase the storage of synthesis of pulmonary surfactant lipids in the avian embryo; results discussed below confirm a stimulation of synthesis.

Two days of treatment (Days 15 and 16) with either CORT or E_2 increased the synthesis of PC and DSPC in the Day 17 embryonic lung; other hormones (including CORT and T_4 in combination) were ineffective. However, administration of CORT or E_2 one day earlier in incubation (beginning on Day 14), was ineffective in stimulating PC synthesis, although a selective and significant stimulation of DSPC synthesis occurred after treatment with CORT. The stimulation of DSPC synthesis after 3 days of CORT treatment must have occurred si-

multaneously with decreased synthesis of other species of PC, since synthesis of total PC (in which DSPC is a major component) was unchanged. Thus, the effect of exogenous adrenal and gonadal steroids upon incorporation of choline into pulmonary PC and DSPC depends upon the specific hormone administered and the duration of treatment.

Increased content of phospholipid, and elevated synthesis of PC or DSPC in response to CORT and E_2, coincided with the earliest period in ontogeny showing rising circulating titers of these two steroids (Kalliecharan and Hall, 1974; Woods and Brazzill, 1981). Tordet and Marin (1976) observed greater amounts of PC in lungs of female embryos (which have higher plasma E_2) than in males. We cannot decide if the stimulation of pulmonary PC or DSPC synthesis by two days of E_2 treatment was greater in male or female embryos since individuals used were not sexed. Our results, and the earlier finding that adrenal glands directly stimulate formation of lamellar bodies (Dameron and Marin, 1978), show that CORT promotes maturation of the surfactant system in the lung of the avian embryo. Its principal action is to specifically stimulate the synthesis of DSPC, the major surface-active component of avian surfactant (Hylka and Doneen, 1982).

Although Dameron and Marin (1978) observed a direct effect of the pituitary upon lamellar body formation, our study appears to exclude PRL (as judged by use of the ovine molecule) as the hypophyseal factor responsible. This conclusion must be considered provisional since oPRL treatment coincided with the rising circulating titer of endogenous PRL in which the prehatching peak occurs on Day 17 of incubation (Harvey *et al.*, 1979). It seemed that PRL might act upon pulmonary lipid since this hormone favors deposition of fat in adults of several avian species (Meier and Burns, 1976). Although PRL allegedly increases pulmonary PC and DSPC in the mammalian fetus (Hamosh and Hamosh, 1977), this result is now thought to have been due to a contaminant in the preparation used (Cox and Torday, 1981), possibly ACTH. The hypophyseal factor(s) that directly (*in vitro*) promoted differentiation of type II pneumocytes in the avian embryo (Dameron and Marin, 1978) remains to be discovered. The possibility that the unperfused pituitary glands used in the coculture experiments contained a biologically significant store of adrenal steroids in blood or pituitary cells must also be eliminated.

Thyroid hormones promote maturation of the mammalian fetal lung, and T_4 can stimulate both morphologic (Wu *et al.*, 1973) and biochemical (Smith and Torday, 1974) differentiation. One action of T_4 is to increase the number of lung glucocorticoid receptors (Morishige, 1982). Peak levels of circulating T_4 in the chicken embryo (Thommes *et al.*, 1977) occur in the late embryonic period, coincident with the highest rates of pulmonary PC and DSPC synthesis (Hylka and Doneen, 1982). However, administration of T_4 did not elevate synthetic rates of PC and DSPC in our study. Thus, T_4 either does not promote this aspect of avian pulmonary maturation, or the presence of high levels of endogenous T_4 eliminated the possibility of further stimulation by added hormone.

Insulin delays maturation of the mammalian fetal lung (Smith *et al.*, 1975; Gross and Walker-Smith, 1978). In contrast, we observed a slight (though insignificant) increase in the synthesis of pulmonary PC and DSPC in the embryonic chicken after treatment with insulin. If insulin increased plasma free fatty acids in the avian embryo, as it does in the adult chicken (Nir and Levy, 1973), the increased precursor pool might itself stimulate synthesis of surfactant phospholipids, as has recently been shown to occur in the rat lung (Feldman *et al.*, 1981).

Results of this study show that CORT, and possibly E_2, are involved in the bio-

chemical maturation of the surfactant system of avian lung, and that CORT also regulates proliferation of some or all types of pulmonary cells. Although glucocorticoids (Giannopoulos, 1974; Ballard and Ballard, 1974; Smith and Bogues, 1980) and estrogens (Morishige and Uetake, 1978; Khosla *et al.*, 1980) have a direct effect upon the mammalian fetal lung, acting through specific receptors, it is not known whether this is also true for the chicken embryo. Our data, together with the coculture experiment of Dameron and Marin (1978) strongly suggest that adrenal steroids, at least, can act directly upon the avian lung.

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