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Glucocorticoid Receptors and the Role of Glucocorticoids in Fetal Lung Development

(fetal rabbit/fetal lamb/dexamethasone/nuclear sites)

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ABSTRACT **The cellular mechanism of glucocorticoid effects upon fetal lung was examined in studies of specific binding activity for corticosteroids. Cytoplasm of fetal rabbit lung contains receptor sites for [^{3}H]dexamethasone at a concentration of 0.43 ± 0.04 pmol/mg of cytosol protein, and the apparent dissociation constant for the binding reaction is 2.7 ± 0.4 nM. The ability of various steroids to compete with labeled dexamethasone for binding to receptor correlates with their biologic potency. The hormone–receptor complex formed *in vitro* at 0° binds with high affinity at 20° to isolated lung nuclei. It is estimated that there are 9500 nuclear binding sites and 12,000 cytoplasmic receptor sites per fetal lung cell. During the last 12 days of gestation in a rabbit, the concentration of cytoplasmic receptor in lung is constant and is 2- to 5-times greater than receptor-site concentration in fetal skin, kidney, heart, muscle, gut, liver, brain, thymus, and placenta. These findings demonstrate that the early steps in the mechanism of glucocorticoid action in target tissues are present in lung cells, and suggest that these hormones accelerate fetal lung differentiation and surfactant production in animals by the induction of new protein synthesis mediated by receptor.**

Adrenal glucocorticoids induce both morphological and enzymic changes in various target tissues. In fetal rabbit and lamb, these steroid hormones cause accelerated lung development and precocious appearance of pulmonary surfactant in whole-lung homogenates and lung washes (1–3). A deficiency of surfactant in lungs of human infants is considered to be the primary cause of idiopathic respiratory distress syndrome (4), a major cause of death in premature infants.

It is not known whether corticosteroids influence the lung directly or as a consequence of their effects in other tissues. In particular, there is little information regarding the molecular mechanism of their action in fetal lung. In many target tissues the first step in the cellular action of steroid hormone involves binding of the hormone to specific cytoplasmic receptors (5, 6). The steroid–receptor complex thus formed migrates to the nucleus, where it binds to chromatin and initiates the characteristic biologic response. In most tissues this occurs by induction of specific new protein synthesis. As an initial approach to understanding the pulmonary effects of glucocorticoids, we examined fetal-rabbit lung for the presence of both specific cytoplasmic receptors and their nuclear binding sites. A preliminary report of these studies has been presented (7) (see note added in proof).

Abbreviations: K_d, equilibrium dissociation constant; HTC, hepatoma tissue culture (cells).

MATERIALS AND METHODS

Stock solutions of nonradioactive dexamethasone (gift of Merck Chemical Co.) and other steroids (Mann Research Labs) were prepared at 5 mM in absolute ethanol. Chromatographically pure (8) [*1,2,3,4*-^{3}H]dexamethasone (Schwarz Bioresearch Co., 5.8 or 12 Ci/mmol) was dissolved in an aqueous solution of 0.02 M *N*-tris-(hydroxymethyl)-methylglycine (Tricine)–2 mM $CaCl_2$–1 mM $MgCl_2$ (pH 7.4) (medium 1).

Pregnant New Zealand white rabbits were anesthetized with intravenous pentobarbitol (20 mg/kg) at 18–30 days of gestation (term is 30–31 days) and their fetuses were obtained by uterotomy. Fetal tissues from 1–3 litters were removed into cold isotonic phosphate-buffered saline (pH 7.6) and frozen at −20° for 0–2 days. Receptor activity was assayed in a cytosol fraction prepared at 2° by mincing tissue in one volume of medium 1 and then homogenizing with 6 strokes of a motor-driven (3000 rpm) Teflon pestle in a Duall Tissue Grinder (Kontes Glass Co.). The homogenate was centrifuged at 600 × *g* for 15 min, then at 100,000 × *g* for 60 min. Aliquots of the supernatant were added immediately to reaction mixtures containing [^{3}H]dexamethasone at concentrations from 1 to 100 nM. Tubes containing in addition an excess of unlabeled dexamethasone (10 μM) were run in parallel for each [^{3}H]dexamethasone concentration to determine "background" binding activity (6). Binding was complete by 90–120 min at 0°, and was proportional to cytosol protein concentration between 2 and 20 mg/ml. Macromolecular bound radioactivity was determined by a charcoal assay (6) that utilizes the property of activated charcoal to absorb free, but not bound, steroid. Results are plotted as [^{3}H]dexamethasone specifically bound (total radioactivity excluded from charcoal minus "background" counts) against free dexamethasone (total dexamethasone added minus the amount bound).

Fetal lung nuclei were prepared from the 600 × *g* pellet by washing in medium 1 twice and once in medium 1 containing 0.25 M sucrose. Pellets of nuclei were combined with aliquots of cytosol previously incubated at 0° with a saturating concentration of [^{3}H]dexamethasone, with or without 10 μM unlabeled dexamethasone, and the nuclear transfer reaction was run in medium 1 containing 0.25 M sucrose at 20° for 1 hr. Nuclei were then sedimented at 600 × *g* for 5 min, and the supernatant cytosol was removed for assay of remaining radioactivity. After two washes in medium 1 containing sucrose, nuclei were suspended in water; an aliquot was counted

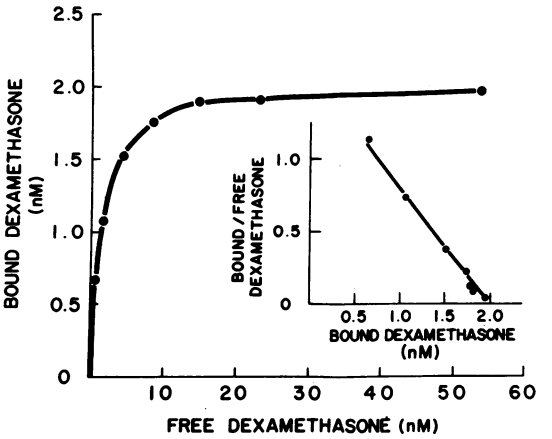

FIG. 1. Specific binding of dexamethasone by cytosol of 27-day fetal-rabbit lung. Reactions were performed as described in *Methods* with [^{3}H]dexamethasone (5.8 Ci/mmol.) Cytosol protein concentration was 4.9 mg/ml. *Inset:* Scatchard plot of binding data.

to determine nuclear-bound radioactivity. Nuclei were counted with a hemocytometer, and protein concentration was assayed by the method of Lowry (9).

RESULTS AND DISCUSSION

Assay of cytoplasmic receptor

Cytosol prepared from 27-day fetal-rabbit lung contains specific receptors for dexamethasone that are saturated at a hormone concentration of about 20 nM (Fig. 1). A Scatchard plot (10) of the binding results (*inset* of Fig. 1) is linear, suggesting that lung cytosol contains a single class of receptor sites. The apparent equilibrium dissociation constant (K_d) of dexamethasone at 0° for the binding reaction is 2.7 nM, and the concentration of receptor sites is 0.43 pmol/mg of cytosol protein (mean values in 11 experiments).

We also measured receptor activity in cytosol prepared in the same manner from the lung of 130- to 140-days gestation fetal lamb, another species in which glucocorticoids influence surfactant production. The mean concentration in four experiments was 0.36 (range 0.18–0.52) pmol/mg of protein, and the mean K_d was 5.8 (range 5.0–6.9) nM. These values for K_d and receptor-site concentration in fetal lung of both rabbit and lamb are similar to those found in rat hepatoma tissue culture (HTC) cells, where the binding of steroid hormone to cytoplasmic receptor mediates the induction of tyrosine aminotransferase (EC 2.6.1.5) and a surface factor (6).

The effect of various unlabeled steroids on the binding of [^{3}H]dexamethasone by cytosol of fetal-rabbit lung and HTC cells is shown in Table 1. 10 μM cortisol, corticosterone, dexamethasone, 11β-hydroxyprogesterone, and 5α-dihydrocortisol each reduced specific binding of 10 nM [^{3}H]dexamethasone by lung cytosol to 0–5% of the control value. These compounds also abolish specific binding in HTC cells (11), where they have either optimal or suboptimal inducing activity (12). Testosterone and its 17α-methyl derivative, both anti-inducers, competed significantly for dexamethasone binding. By contrast, the inactive steroids epicortisol or androstenedione only decreased binding to 82 and 91% of the control value, respectively. These results suggest that the specific cytoplasmic receptors of fetal-lung and HTC cells are similar. In addition, they demonstrate a correlation between the affinity of steroids for receptor and their biologic potency.

Nuclear binding of receptor–steroid complex

Migration of cytoplasmic receptor–steroid complex to nuclei is an early step in the action of steroid hormones in many target cells (5, 13). If the effects of glucocorticoids in fetal lung also involve regulation at the nuclear level, transfer of steroid by receptor to the nucleus should occur. We examined the ability of fetal-lung receptor to bind to lung nuclei in experiments where isolated nuclei were incubated *in vitro* with cytosol containing [^{3}H]dexamethasone–receptor complex. In the complete system (line 1 of Table 2), under the conditions used, about 56% of the labeled dexamethasone was transferred from cytosol to nucleus. No transfer of dexamethasone above "background" levels occurs in the absence of cytosol. If incubation is performed at 0°, the transfer is less than 10% of that at 20°. Cytosol heated to 50° loses more than 90% of its receptor activity, resulting in a similar decrease in the transfer of steroid to nuclei. Nuclear transfer is also reduced when there is dissociation of steroid from receptor, produced by treatment of the cytosol with charcoal.

The demonstration of this step in glucocorticoid action *in vitro* allowed us to determine the amount of binding of steroid–receptor complex to nuclei as a function of bound receptor concentration. Results of such an experiment with

TABLE 1. *Effect of various steroids on binding of [^{3}H] dexamethasone by cytoplasmic extracts*

Nonradioactive steroid added (10 μM)	Biological activity*	[^{3}H] Dexamethasone bound by cytosol (% of control)	
		Fetal lung	HTC†
None	—	100	100
Dexamethasone	Optimal inducer	0	0
Cortisol	Optimal inducer	2	0
Corticosterone	Optimal inducer	3	0
11β-Hydroxyprogesterone	Suboptimal inducer	0	0
5α-Dihydrocortisol	Suboptimal inducer	5	10
Testosterone	Anti-inducer	24	27
17α-Methyltestosterone	Anti-inducer	18	11
Epicortisol	Inactive	82	99
Androstenedione	Inactive	91	79

Cytosol (5.8 mg of protein per ml) prepared from 29-day gestation fetal-rabbit lung was incubated at 0° for 90 min with 10 nM [^{3}H]dexamethasone (5.8 Ci/mmol) in the presence of 10 μM unlabeled steroid. The control value (100%) in the absence of unlabeled steroid represents 6425 cpm/ml bound; "background" binding (see *Methods*) in the presence of 10 μM unlabeled dexamethasone was 570 cpm/ml.

* Classified by capacity to act as an inducer in hepatoma tissue culture cells (12).

† Data of Baxter (6) from hepatoma tissue culture cells.

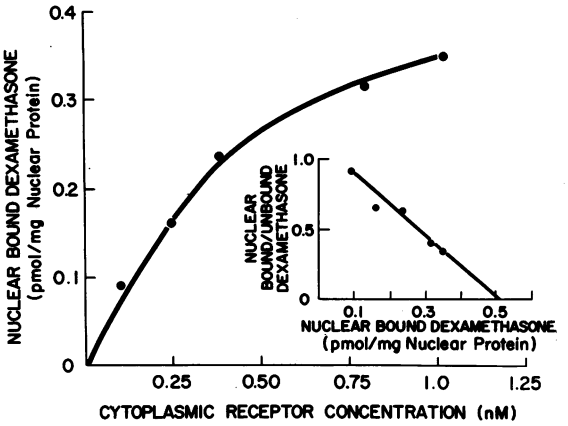

FIG. 2. Binding of dexamethasone by isolated lung nuclei as a function of the concentration of dexamethasone–receptor complex. Different amounts of cytosol from 23-day fetal-rabbit lung exposed to 20 nM [^{3}H]dexamethasone (12 Ci/mmol) were incubated with nuclei (1.7 mg of protein) prepared from the same tissue. Transfer of specifically bound dexamethasone to nuclei was assayed as described in *Methods*. *Inset:* Scatchard analysis of the data.

23-day fetal-rabbit lung are shown in Fig. 2. Nuclear binding of dexamethasone is proportional to receptor concentration at low concentrations, and approaches saturation at concentrations greater than 1 nM. At the lowest concentration of receptor complex, transfer from the cytosol amounted to more than 70% of the specifically bound dexamethasone. This result indicates that under appropriate conditions most of the bound steroid migrates to the nucleus. When the data of Fig. 2 are plotted by the method of Scatchard (inset), a linear relationship is obtained that extrapolates to a value of 0.51 pmol of dexamethasone bound per mg of nuclear protein. The apparent K_d is 0.46 nM under the conditions used, indicating a very high affinity of steroid–receptor complex for the nuclear acceptor sites. Similar values were found in lungs of older fetal rabbits, and also in preliminary experiments with 140-day fetal-lamb lung.

TABLE 2. *Properties of the binding of dexamethasone by isolated lung nuclei*

Assay	Dexamethasone specifically bound after incubation (pmol/reaction mixture)	
	Nuclei	Cytosol
Complete	0.508	0.393
Minus cytosol	0	0
Incubation at 0°	0.040	1.062
Heat-inactivated cytosol	0.027	0.033
Charcoal-treated cytosol	0.297	0.183

The complete reaction mixture contained nuclei from 27-day fetal-rabbit lung (1.8 mg of nuclear protein), 20 nM [^{3}H]dexamethasone (12 Ci/mmol), lung cytosol (2.8 mg of protein) incubated for 150 min at 0° with dexamethasone, and medium 1 containing 0.25 M sucrose, in a total volume of 0.8 ml. Incubation was at 20°. Total nuclear binding was 7733 cpm, which was corrected for 1773 cpm of "background" binding. Heat-inactivated cytosol was incubated for 20 min at 50° before incubation with dexamethasone, and charcoal-treatment of cytosol was performed after the incubation at 0°.

TABLE 3. *Concentration and apparent dissociation constant of receptor in tissues of fetal rabbit*

Tissue	Gestational age (days)	Number of experiments	Receptor site concentration (pmol/mg of protein)	Apparent dissociation constant for dexamethasone (nM)
Lung	18–29	11	0.43 ± 0.04 (0.28–0.72)	2.7 ± 0.4 (1.2–5.5)
Fetal placenta	16–26	4	0.26 ± 0.02 (0.22–0.33)	3.7 ± 0.5 (3.0–5.1)
Skin	18–28	3	0.22 (0.17–0.28)	2.5 (1.8–3.0)
Kidney	28	1	0.19	2.2
Heart	27–29	3	0.17 (0.15–0.19)	6.3 (4.2–8.4)
Muscle	19–29	6	0.14 ± 0.01 (0.10–0.20)	2.6 ± 0.4 (1.0–3.2)
Small intestine	23–28	2	0.12 (0.11–0.13)	8.0 (7.5–8.5)
Liver	18–30	10	0.12 ± 0.01 (0.09–0.17)	5.0 ± 0.4 (3.5–7.2)
Brain	18–28	4	0.08 ± 0.01 (0.06–0.13)	5.7 ± 0.4 (4.6–6.7)
Thymus	29	2	0.07 (0.06–0.08)	5.5 (3.0–8.0)
Maternal placenta	16–26	4	0	—

Binding reactions of the type shown in Fig. 1 were performed with dexamethasone, as described in *Methods*. Mean value, standard error, and range are shown for receptor concentration and K_d.

If it is assumed that most types of lung cells contain the glucocorticoid receptor, we estimate that each cell contains on the average 9500 nuclear sites and 12,000 cytoplasmic receptor sites. The observation that there are about equal numbers of cytoplasmic and nuclear binding sites is in agreement with the findings in cultured hepatoma cells, and supports a proposed model for the cellular mechanism of glucocorticoid action (13).

Receptor in other fetal tissues

Specific cytoplasmic receptors have been found in all steroid hormone-responsive tissues examined thus far; furthermore, the development of hormone resistance in certain tissues is associated with a decreased concentration of receptor (14, 15). Therefore, we examined receptor concentration in other fetal tissues for comparison with lung, and as an indicator of which fetal tissues potentially might respond to glucocorticoids. Fetal lung contained 2- to 5-fold greater concentration of receptor sites than any of the other tissues examined. (Table 3). The maternal placental site did not have detectable re-

ceptor activity. The apparent K_d for the tissues ranged from 2.2 to 8.0 nM. The higher apparent K_d observed in some experiments may reflect increased levels of endogenous glucocorticoids, which would compete to some extent with labeled dexamethasone. Scatchard plots for dexamethasone binding in tissues other than lung also were linear. Thus, it appears that many fetal tissues contain similar cytoplasmic receptor systems, although at lower concentrations than observed in lung. It is not yet established, however, whether these receptors also undergo translocation to nuclear binding sites.

Glucocorticoid hormones induce specific enzymes in embryonic retina (*16*) and pancreas (17), fetal gut (18), and newborn liver (19); however, effects of corticosteroids have not been described in most fetal tissues. The presence of specific cytoplasmic receptors in many fetal tissues of rabbits suggests that glucocorticoids may exert numerous yet unrecognized developmental effects. It is possible that the concentration of receptor determines the responsiveness of a tissue to glucocorticoids. Lower concentrations of receptor in some fetal tissues, however, could result from the relative deficiency or absence of receptor in certain cell types. In an adult rat, for example, McEwen (20) finds a severalfold variation in the extent of corticosterone uptake in different regions of the brain. In other tissues with relatively homogeneous cell populations, such as skeletal muscle and heart, this possibility seems less likely. Cytosol made from whole fetal lung has receptor levels similar to those found in homogeneous populations of cultured hepatoma cells and lymphoma cells (11, 15), suggesting that most or all of the constituent cell types of lung contain adequate levels of cytoplasmic receptors for biologic activity.

Ontogeny of lung receptor

A wide distribution of receptor among different pulmonary cell types would be consistent with the observation that administration of glucocorticoids to fetal rabbits stimulates morphological development of many lung cell types, including the type II alveolar cell that is thought to be the site of surfactant synthesis (1). These effects of adrenal hormone on cell differentiation occur as early as 19 days of gestation; however, there is no evidence for stimulation of alveolar surfactant by exogenous glucocorticoids before 26 days (2). We find that cytoplasmic receptor is present at 18 days of gestation, and that its concentration remains constant until birth; values at 18 and 29 days, for example, were identical (0.38 pmol/mg of protein). It was not technically possible to obtain lung tissue from younger fetuses; however, cytosol prepared from whole 16-day rabbit fetus contained receptor at a concentration of 0.13 pmol/mg of protein. These data indicate that cytoplasmic receptor appears early in gestation and probably is not normally the limiting factor in development of fetal-lung responsiveness to either endogenous or administered glucocorticoids.

Biological implications

We have presented evidence that fetal lung contains specific cytoplasmic receptors that bind glucocorticoids, then migrate to the nucleus. These findings are consistent with a direct role for glucocorticoids in both normal and accelerated pulmonary development. Furthermore, they suggest that adrenal hormones induce specific proteins in fetal-lung cells via the receptor system in a manner analogous to the induction of specific enzymes in adult liver. The observation that glucocorticoids stimulate appearance of pulmonary surfactant in animals is of particular interest with regard to human-lung development and the occurrence of idiopathic respiratory distress syndrome. In a preliminary study of fetal-human lung (data not shown), we found cytoplasmic receptors and nuclear binding sites present at 14 weeks of gestation, several weeks before tissue surface-active material has been detected (21). Since tests are available to estimate the amount of surfactant present before birth (22), preventive *in utero* therapy with glucocorticoids for infants deficient in surfactant is an exciting possibility.

NOTE ADDED IN PROOF

In a recent report, Giannopoulos G., Mulay, S. & Solomon, S. (1972) *Biochem. Biophys. Res. Commun.* **47**, 411–418, demonstrate specific nuclear uptake of [^{3}H]cortisol by minces of fetal-rabbit lung. Preliminary evidence for binding of cortisol to lung cytoplasmic macromolecules distinct from cortisol-binding globulin on gel filtration is also presented.

The method for assay of nuclear transfer was generously provided by G. G. Rousseau and J. D. Baxter before publication (13). We thank G. T. Tomkins, J. A. Clements, and W. H. Tooley for their advice and support and A. C. G. Platzker for specimens of fetal-lamb lung. This work was supported in part by Grants HL-06285, HL-5251, and HL-14201 from the National Heart and Lung Institute.

1. Kikkawa, J., Kaibara, M., Motoyama, E. K., Orzalesi, M. M. & Cook, C. D. (1971) "Morphologic development of fetal rabbit lung and its acceleration with cortisol," *Amer. J. Pathol.* **64**, 423–433.
2. Motoyama, E. K., Orzalesi, M. M., Kikkawa, Y., Kaibara, M., Wu, B., Zigas, C. J. & Cook, C. D. (1971) "Effect of cortisol on the maturation of fetal rabbit lungs," *Pediatrics* **48**, 547–555; Kotas, R. V. & Avery, M. E. (1971) "Accelerated appearance of pulmonary surfactant in the fetal rabbit," *J. Appl. Physiol.* **30**, 358–361.
3. Platzker, A. C. G., Kitterman, J. A., Tooley, W. H. & Clements, J. A. (1972) "Surfactant appearance and secretion in fetal lamb lung in response to dexamethasone," *Pediat. Res.* **6**, 406; DeLemos, R. A., Shermeta, J. W., Knelson, J. H., Kotas, R. & Avery, M. E. (1970) "Acceleration of appearance of pulmonary surfactant in the fetal lamb by administration of corticosteroids," *Amer. Rev. Resp. Dis.* **102**, 459–461.
4. Avery, M. E. & Mead, J. (1959) "Surface properties in relation to atelectasis and hyaline membrane disease," *Amer. J. Dis. Child.* **97**, 517–523.
5. Jensen, E. V., Numata, M., Brecher, P. I. & DeSombre, E. R. (1971) in *The Biochemistry of Steroid Hormone Action*, ed. Smellie, R. M. S. (Academic Press, London), pp. 133–159; Raspé, G. (Ed.) (1971) "Schering Workshop on Steroid Hormone Receptors," in *Advan. Biosci.* (Pergamon Press, Vieweg), Vol. 7.
6. Baxter, J. D. & Tomkins, G. M. (1971) "Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells," *Proc. Nat. Acad. Sci. USA* **68**, 932–937.
7. Ballard, P. L. & Ballard, R. A. (1972) "Glucocorticoid receptors in the fetal lung," *Pediat. Res.* **6**, 338.
8. Baxter, J. D. & Tomkins, G. M. (1970) "The relationship between glucocorticoid binding and tyrosine aminotransferase induction in hepatoma tissue culture cells," *Proc. Nat. Acad. Sci. USA* **65**, 709–715.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) "Protein measurement with the folin phenol reagent," *J. Biol. Chem.* **193**, 265–275.
10. Scatchard, G. (1949) "The attractions of proteins for small molecules and ions," *Ann. N.Y. Acad. Sci.* **51**, 660–672.
11. Rousseau, G. G., Baxter, J. D. & Tomkins, G. M. (1972) "Glucocorticoid receptors: relations between steroid binding biological actions," *J. Mol. Biol.* **67**, 99–115.

12. Samuels, H. H. & Tomkins, G. M. (1970) "Relation of steroid structure to enzyme induction in hepatoma tissue culture cells," *J. Mol. Biol.* **52**, 57–74.
13. Baxter, J. D., Rousseau, G. G., Benson, C., Garcea, R. L., Ito, J. & Tomkins, G. M. (1972) "Role of DNA and specific cytoplasmic receptors in glucocorticoid action," *Proc. Nat. Acad. Sci. USA*, **69**, 1892–1896.
14. Gehring, U., Tomkins, G. M. & Ohno, S. (1971) "Effect of the androgen-insensitivity mutation on a cytoplasmic receptor for dihydrotestosterone," *Nature New Biol.* **232**, 106–107; McGuire, W. L., Huff, K., Jennings, A. & Chamness, G. C. (1972) "Mammary carcinoma: a specific biochemical defect in autonomous tumors," *Science* **175**, 335-336; Kirkpatrick, A. F., Milholland, R. J. & Rosen, F. (1971) "Stereospecific glucocorticoid binding to subcellular fractions of the sensitive and resistant lymphosarcoma P1798," *Nature New Biol.* **232**, 216–218; Hackney, J. F., Gross, S. R., Aronow, L. & Pratt, W. B. (1970) "Specific glucocorticoid binding macromolecules from mouse fibroblasts growing in vitro," *Molec. Pharmacol.* **6**, 500–512.
15. Rosenau, W., Baxter, J. D. & Tomkins, G. M. (1972) "Mechanism of resistance to steroids: glucocorticoid receptor defect in lymphoma cells," *Nature New Biol.* **237**, 20–24.
16. Piddington, R. & Moscona, A. A., (1967) "Precocious induction of retinal glutamine synthetase by hydrocortisone in the embryo and in culture. Age-dependent differences in tissue response," *Biochim. Biophys. Acta* **141**, 429-432.
17. Yalonsky, U., Zelikson, R. & Kulka, R. G. (1969) "The effect of hydrocortisone on the accumulation of amylase in embryonic chick pancreas," *FEBS Lett.* **2**, 323-326.
18. Moog, F. (1971) in *Hormones in Development*, eds. Hamburgh, M. & Barrington, E. J. W. (Appleton-Century-Crofts, New York), pp. 143–160.
19. Jacquot, R. (1971) in *Hormones in Development*, eds. Hamburgh, M. & Barrington, E. J. W. (Appleton-Century-Crofts, New York), pp. 587–599.
20. McEwen, B. S., Magnus, C. & Wallach, G. (1972) "Soluble corticosterone-binding macromolecules extracted from rat brain," *Endocrinology* **90**, 217–226.
21. Platzker, A. C. G., Clements, J. A. & Tooley, W. H., (1971) "Surfactant development in the human fetal lung," *Clin. Res.* **19**, 232.
22. Clements, J. A., Platzker, A. C. G., Tierney, D. F., Hobel, C. J., Creasy, R. K., Margolis, A. J., Thibeault, D. W., Tooley, W. H. & Oh, W. (1972) "Assessment of the risk of the respiratory distress syndrome by a rapid new test for surfactant in amniotic fluid," *N. Engl. J. Med.* **286**, 1077; Gluck, L., Kulovich, M. V., Borer, R. C., Brenner, P. H., Anderson, G. G. & Spellacy, W. N. S. (1971) "Diagnosis of the respiratory distress syndrome by amniocentesis," *Amer. J. Obstet. Gynecol.* **109**, 440–445.