Proceedings of the National Academy of Sciences
Vol. 65, No. 3, pp. 709–715, March 1970

The Relationship between Glucocorticoid Binding and Tyrosine Aminotransferase Induction in Hepatoma Tissue Culture Cells

John D. Baxter and Gordon M. Tomkins*

LABORATORY OF MOLECULAR BIOLOGY, NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated by E. R. Stadtman, August 25, 1969

Abstract. Glucocorticoid receptors with many properties suggesting their involvement in the hormonal induction of tyrosine aminotransferase have been detected in rat hepatoma tissue culture cells. These "specific receptors" approach saturation as the steroid concentrations required for maximal induction are reached. Inducer analogs influence cortisol binding to the specific receptors in direct relation to their influence on cortisol induction. The inducer association with and dissociation from specific receptors are rapid enough to account for the kinetics of induction and deinduction. Chromatographic studies suggest the specific receptors bind unaltered dexamethasone and cortisol. "Nonspecific" association of steroids with the cells also occurs and can be distinguished from specific binding.

Introduction. Steroid hormones induce the synthesis of tyrosine aminotransferase by rat hepatoma tissue culture cells.[1–3] A cytoplasmic macromolecule which binds cortisol and other steroids has been found[4] in these cells, but certain of its properties suggested that it does not mediate the enzyme induction.[4] Therefore, we examined cellular inducer binding in a more general way and report here the presence of steroid receptors with properties more consistent with their involvement in the induction.

Materials and Methods. Dexamethasone[5] (a gift of Merck, Sharp and Dohme) and other steroids (Mann Research Laboratories, Inc.), were of satisfactory purity.[6] Dexamethasone-1,2-^{3}H (Schwarz BioResearch Co., 4 Ci/mM) and cortisol-1,2-^{3}H (New England Nuclear Corp., 44 Ci/mM) were at least 97% radiochemically pure by thin-layer chromatography (see below). Radioactive steroids in benzene:ethanol, 9:1, were added to (serum free) induction medium[6] and the benzene removed by evaporation under N_2. Nonradioactive steroids in ethanol, or ethanol alone were added to induction medium. The final ethanol concentration was usually about 0.05%.

Rat hepatoma tissue culture cells were grown in spinner culture.[1, 6] The serum in the media had been heated at 56° for 30 min which largely destroys the binding capacity of transcortin.[7] For steroid uptake experiments, cells were harvested by centrifugation at densities of 10^5 to 1.9×10^6/ml and were then washed once with and resuspended in induction medium at 37° before addition of the steroid. Incubations were performed in a gyrotory shaker bath (75–125 rpm) at 37° for 30 min at cell densities from 5×10^5 to 2×10^7 cells/ml, unless stated otherwise. For kinetic experiments lasting more than 30 min, a cell density less than 10^6/ml was used, since both steroid uptake and tyrosine

aminotransferase induction were markedly decreased at higher densities during longer incubations.

When ^{3}H-steroid uptake to whole cells was studied, ^{14}C-inulin (New England Nuclear Corp.), which is excluded from the cells,[6] was added to the cell suspension near the end of the incubation period. The samples were then centrifuged at 600 to 800 $\times$ g for 3 to 5 min. The ^{3}H cpm in the supernatant medium was used to calculate the steroid concentration in the incubation medium at equilibrium. Cell-associated radioactivity was determined by subtracting the ^{3}H cpm due to extracellular steroid in the cell pellet (estimated by multiplying the ^{14}C in the pellet by the ratio of ^{3}H to ^{14}C in the supernatant medium) from the total ^{3}H in the pellet. Extracellular ^{3}H trapped in the cell pellet was always less than 14% of the total.

When uptake by cell fractions was examined, cell suspensions, after incubation with steroid, were chilled for about 15 min and centrifuged at 600 to 800 $\times$ g for 3 to 5 min. Further procedures were performed at 0 to 4°. The chilled pellet was washed once with phosphate-buffered saline,[8] resuspended in at least 10 vol of 20 mM N-Tris-(hydroxymethyl)-methyl glycine (Tricine), 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 6.1), and homogenized in a Duall Tissue Grinder using a motor driven Teflon pestle (Kontes Glass Co.) at 3000 rpm for 8 to 10 strokes. The homogenate was centrifuged at about 800 $\times$ g for 5 min to separate the supernatant layer, "homogenate supernate," from the pellet, "crude nuclei." The pellet, examined microscopically after staining with 0.1% Azure C, contained nuclei with many cytoplasmic tags and some debris, most of which could be removed by washing the crude nuclei twice with 0.25 M sucrose, 3 mM $MgCl_2$, 20 mM Tricine (pH 7.6) to yield "purified nuclei." Variations from these procedures are mentioned in the individual experiments.

Aliquots of supernatant fractions and pellets (resuspended in water) were counted for radioactivity in 10 ml of toluene with 8 gm/liter Butyl-PBD (Ciba Corp.) and 10% Biosolv (Formula BBS-3, Beckman Instrument Co.). Protein concentrations were determined by either a modified biure method[9] or by the method of Lowry *et al.*[10] Tyrosine aminotransferase was assayed as described[6] by the method of Diamondstone.[11]

^{3}H-steroid stock solutions and extracts of various experimental fractions were examined by thin-layer chromatography. Extractions were performed with methylene chloride twice before and a third time after heating samples at 65 to 75° for 5 to 10 min. Extraction yields were at least 92–96%. The solvent systems used,[6] (1) ethyl acetate:chloroform (65:35 or 50:50) and (2) acetone:methylene chloride (30:70), separate the major cortisol metabolites and dexamethasone migrates about 0.25 in system 1 and about 0.5 in system 2 relative to the solvent front.

Results. Uptake of steroid by hepatoma tissue culture cells: Dexamethasone uptake by whole cells increases with increasing steroid concentration (Fig. 1*A*). Above 5 $\times$ 10^{-8} M, uptake is approximately linear, but below that concentration there appear to be components which become saturated. To illustrate this, these data are replotted in Figure 1*B* as the distribution (Q) of steroid between cells and incubation medium (see the legend of Fig. 1) as a function of the incubation concentration of steroid. For a class of receptors which reversibly binds steroids and approaches saturation at physiologic steroid concentration, a plot of Q as a function of the steroid concentration ($\log_{10}$M) would be almost horizontal at very low concentrations and would then decrease as M approaches the dissociation constant for the steroid-receptor complex. Q would finally approach zero at high steroid concentrations. However, if this class of specific receptors exists in the presence of other "nonspecific" compartments which take up steroid linearly over the concentration range studied, Q, after descending, would level off at a value greater than zero as the sites of higher affinity become saturated and uptake by the other compartments proceeds.

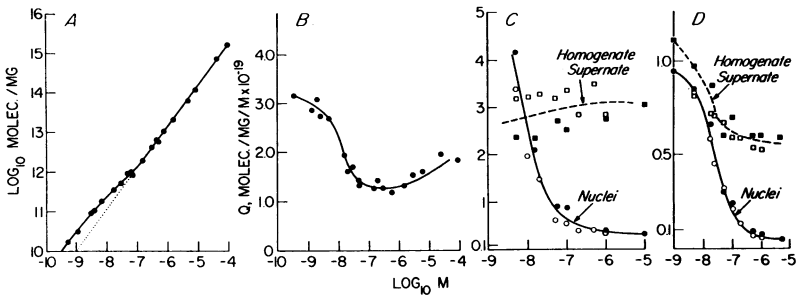

FIG. 1.—(*A*). Uptake of ^{3}H-dexamethasone by hepatoma tissue culture cells. Uptake was measured as described in the text and is shown as $\log_{10}$ molecules of cell-associated steroid/mg cell protein ($\log_{10}$ molecule/mg) vs molar steroid concentration at equilibrium ($\log_{10}$M). The approximate straight line above 5×10^{-8}–10^{-7} *M* is extrapolated (*dotted line*) to the baseline in order to demonstrate the difference in slope of the uptake curve below 5×10^{-8} *M* steroid concentration.

(*B*) The data shown in Fig. 1*A* replotted as the distribution of steroid between cells and medium at equilibrium, Q (Q = number of steroid molecules taken up per milligram protein in the sample divided by the molar concentration of steroid in the medium at equilibrium, i.e., molecule/mg/M) as a function of the $\log_{10}$ of steroid concentration at equilibrium ($\log_{10}$M).

(*C and D*). Uptake of radioactive steroid (^{3}H-dexamethasone in 1*C* and ^{3}H-cortisol in 1*D*) by hepatoma tissue culture cells measured in cell fractions and plotted as Q vs $\log_{10}$M. Other details are given in the text. Shaded and open circles or squares refer to different experiments. ○,● nuclear uptake. □,■, homogenate supernate uptake.

The data in Figure 1*B* conform reasonably well to the latter prediction[12] and suggest that there are dexamethasone receptors (referred to as "specific" receptors) which approach saturation between 5×10^{-8} and 10^{-7} *M*, the same concentration range as that required for the maximal induction of tyrosine aminotransferase by dexamethasone.[6] On the other hand, the amount of "nonspecifically" associated steroid increases linearly with steroid concentration and accounts for most of the cell-associated hormone when more than 10^{-8} *M* dexamethasone was used in the incubations. Such nonspecific cell-steroid interaction has also been observed in other studies of steroid binding to specific sites in target tissues.[13–15]

To characterize the specific receptors further, cells were incubated with dexamethasone or cortisol and fractionated into purified nuclear and homogenate supernate fractions as described in the *Materials and Methods* (Figs. 1*C* and *D*). In the nuclear fraction, Q decreased markedly with increasing M and this was irregularly seen with the homogenate supernate.[16] Therefore, specific receptors appear to be located in the nuclear fraction, although these data do not demonstrate that they are located in the nucleus itself. Note that Q for the nuclear fraction levels off around 5×10^{-8} *M* dexamethasone and around 5×10^{-7} to -10^{-6} *M* cortisol which in both cases roughly corresponds to the maximally effective concentration of these steroids as inducers.[6]

The estimated specific binding using uptake by whole cells was examined by the Scatchard technique,[17] and although the scatter of the points did not allow accurate determinations, if single site binding were assumed, the dissociation constant for the steroid-specific receptor complex was estimated to be about 10^{-8}

M for dexamethasone and the number of specific receptor sites about 10^5 per cell.

Since the specific receptors are easily saturated, their association with hormone was subsequently approximated by comparing the difference in binding of radioactive steroid in the absence or presence of excess nonradioactive steroid. The latter competes only for specifically bound steroid since the nonspecific association increases linearly with increasing concentration (Fig. 1*A*). Usually, 5×10^{-9} M dexamethasone or cortisol was used for these competition experiments, since at this concentration, most of the nuclear uptake (cf. Figs. 1*C* and *D*) is to specific receptors. Competition for specific binding can be demonstrated even when the nonradioactive steroid is added after incubation of cells with the radioactive steroid which suggests an equilibrium between free and bound steroid.

Specific binding was also demonstrated when cells were incubated with cycloheximide (10^{-4} M) for 30 minutes or actinomycin D (7.5 μg/ml) for 2.5 hours or suspended in phosphate-buffered saline for 2.5 hours before adding radioactive steroid. In other preliminary experiments, purified nuclei incubated with ^{3}H-dexamethasone showed less than 20 per cent of the specific binding of nuclei prepared from whole cell incubations.

Kinetics of association and dissociation: The effect of steroid hormones on tyrosine aminotransferase induction can be detected within 30 minutes.[18] Figure 2*A* shows that at 5×10^{-9} M, uptake of ^{3}H-dexamethasone is maximal in about 30 minutes and at 10^{-6} M within about 10 minutes. Binding to specific nuclear receptors at 5×10^{-9} M dexamethasone was also maximal within 30 minutes, while the homogenate supernate uptake was maximal within 5 minutes (Fig. 2*B*). Therefore, steroid uptake is rapid enough to account for the kinetics of enzyme induction. Curiously, after 24 hours, a time when the tyrosine aminotransferase concentration was high, the steroid bound to specific receptors was somewhat less than after 30 minutes, and as shown below this result could not be attributed to dexamethasone metabolism. The rate of association of steroid with specific nuclear receptors is diminished when cells are incubated at 0 to 4°. A temperature dependence also exists for glucocorticoid binding to thymocyte receptors.[19]

When tyrosine aminotransferase is induced and steroid concentration is then decreased (by washing or dilution) or an "anti-inducing" steroid is added, the rate of enzyme synthesis decreases within 30 minutes.[6, 18, 20] Figure 2*C* shows that dissociation of bound steroid is also rapid when cells after incubation with steroid are resuspended in steroid-free medium. Most of the steroid bound after incubation at 5×10^{-9} M dexamethasone dissociates within 30 minutes, while that from the incubation with the additional nonradioactive steroid at 10^{-6} M dissociates within 5 minutes. Also, the efflux at 0–4° is slower than at 37°.

Effect of analogs on inducer uptake by specific receptors: The data in Figure 1 shows the relative affinity for the specific receptors for two inducers, dexamethasone and cortisol, is similar to their relative effectiveness as inducers. Certain other steroids inhibit cortisol induction and may either induce tyrosine aminotransferase to a lesser degree ("sub-optimal inducers"), or not at all ("anti-inducers").[6] Other "inactive" steroids neither induce nor inhibit cortisol induction.[6] We studied two optimal inducers, dexamethasone and cortisol; one

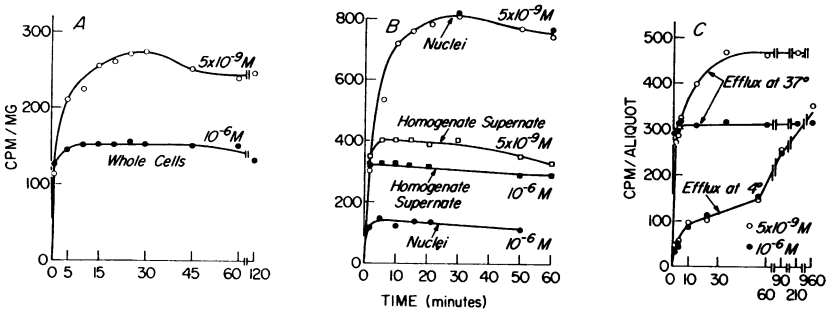

FIG. 2—(*A and B*). Kinetics of uptake of ^{3}H-dexamethasone by hepatoma tissue culture cells measured in whole cells and in cell fractions.

(*A*). Cells were incubated with 5×10^{-9} *M* ^{3}H-dexamethasone in the absence (○—○) or presence (●—●) of 10^{-6} *M* nonradioactive dexamethasone (see text), and aliquots were taken at various times and centrifuged for 15 sec (Sorvall cell-washing centrifuge) and the supernatant medium was removed immediately. Radioactive steroid in the cell pellet was then assayed as described in the *Materials and Methods* (utilizing ^{14}C-inulin to estimate extracellular steroid).

(*B*). Uptake by nuclear and homogenate supernate fractions. Cells were incubated with 5×10^{-9} *M* ^{3}H-dexamethasone in the absence (○—○, □—□) or presence (●—●, ■—■) of 10^{-6} *M* nonradioactive dexamethasone at 37° and aliquots were taken at various times and the cells immediately were centrifuged for 15 sec. The supernatant medium was removed immediately, the pellet was chilled, and the cells were fractionated and assayed as described in the *Materials and Methods.*

(*C*). Dissociation of steroid from hepatoma tissue culture cells. Cells were incubated with 5×10^{-9} *M* ^{3}H-dexamethasone, in the absence (○—○) or presence (●—●) of 10^{-6} *M* nonradioactive dexamethasone, for 30 min at 37° and then were centrifuged and the cell pellet rediluted to about 10^7 cells per milliliter in 20 ml of steroid-free phosphate-buffered saline. Aliquots of 2 ml were taken at various times and centrifuged for 15 sec and the radioactivity which remained in the cells and that which appeared in the supernatant medium was measured. Although the efflux into the media followed the decrease in radioactivity associated with the cells, the efflux was found to be the most accurately reproducible and is shown as cpm per 0.2 ml aliquot in the figure. Efflux at 37° and at 0 to 4° is shown. Other experiments showed that centrifugation and resuspension of cells in the original or fresh media did not themselves cause further steroid efflux.

sub-optimal inducer, 17 α-hydroxyprogesterone; one anti-inducer, testosterone; and two inactive steroids, epicortisol and androstenedione. The data in Table 1, demonstrate that these analogs do affect cortisol (or dexamethasone) binding to the specific receptors in direct relation to their effect on cortisol induction.

Metabolic transformation of steroid inducers: Rat hepatoma tissue culture cells were incubated with various concentrations of radioactive dexamethasone or cortisol for 30 minutes (when "specific" binding is maximal) and for 14 hours, when the levels of tyrosine aminotransferase were high (by this time the rate of its synthesis is maximal[18]). Steroids were extracted from cell fractions and chromatographed as described in the *Materials and Methods.*

After incubations with 5×10^{-9} and 10^{-5} *M* ^{3}H-dexamethasone, no significant amount of metabolites were detected in any extracts.

From cells incubated with 5×10^{-9} *M* cortisol (mostly specifically bound steroid), most of the radioactivity from nuclear extracts migrated with cortisol while less than 20 per cent migrated with di- and tetrahydrocortisol. At higher cortisol incubation concentrations where most of the radioactivity is nonspe-

TABLE 1. *The effect of inducer analogs on uptake of radioactive cortisol or dexamethasone to the nuclear receptors.**

^{3}H-cortisol or ^{3}H-dexamethasone uptake to crude or purified nuclei in presence or absence of additional nonradioactive steroid. Values presented are percentage of control (control = uptake of ^{3}H-steroid in the absence of excess nonradioactive steroid).‡

Nonradioactive Analog Added† (10^{-5} *M*)	^{3}H-Cortisol		^{3}H-Dexamethasone
	5×10^{-9} *M*, purified nuclei§	10^{-7} *M*, crude nuclei	5×10^{-9} *M*, purified nuclei
None	100	100	100
Epicortisol	88	106	107
Androstenedione	65	105	100
Testosterone	30	64	75
17α-Hydroxyprogesterone	18	35	43
Cortisol	10	20	
Dexamethasone			10

* Uptake by crude or purified nuclear fractions was measured after incubation of whole cells with ^{3}H-steroid as described in the *Materials and Methods*.

† The analogs are listed in increasing order of expected affinity for the specific receptors.[6] Induction by 10^{-7} *M*, but not by 10^{-5} *M* cortisol is inhibited by 10^{-5} *M* 17α-hydroxyprogesterone or testosterone.[6] Therefore, the binding of cortisol to specific receptors should be stronger than that of 17α-hydroxyprogesterone or testosterone. Testosterone at 10^{-5} *M* does not inhibit 10^{-7} *M* cortisol induction to the same extent as does 10^{-5} *M* 17α-hydroxyprogesterone[6] and therefore should not inhibit cortisol binding to specific receptors as well as does 17α-hydroxyprogesterone. Epicortisol and androstenedione at 10^{-5} *M* do not influence 10^{-7} *M* cortisol induction.[6]

‡ At 5×10^{-9} *M* ^{3}H-cortisol (44 Ci/mM) 100% = 2920 cpm/mg protein, at 10^{-7} *M* ^{3}H-cortisol (3.85 Ci/mM) 100% = 1580 cpm/mg protein, and at 5×10^{-9} *M* ^{3}H-dexamethasone (4 Ci/mM) 100% = 710 cpm/mg protein.

§ The means of two experiments are given and the separate determinations differed by less than 25%.

cifically associated, 44–78 per cent of the radioactivity migrated with cortisol and correspondingly more appeared as metabolites. In extracts from the homogenate supernate from cortisol experiments, as much as 11 per cent of the counts migrated more rapidly than cortisol.

Chromatograms of extracts from the medium in which cells were incubated followed the profile of stock solutions of radioactive steroid.

We conclude that, although some metabolism of cortisol was observed, the specific nuclear receptors bind primarily the unaltered steroid.

Discussion. The strongest evidence that the specific receptors reported here are involved in the glucocorticoid-mediated induction of tyrosine aminotransferase apears to us to be the correlation between the effect of inducer analogs on induction and on inducer binding to the specific receptors. The kinetics of association and dissociation of inducers with specific receptors are likewise consistent with the kinetics of induction and deinduction. In other hormone sensitive systems indications of a similar correlation between the binding of steroids and their biological actions have also been found.[13–15, 19, 21–26]

The chemical nature of the specific receptors in hepatoma tissue culture cells is not known. They differ from other macromolecules reported to bind glucocorticoid hormones (e.g., the cytoplasmic steroid-binding macromolecule in these cells,[4] the cortisol-binding macromolecule of human plasma, transcortin,[27] and the histones[28–30]) mainly in their relative affinity for various steroids.

Our current model for the regulation of enzyme synthesis includes the existence

of a labile post-transcriptional repressor which decays in the absence of continued RNA synthesis.[31] Since the specific receptors reported here do not disappear after incubation of the cells with actinomycin D or cycloheximide, these receptors do not appear to be the labile repressor predicted by the model (actinomycin D or puromycin neither block estrogen uptake by the uterine receptors[23] nor block aldosterone uptake by toad bladder[14]). Thus, the way in which the association of steroids with specific receptors ultimately controls the synthesis of specific proteins remains unclear.

* Present address: Department of Biochemistry, University of California Medical Center, San Francisco, California 94102.

[1] Thompson, E. B., G. M. Tomkins, and J. F. Curran, these PROCEEDINGS, **56**, 296 (1966).

[2] Granner, D. K., S. Hayashi, E. B. Thompson, and G. M. Tomkins, *J. Mol. Biol.*, **35**, 291 (1968).

[3] Tomkins, G. M., E. B. Thompson, S. Hayashi, T. D. Gelehrter, D. K. Granner, and B. Peterkofsky, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), p. 349.

[4] Gardner, R. S., and G. M. Tomkins, *J. Biol. Chem.*, **244**, 4761 (1969).

[5] We used dexamethasone whenever possible for the uptake studies because it is more resistant to metabolic conversion than is cortisol. Dexamethasone may be transformed in mammals to the glucuronide (R. H. Silber, personal communication) which would neither be extracted by methylene chloride nor would migrate in the solvent systems used in this study.

[6] Samuels, H. H., and G. M. Tomkins, *J. Mol. Biol.*, in press.

[7] Sandberg, A. A., H. Rosenthal, S. L. Schneider, and W. R. Slaunwhite, Jr. in *Steroid Dynamics*, ed. by G. Pincus, T. Makao, and J. T. Tait (New York: Academic Press, 1966), p. 9.

[8] Dulbecco, R., *J. Exptl. Med.*, **99**, 167 (1954).

[9] Gornall, A. G., C. J. Bardawill, and M. M. David, *J. Biol. Chem.*, **177**, 751 (1949).

[10] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[11] Diamondstone, T. I., *Anal. Biochem.*, **16**, 395 (1966).

[12] Figure 1*B* also shows that at higher steroid concentrations Q increases. The significance of this observation is presently unclear, although it may represent cooperative steroid uptake by some sites. Since the concentrations at which the increase in Q is observed are above that required for the maximal steroid effect on induction no systematic investigation of it has yet been made.

[13] Fanestil, D. D., and I. S. Edelman, these PROCEEDINGS, **56**, 872 (1966).

[14] Sharp, G. W. G., C. L. Komack, and A. Leaf, *J. Clin. Invest.*, **45**, 450 (1966).

[15] Munck, A., and T. Brinck-Johnsen, *J. Biol. Chem.*, **243**, 5556 (1968).

[16] More recently, we have demonstrated by other techniques that specific receptors are present in the homogenate supernate and may in fact account for most of the specific receptors in hepatoma tissue culture cells (Baxter, J. D., and Tomkins, G. M., in preparation).

[17] Scatchard, G., *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).

[18] Granner, D. K., E. B. Thompson, and G. M. Tomkins, *J. Biol. Chem.*, in press.

[19] Schaumburg, B. P., and E. Bojesen, *Biochim. Biophys. Acta*, **170**, 172 (1968).

[20] Auricchio, F., D. Martin, Jr., and G. M. Tomkins, *Nature*, **224**, 806 (1969).

[21] Ausiello, D. A., and G. W. G. Sharp, *Endocrinology*, **82**, 1163 (1968).

[22] Herman, T. S., G. M. Fimognari, and I. S. Edelman, *J. Biol. Chem.*, **243**, 3849 (1968).

[23] Jensen, E. V., in *Proceedings of the 2nd International Congress of Endocrinology, London, 1964* (Amsterdam: Exerpta Medica Foundation, 1965) p. 420.

[24] Jensen, E. V., T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut, and E. R. DeSombre, these PROCEEDINGS, **59**, 632 (1968).

[25] Melnykovych, G., and C. F. Bishop, *Biochim. Biophys. Acta.*, **177**, 579 (1969).

[26] Beato, M., J. Homoki, and C. E. Sekeris, *Exptl. Cell Res.*, **55**, 107 (1969).

[27] Florini, J. R., and D. A. Buyske, *J. Biol. Chem.*, **236**, 247 (1961).

[28] Sluyser, M., *Biochim. Biophys. Acta.*, **182**, 235 (1969).

[29] Sluyser, M., *J. Mol. Biol.*, **19**, 591 (1966).

[30] Sunaga, K., and S. S. Koide, *Biochem. Biophys. Res. Commun.*, **26**, 342 (1967).

[31] Tomkins, G. M., T. D. Gelehrter, D. Granner, D. Martin, Jr., H. H. Samuels, and E. B. Thompson, *Science*, **166**, 1474 (1969).