J. Biosci., Vol. 1, Number 3, September 1979, pp. 317–326. © Printed in India.

Isolation of sheep anterior pituitary messenger RNA and its translation in a heterologous cell-free system

M. S. K. PRASAD* and P. R. ADIGA
Department of Biochemistry, Indian Institute of Science, Bangalore 560 012
* Present address: Department of Biological Sciences, Oakland University, Oakland, Michigan, USA

MS received 7 April 1979; revised 23 June 1979

Abstract. A preparation rich in the specific messenger RNA involved in the synthesis of prolactin from sheep anterior pituitary glands was obtained by employing both the immunochemical and affinity techniques. A dose-dependent and efficient stimulation of protein synthesis by the isolated total pituitary RNA as well as poly (A) rich RNA were achieved with the reticulocyte system. The synthesis of prolactin as one of the translational products of this cell-free system was established by specific immunoprecipitation followed by resolution on sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Keywords. Pituitary RNA; oligo (dT)-cellulose; prolactin-mRNA; reticulocyte lysate; immunoprecipitation.

Introduction

Investigations on the factors influencing the synthesis and secretion of the endocrine principles of the anterior pituitary, besides being of inherent interest, are important in understanding the molecular basis of several endocrine disorders. Earlier, Adiga *et al*., (1965, 1966, 1968) probed into the mode of elaboration of some of the pituitary hormones at the molecular level using the homologous cell-free systems synthesising pituitary hormones. These studies are now extended to the isolation and translation of sheep pituitary RNA in a heterologous cell-free system with a view to quantitate directly the messenger activity and translational fidelity of the unfractionated total RNA as well as poly (A)-rich RNA. Furthermore, an elegant method for enriching the specific polysomes engaged in the synthesis of prolactin (PRL) in relatively larger amounts by the isolated pituitary has been described in this paper.

Materials and methods

Ovine pituitary prolactin (PRL, NIH–P–S 12) was obtained through the courtesy of the Hormone Distribution Officer, NIAMDD, National Institute of Health, Bethesda, MD, U.S.A. Diethyl pyrocarbonate, heparin, ethylene glycol-bis (2-aminoethyl ether)-N,N^1-tetra acetic acid (EGTA), dithiothreitol (DTT), β-mer-

captoethanol, N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid (HEPES), creatine phosphate, creatine phosphokinase (EC 2.7.3.2), pyruvate kinase (EC 2.7.1.40) and micrococcal nuclease (EC 3.1.4.7) were products of Sigma Chemical Co., St. Louis, MO, USA. Phenylhydrazine hydrochloride was purchased from Centron Research Laboratories, Bombay, India. Oligo (dT)-cellulose was procured from the Collaborative Research Inc., Waltham, MA, USA. [^{3}H]-leucine (sp. act. 5 Ci/mmol), [^{3}H]-phenylalanine (sp. act. 3 Ci/mmol), [^{3}H]-proline (sp. act. 5 Ci/mmol) and [^{14}C]-leucine (sp. act. 300 Ci/mmol) were purchased from New England Nuclear Corporation, Boston, MA, USA. All other chemicals were of analytical grade.

Preparation of antibodies

Specific antiserum to ovine PRL was raised in rabbits by four weekly subcutaneous injections at multiple sites of the hormone (1 mg|ml) emulsified with an equal volume of Freund's complete adjuvant (Difco). The rabbits were bled 5 days after the last injection and serum prepared. The antiserum gave a single precipitin band on immunodouble diffusion agar plates, did not bind significant amounts of ^{125}I-labelled ovine luteinising hormone or growth hormone and precipitated 400 μg of PRL/ml at equivalence point. This antiserum was further purified by precipitation with ammonium sulphate at 40% saturation and chromatography on DEAE- and CM-cellulose to remove RNase (Palacios *et al*., 1972).

Preparation of components from sheep anterior pituitaries for cell free protein synthesis

Anterior pituitary lobes were dissected within 15 min from pituitary glands of freshly slaughtered sheep. Total polysomes, 'pH 5·0 enzymes' and transfer factors were prepared essentially as described by Adiga *et al*. (1966).

Procedure for isolation of prolactin-specific polysomes

Total polysomes at a concentration of 5 A_{260} units/ml of 50 mM Tris-HCl, pH 7·6 buffer containing 25 mM KCl, 5 mM $MgCl_2$, 100 μg/ml heparin were incubated with pure PRL-IgG at 4° C for 60 min. The soluble polysome-antibody complex was then passed through a RPL-Sepharose column (0·5 × 5 cm) prepared by coupling 10 mg of ovine PRL to 2 g of CNBr-activated Sepharose 4B (March *et al*., 1974). This affinity matrix contained antigenic sites that reacted with the antibody molecules which have previously bound univalently to the nascent PRL chains. The specific polysomes were retained by the matrix. The unbound polysomes were washed off with excess of the buffer and this was followed by the elution of the retained polysomes with 0·01 M Tris-HCl, 0·05 M EDTA buffer containing pH 7·6 and 100 μg heparin/ml as described by Palacios *et al*. (1973).

Resolution of poly (A)-rich RNA by oligo (dT)-cellulose chromatography

Pituitary polysomal and cytoplasmic RNA were prepared using phenol : chloroform extraction as described by Penman (1966). Poly (A)-rich RNA was obtained by affinity chromatography on oligo (dT)-celluiose (Prasad *et al*., 1978).

Cell free protein synthesis in an mRNA-dependent reticulocyte system

Nuclease-treated rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (1976). Briefly, the lysate was supplemented with 25 μM. hemin and treated with $CaCl_2$ and micrococcal nuclease to digest endogenous globin mRNA. After a 15 min incubation at 28° C, excess of EGTA was added to inactivate the nuclease. The translational assay was performed essentially as described for an untreated lysate system (Taylor and Schimke, 1973).

The assays for cell-free protein synthesis were carried out at 30° C for 90 min (unless otherwise stated) and the reactions terminated by addition of 10% trichloroacetic acid containing 0·2% (w/v) unlabelled amino acids. The precipitate was washed twice with 5% trichloroacetic acid, once with 5% trichloroacetic acid at 90° C for 15 min, once with cold 5% trichloroacetic acid and then with 2 : 1 (v/v) mixture of ethanol and diethyl ether and finally with ether. The dry precipitate was dissolved in 0·5 ml of 1 M KOH and radioactivity measured in a Beckman LS–100 liquid scintillation spectrometer (Prasad *et al*., 1978).

Incubation of anterior pituitary slices with [^{3}H]-amino acids

The anterior pituitary glands were processed with a tissue slicer to get approximately 2 mm thick slices. Three to four slices were incubated at 37° C for 4 h with 2 ml of Krebs-Ringer bicarbonate buffer containing 10 mM HEPES, 0·2% glucose and 2 μCi of [^{3}H] -leucine.

Analysis of immuno precipitates

Pituitary slices were homogenised in 0·01 M sodium phosphate (pH 7·4) containing 0·15 M NaCl and centrifuged at 10,000 g for 10 min. Aliquots of this supernatant or cell-free reactions were treated with the antiserum raised against ovine PRL. The immunoprecipitates were processed by the method of Rhoads *et al*. (1971) and then analysed by the sodium dodecyl sulphate (sod. dod. SO_4) polyacrylamide gel electrophoresis (Palmiter *et al*., 1971).

Results

In vitro amino acid incorporation in the homologous cell free system by sheep pituitary polysomes

The functional integrity of the isolated sheep anterior pituitary polysomes was monitored by assaying their ability to incorporate amino acids in a homologous cell-free system. From figure 1 it is evident that protein synthesis occurred efficiently and increased linearly upto 30 min.

Enrichment of prolactin mRNA using PRL-Sepharose

Freshly isolated total pituitary polysomes were incubated with purified anti PRL-IgG and the soluble polysome-antibody complex was passed through PRL-Sepharose. It is evident from figure 2 that the vast majority of polysomes was not retained by this affinity matrix. However, a small fraction (0·6%) of the loaded polysomes was selectively adsorbed by the column and could be eluted with 0·01 M

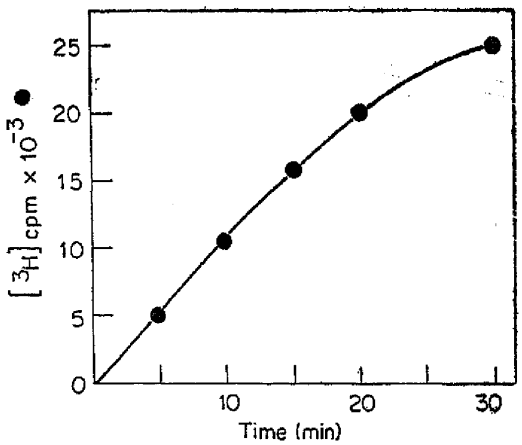

Figure 1. Time-course of protein synthesis in the sheep anterior pituitary cell-free system.
Pituitary polysomes (0·5 mg of RNA) were incubated at 37° C in a total volume of 0·5 ml with the following: 2·0 mg 'pH 5·0 enzyme,' protein, 2 5 mM ATP, 0·25 mM GTP, 2·5 mM phosphoenol pyruvate, 6 μg pyruvate kinase, 5mM magnesium acetate, 6 mM β-mercaptoethanol, 10 mM Tris-HCI buffer pH 7·4, 20 μM of each of [^{12}C]-amino acids other than [^{3}H]-labelled amino acids and 5 μCi of each of [^{3}H] phenylalanine, leucine and proline. At different time periods, aliquots (100 μl) were removed and radioactivity incorporated into protein determined.

tris-HCl buffer pH 7·6 plus 0·05 M EDTA. The presence of poly (A)-rich RNA (6%) could be determined in such PRL-Sepharose bound and EDTA eluted polysomal components by the oligo (dT)-cellulose column chromatography (figure 3).

Micrococcal nuclease treatment of rabbit reticulocyte lysates

The data presented in figure 4 clearly show that in the absence of the nuclease, translation was achieved efficiently and linearly upto 60 min. Incubation with nuclease for a short duration of 5–10 min was sufficient to completely destroy the endogenous messenger activity of the lysate. Interestingly prior addition of EGTA to the lysate could effectively inactivate the enzyme (table 1).

Stimulation of protein synthesis in mRNA-dependent reticulocyte lysate by added pituitary total RNA and poly (A)-rich RNA.

The translation of pituitary total RNA with the conventional reticulocyte lysate resulted in inhibition of total protein synthesis, rendering the system unsuitable for the direct assay of mRNA. In contrast, a dose-dependent stimulation of protein synthesis by added pituitary cytoplasmic RNA and poly (A)-rich RNA could be achieved by using the nuclease-pretreated lysate. Thus, this system appeared to be better suited for the direct assay of the messenger activity in total unfractionated pituitary RNAs since even the highest concentration of RNA (120 μg) tested was not inhibitory and considerable stimulation was achieved with less than 1 μg of poly (A)-rich RNA (figure 5).

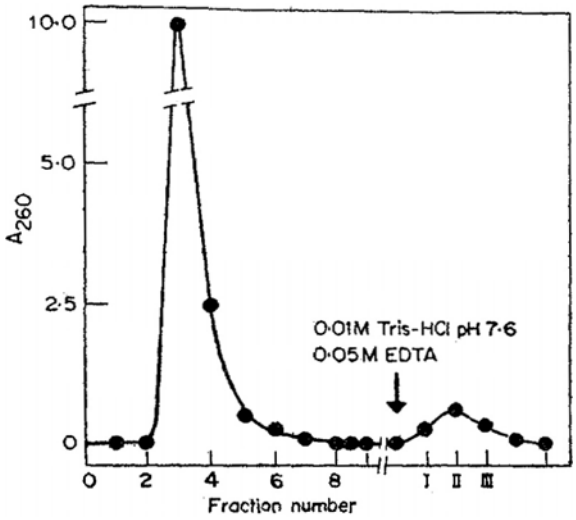

Figure 2. Enrichment, of prolactin synthesising mRNA in the sheep pituitary polysomes by using PRL-Sepharose affinity matrix.
Freshly isolated sheep pituitary polysomes (30 A26 0 units) were treated with l·2mg of ovine PRL-specific IgG, stirred for 1 h in cold and passed through PRL-Sepharose column (1·5 × 8 cm). The effluent collected in 2 ml fractions. Polysomes adsorbed on the column were eluted with the buffer containing 0·05 M EDTA.

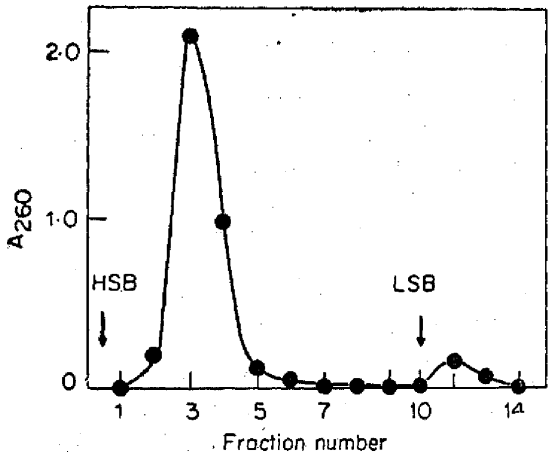

Figure 3. Isolation of PRL specific mRNA.
Fractions I–III from figure 2 were pooled. Extraction of RNA and subsequent oligo (dT)-cellulose fractionation have been described in materials and methods section. The amount of RNA charged to Oligo (dT)-cellulose column was equivalent to approx. 10^5 μg.
HSB 0·02M Tris-HCl, pH 7·6 containing 0·5M KCl and 0·00l M EDTA; LSB – 0·01 M Tris-HCl buffer, pH 7·6.

Table 1. Effect of $CaCl_2$ and EGTA on protein synthesis in the rabbit reticulocyte lysate.

Additions	[^{14}C]-Leucine incorporated (c.p.m.)
None	12400
Nuclease + $CaCl_2$[a]	5600
Nuclease + $CaCl_2$ + EGTA[b]	11200
EGTA	12000

Freshly prepared rabbit reticulocyte lysates (150 μl) containing 1 μg micrococcal nuclease, EGTA (2 mM) and/or $CaC1_2$ (1 mM) were incubated in triplicate with [^{14}C]-leucine-(l μCi) at 30° C for 30 rnin and processed for estimation of the radioactivity incorporated into protein as described under materials and methods.

[a] EGTA was omitted during preincubation period of 10 min with nuclease.

[b] EGTA was added first to the lysate, followed by $CaCl_2$ before preincubation at 28° C.

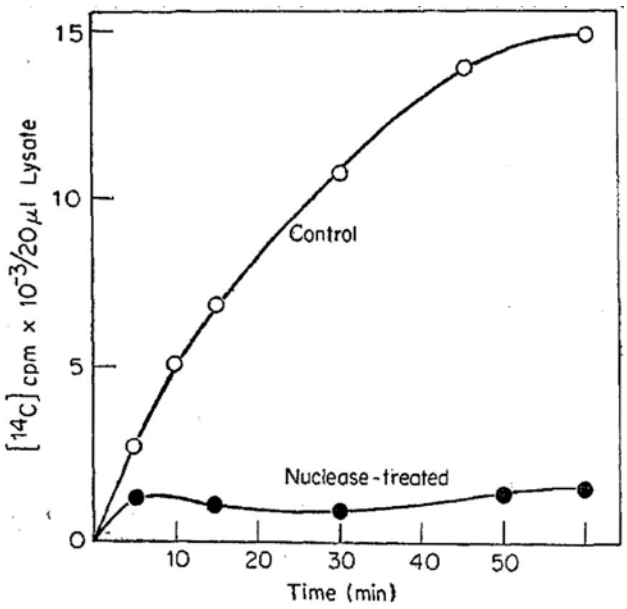

Figure 4. Effect of nuclease digestion on globin mRNA activity.
Lysates (150 μl/tube 2·0mg protein) were incubated along with [^{14}C]-teucine (1 μCi/tube) and 1 μg nuclease + 1 mM $CaCl_2$ at 30° C for different periods. A control experiment was done in the absence of nuclease .At the end of the reaction period aliquots (20 μ1) were withdrawn and precipitated with 5% trichloroacetic acid and radioactivity in the precipitate was determined.

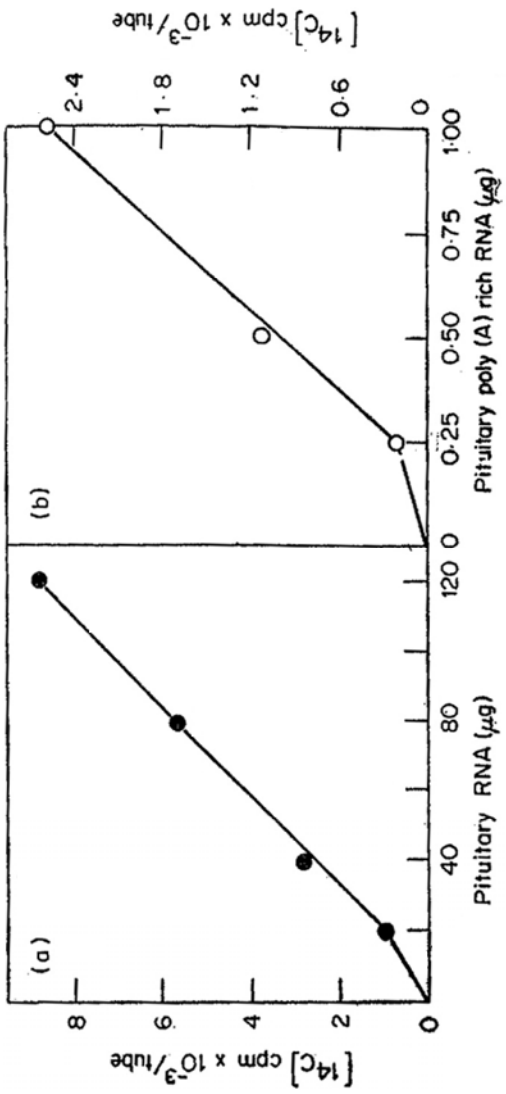

Figure 5. Protein synthesis in the reticulocyte lysate directed by sheep pituitary RNA.

Messenger RNA-dependent reticulocyte lysate (nuclease treated) was prepared as described under materials and methods. Aliquots (150 μl) of such lysate were incubated with [^{14}C]-leucine (1 μCi/tube) and varying amounts of pituitary cytoplasmic total RNA (*a*) or poly (A)-rich RNA (*b*). Incubations were carried out in duplicate at 30° C for 30 min and the radioactivity incorporated was plotted against the amount of RNA used in the assay after deducting that obtained in the absence of added RNA.

Analysis of the translation product

To identify PRL among the translational products in the RNA-stimulated heterologous cell-free system, the lysate volume was scaled upto 0·5 ml with proportionate increase in added RNA. After incubation, specific antiserum to PRL was added along with carrier PRL. Final proof that PRL was in fact a translation product was obtained by SDS-polyacrylaraide gel electrophoresis of the immunoprecipitate (figure 6). This conclusion was further reinforced by the data obtained by analysing the immunoprecipitate of the radioactive PRL synthesised by the pituitary slices *in vitro*.

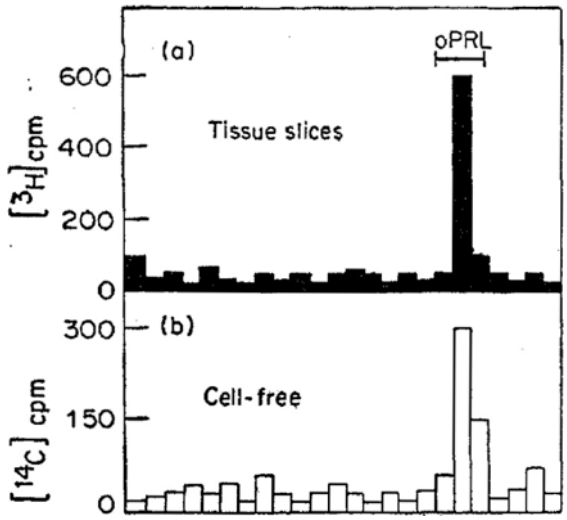

Figure 6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of immunoprecipitates from sheep anterior pituitary homogenates and cell-free reaction.
A. Pituitary slices were incubated with 2 μCi of [^{3}H]-leucine in Krebs-Ringer bicarbonate buffer containing 0·2% glucose and 20 mM HEPES, pH 7·4, at 37° C for 4h. (^{3}H)-labelled PRL was immunoprecipitated with specific ovine PRL-antiserum along with carrier PRL (10 μg). The washed immunoprecipitated protein was subjected to electrophoresis. Direction of electrophoresis is from left (+) to right (–).

B. [^{14}C]-Prolactin synthesised in the rabbit reticulocyte cell-free system in the presence of pituitary cytoplasmic RNA was precipitated with ovine PRL antiserum. The other details as in A.

Discussion

As a first step towards a complete understanding of the molecular mechanisms of biosynthesis of various pituitary hormones, various techniques for the isolation and translation of mRNA for prolaotin, from sheep anterior pituitary glands are described in this paper. From sheep pituitary glands, initially we could isolate functionally active polysomes containing poly (A)-rich RNA, identified by oligo (dT) cellulose chromatography and Millipore filter binding. Their identity was confirmed by hybridisation with [^{3}H]-poly U (data not shown).

In recent years, several laboratories have successfully attempted the isolation of mRNAs coding for specific proteins. Some of these studies were rendered possible by such unique features as (i) the mRNA for the protein in question (*e.g.*, globin) constituted a large portion of the total poly (A)-rich RNA with a predictable size (Lebleu, 1974) thus facilitating its isolation; (ii) the unique physical properties resulting from unusual base composition (*e.g.*, silk fibroin) (Suzuki and Brown, 1972). These exploitable properties could not be used for PRL-mRNA, as the mammalian anterior pituitary elaborated several protein hormones of similar size (*e.g.*, PRL, growth hormone, gonadotropins). Further, PRL has no unusual amino acid composition. A more recent approach to isolate mRNA involved prior identification of specific polysomes engaged in the synthesis of the particular protein. The interaction of [^{125}I]-labelled, specific antibody to polysomes was made use of as a probe to identify the polysomes concerned (Vussart and Dumont, 1973) and quantitative recovery achieved by the double-antibody technique. More versatile among these immunochemical techniques was that of Palacios *et al*. (1972), who employed glutaraldehyde polymerised ovalbumin to selectively enrich the soluble antibody-ovalbumin-polysome complex. In the present study, a modification of their elegant technique was employed with a view to minimise the requirement for PRL as an insoluble immunoaffinity matrix. However, the extremely low recovery of specific PRL mRNA achieved by this technique was a severe constraint for further investigations on the physicochemical and functional characterisation. In spite of this limitation, it may prove useful in attempts to synthesise complementary DNA (cDNA), essential for the detection and estimation of the minute amounts of mRNA for PRL, not possible by direct assay in heterologous cell-free systems. This has been accomplished in the case of ovalbumin mRNA (Schimke *et al*., 1975) resulting in the unravelling of the various regulatory features governing the elaboration of this egg white protein in the chick oviduct (Rosen and O'Mally, 1975).

Inability to assay pituitary RNA for mRNA content using the unmodified reticulocyte lysates presumably due to its high endogenous activity is in conformity with several earlier observations (Rosenfeld *et al*., 1972; Means *et al*., 1972). Earlier demonstration of translation of specific protein in this system, presumably at the expense of endogenous mRNA was possible only after isolation of the product by immunoprecipitation (Means *et al*., 1972).

Using the modified reticulocyte cell-free system having low endogenous mRNA activity, a dose-dependent, satisfactory stimulation of protein synthesis in response to exogenous total RNA as well as pituitary poly (A)–RNA could be achieved. The greater efficiency of translational fidelity of this system with particular reference to PRL-mRNA could be unequivocally demonstrated using specific immunoprecipitation followed by sod. dod. SO_4 polyacrylamide gel electrophoresis. When this work was in progress, Maurer *et al*. (1976) with rat pituitary total RNA and poly (A)-RNA and Evans and Rosenfeld (1976) using GH_3 cell RNA employing the wheat germ system demonstrated the presence of a larger protein species (precursor) possessing both immunological cross-reactivity with and sequence homology to rat PRL. Our inability to detect such a prehormone may be either due to inefficient resolution of the translated products on Polyacrylamide gels or to the possible presence of a soluble 'signal peptidase' (Goldman and Blobel, 1978) capable of processing the hormone precursor to prolactin in the reticulocyte lysate system.

Acknowledgements

Our thanks are due to Capt. Lakshman Singh, Military Slaughter House, Bangalore for providing facilities for obtaining fresh sheep pituitaries. MSK thanks the University Grants commission, New Delhi for financial assistance.

References

Adiga, P. R., Hussa, R. O. and Winnick, T. (1968) *Biochemistry* **7,** 1808.
Adiga, P. R., Rao, P. M., Hussa, R. O. and Winnick, T. (1966) *Biochemistry* **5,** 3850.
Adiga, P. R., Uomura, I. and Winnick, T. (1965) *Biochemistry* **4,** 246.
Evans, G. A. and Rosenfeld, M. G. (1976) *J. Biol. Chem.,* **251,** 2842.
Goldman, B. M. and Blobel, G. (1978), *Proc. Natl. Acad. Sci. USA*., **75,** 5066.
Lebleu, B. (1974) *Methods Enzymol.* ***F*30,** 613.
March, S. C., Parikh, I. and Cuatrecasas, P. (1974) *Anal. Biochem*., **60,** 149.
Maurer, R. A., Stone, R. and Gorski, J. (1976) *J. Biol. Chem*., **251,** 2801.
Means, A. R., Comstock, J. P., Rosenfeld, G. C. and O'Malley, B. W. (1972) *Proc. Natl. Acad. Sci. USA*., **69,** 1146.
Palacios, R., Palmiter, R. D. and Schimke, R. T. (1972) *J. Biol. Chem*., **247,** 2316.
Palacios, R., Sullivan, D., Summers, N. M., Kiely, M. L. and Schimke, R. T. (1973) *J. Biol. Chem*., **248,** 540.
Palmiter, R. D., Oka, T. and Schimke, R. T. (1971) ***J.*** *Biol. Chem*., **246,** 724.
Pelham, H. R. B. and Jackson, R. J. (1976) *Eur. J. Biochem*., **67,** 247.
Prasad, M. S. K., Muralidhar, K., Moudgal, N. R. and Adiga, P. R. (1978) *J. Endocrinol*., **76,** 283.
Rhoads, R. E., Mcknight, G. S. and Schimke, R. T. (1971) *J. Biol. Chem*., **246,** 7407.
Rosen, J. M. and O'Malley, B. W. (1975) in *Biochemical actions of hormones,* ed. G. Litwack (New York: Academic Press) Vol. **3,** p. 271.
Rosenfeld, G. C., Comstock, J. P., Means, A. R. and O'Malley, B. W. (1972) *Biochem. Biophys. Res. Commun*., **46,** 1695.
Schimke, R. T., Stanley McKnight, G. and Shapiro, D. J. (1975) in *Biochemical actions of hormones*, ed. G. Litwack (New York: Academic Press), Vol. 3, p. 245.
Suzuki, Y. and Brown, D. D. (1972) *J. Mol. Biol*., **63,** 409.
Taylor, J. M. and Schimke, R. T. (1973) *J. Biol. Chem.,* **248,** 7661.
Vussart, G. and Dumont. P. (1973) *Eur. J. Biochem*., **32.** 322.