RAPID ISOLATION OF MOUSE LEYDIG CELLS BY CENTRIFUGATION IN PERCOLL DENSITY GRADIENTS WITH COMPLETE RETENTION OF MORPHOLOGICAL AND BIOCHEMICAL INTEGRITY

M. SCHUMACHER, G. SCHÄFER, A. F. HOLSTEIN+ and H. HILZ

Institut für Physiologische Chemie und +Anatomisches Institut der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, FRG

Received 9 May 1978

1. Introduction

Partial purification of rat Leydig cells from crude interstitial cell suspensions by Ficoll gradients [1], and isolation of rather pure Leydig cells by Metrizamide gradients [2] have been described. Application of the latter procedure [2] to the purification of mouse Leydig cells resulted in a high contamination with other testicular cells as well as in a loss of viability due presumably to the penetration of Metrizamide into the cells. This paper describes the isolation of 90–95% pure Leydig cells from a crude testicular cell suspension prepared by non enzymic tissue disintegration and containing 2–4% Leydig cells. The cells showed excellent preservation of all morphological criteria as revealed by electron microscopy. They could be stimulated to half-maximal cAMP levels and testosterone production by the same hCG concentration as the crude cell suspension, and the increase in testosterone formation per cell (25–50-fold) corresponded to the purification factor for the Leydig cells. The integrity of the cells was also documented by an immediate, overshooting cAMP accumulation (up to 120-times control levels) in the absence of any phosphodiesterase inhibitor (cf. [3]). This was accompanied by a maximal and persistent activation of protein kinase leading to a linear increase in testosterone synthesis and in ornithine decarboxylase activity for several hours. It is also shown that mouse Leydig cells can be distinguished from other testis cells by phase contrast microscopy.

2. Materials and methods

Highly-purified hCG (12 000 'LH' U/mg, homogeneous in gel electrophoresis) was a generous gift of Dr D. Grässlin (Hamburg), ^{125}I-labeled hCG and testosterone antiserum were kindly provided by V. Lichtenberg (Hamburg).

2.1. *Preparation of crude Leydig cell suspensions*

Non-enzymic disaggregation of mouse testis cells was performed as in [4], with slight modifications. The testes of adult mice (NMRI, 3 months old) were decapsulated and cut with scissors into small pieces, and suspended in minimal essential medium (MEM) containing 0.07% serum albumin, 100 U penicillin/ml, 50 μg streptomycin/ml, and 25 mM Hepes, pH 7.4 (10 ml/testis). The mixture was gently stirred for 10 min at 24°C, filtered through two layers of nylon gauze, and centrifuged at 80 g (10 min, 20°C). The cells were washed once in MEM, resuspended in MEM (cells from 1–2 testes/ml), and counted in a haemocytometer.

2.2. *Fractionation of testis cells in Percoll density gradients*

Nine parts Percoll (Pharmacia) were mixed with 1 part 10-fold conc. Earle's balanced salt solution containing 0.7% serum albumin and 250 mM Hepes, pH 7.4, to give an osmolality of 300 mOsmol/kg. Linear density gradients (0–90%, 45 ml) were prepared by diluting the Percoll solution with isotonic Earle's

salt buffer containing 0.07% serum albumin. Crude cell suspensions, 3 ml containing up to 10^8 cells, were layered on top of the gradient and centrifuged for 20 min at 800 × g_{av} (20°C). Four visible bands of testicular cells were obtained, which were diluted with 3 vol. MEM and sedimented at 150 × g (15 min, 20°C). Highly purified Leydig cells were found in the third band, corresponding to a Percoll concentration of 38–52% (v/v). The sedimented cells were washed twice with MEM-serum albumin, using low speed centrifugation (80 × g, 8 min, 20°C).

2.3. *Determination of cAMP levels and of steroidogenesis*

Cells, 5–20 × 10^4, were routinely incubated in 400 μl medium ± hCG at 36°C in a shaking water bath for an appropriate time. Cells were then rapidly sedimented, the supernatant used for testosterone determination [5], and the pellet extracted either with 5% trichloroacetic acid for quantification of intracellular cAMP [6], or stored at −80°C for the assay of protein kinase [7] and ornithine decarboxylase activity [8].

2.4. *^{125}I-labeled hCG binding to cells*

About 1.5 × 10^7 crude testicular cells were incubated in 1.5 ml medium containing 80 ng ^{125}I-labeled hCG (1.2 × 10^6 cpm) for 1 h (36°C) in the presence and absence of excess unlabeled hormone (200 U hCG). The suspensions were washed twice with 10 ml medium (150 × g, 10 min, 20°C) and layered on top of the Percoll gradient (see above). After centrifugation, 0.74 ml fractions were taken from the bottom of the tube, and the cells washed once with 2.5 ml isotonic Hepes buffer, pH 7.3 (2000 × g, 20 min, 4°C). The resulting pellet was analyzed for bound radioactivity.

2.5. *Morphology of testicular cells*

Testicular cells sedimented in Percoll solution were processed for light and electron microscopical investigations as in [9]. For comparison small tissue pieces of the testis were rapidly dissected out supravitally and fixed [9].

2.6. *Phase contrast microscopy*

Phase contrast microscopy (Zeiss photomicroscope) of Leydig cells was performed with cells suspended in MEM containing phenol red (10 mg/l).

3. Results and discussion

A crude suspension of mouse interstitial cells was prepared by non-enzymic dispersion as in [4]. When the suspensions were layered on Percoll gradients containing Earle's salts with 0.07% serum albumin, and centrifuged for 20 min at room temperature, separation into 4 visible bands was obtained (fig.1) The upper band (I) consisted mainly (~80%) of damaged cells as shown by dye-exclusion test. Band II contained 90% of the total cell number. Leydig cells were concentrated in band III as shown by the ability to bind labeled hCG specifically, and to respond to the trophic hormone by an increase in cAMP levels and in testosterone formation. Sedimentation and washing of the Leydig cell fraction at 150 × g for 15 min usually resulted in contamination with 15–25% spermatozoa, which could be avoided (at the expense of the yield)

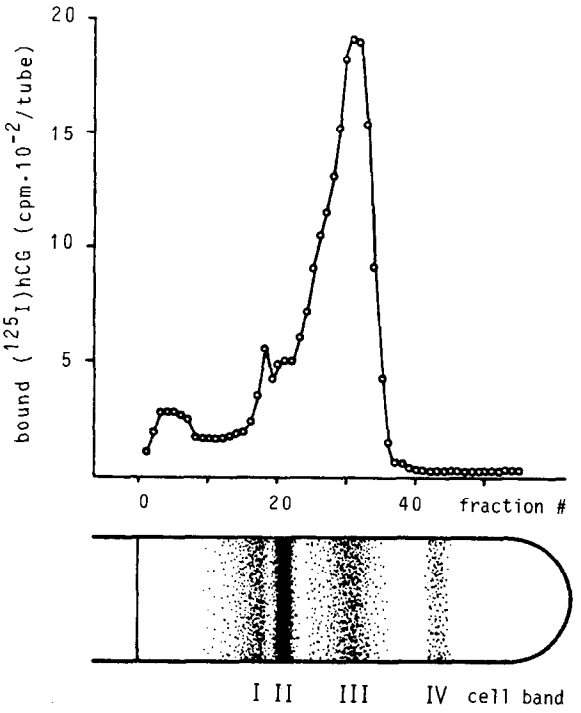


Fig.1. Fractionation of testicular cells by Percoll density gradient centrifugation and distribution of ^{125}I-labeled hCG binding capacity. Crude testicular cells were incubated with ^{125}I-labeled hCG and fractionated as described in section 2. Radioactivity bound in the presence of excess unlabeled hormone was subtracted.

Table 1
Response to hCG of unfractionated testicular cells and of cell bands obtained by gradient centrifugation

Cell fraction	hCG (10 ng/ml)	cAMP content (pmol/10^6 cells)	Testosterone formation (ng/10^6 cells)
Unfractionated cells	–	0.40 ± 0.91	2.2 ± 0.2
	+	2.11 ± 0.06	51.4 ± 1.4
Fractionated cells			
band I	–	0.32 ± 0.02	0.4 ± 0.2
	+	0.49 ± 0.03	2.9 ± 0.2
band II	–	0.50 ± 0.03	< 0.05
	+	0.72 ± 0.06	3.2 ± 0.02
band III	–	0.88 ± 0.02	18.9 ± 1.9
	+	53.50 ± 3.30	1350.0 ± 20.0
band IV	–	< 0.05	< 0.05
	+	0.42 ± 0.07	8.4 ± 0.8

Cells were incubated for 2.5 h and analyzed as described in section 2. Mean values from 3 determinations ± SEM

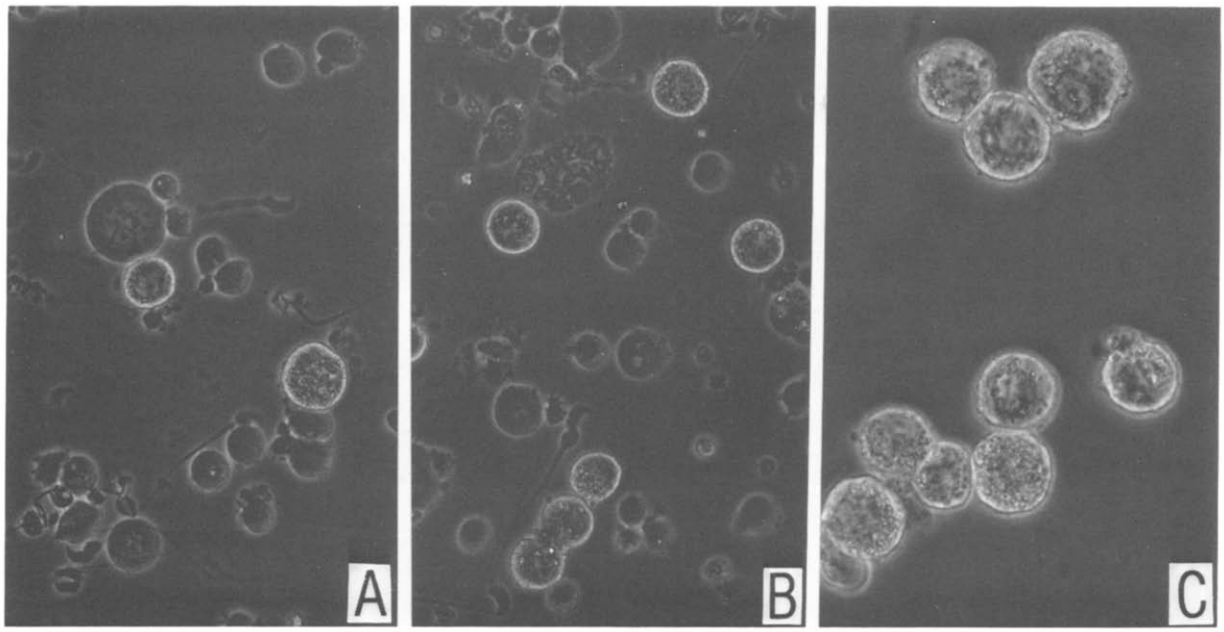
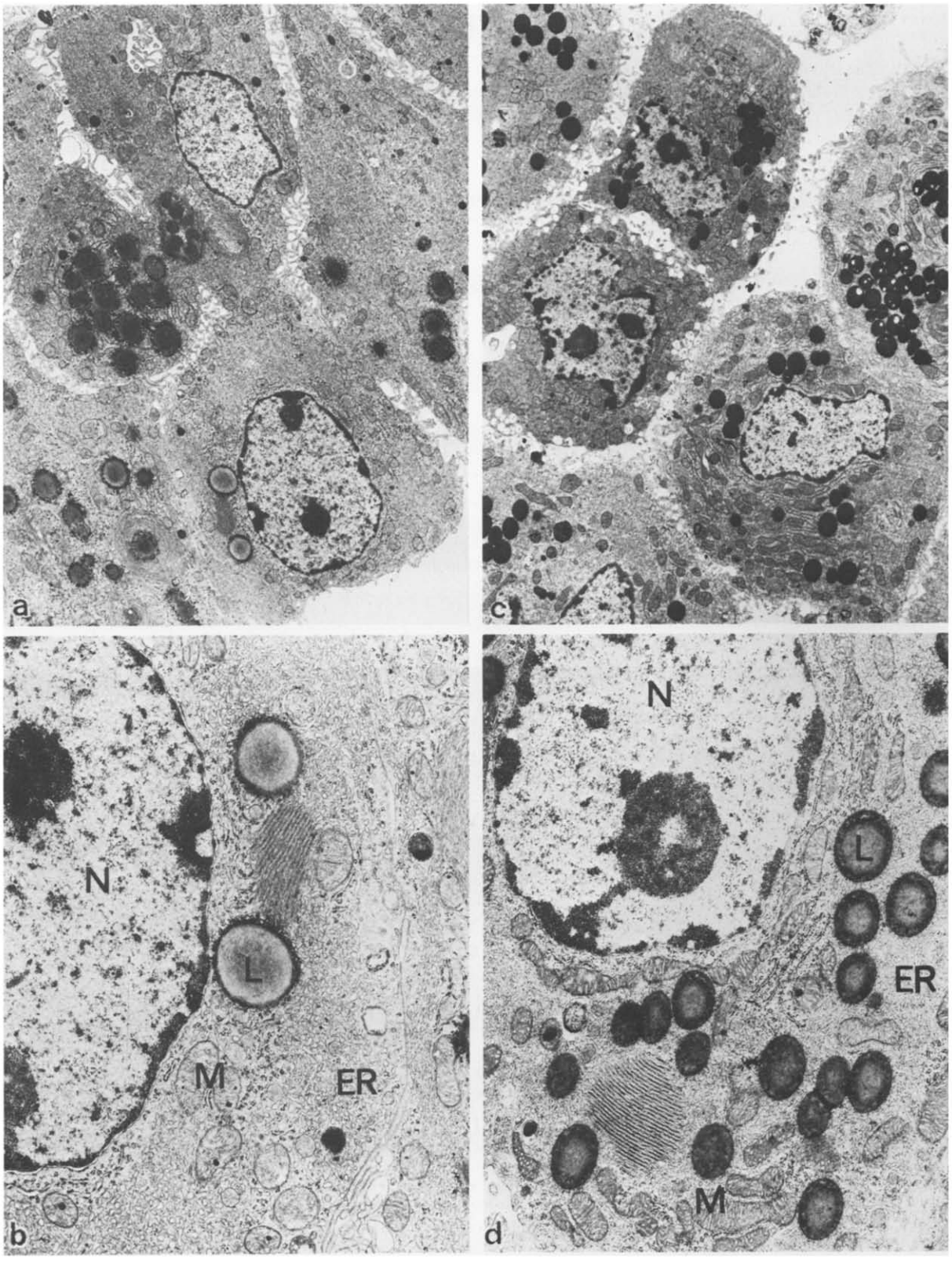


Fig.2. Detection of mouse Leydig cells by phase contrast microscopy. (a) crude suspension of Leydig cells; × 380. (b) mixture of purified and crude Leydig cell suspensions; × 380. (c) Leydig cell suspension purified by Percoll gradient centrifugation. 95% of the cells showed positive dye exclusion; × 608. Short exposure time allowed better discrimination of cells.


a
c
N
L
M
ER
b
N
L
ER
M
d

by centrifugation at 80 × *g* for 8 min. The Percoll gradient centrifugation also separated out all erythrocytes which were concentrated in band IV.

One single density gradient centrifugation step was apparently sufficient to purify the Leydig cells 25-fold. This is shown by a corresponding increase in the hCG response/cell from band III (table 1).

In the course of these studies it was discovered that intact mouse Leydig cells could be clearly distinguished from other testicular cells by their bright yellowish appearance under the phase contrast microscope (fig.2 lighter cells). When this property was used to determine the number of Leydig cells in crude and purified suspensions, a similar purification factor was obtained as by the biochemical parameters (cf. table 1).

3.1. *Morphological integrity*

Morphological integrity of the Leydig cells obtained by the Percoll procedure is documented in fig.3. It shows a well-preserved shape of cells and cell organelles, thus demonstrating the excellent preservation of the Leydig cells during disaggregation and fractionation (cf. [10]). The purified cells were definitely identified as Leydig cells by electron microscopy when compared with interstitial cells from intact mouse testis (fig.3a,b), exhibiting the typical structures (folded plasma membrane, lipid droplets, smooth endoplasmic reticulum, mitochondria with tubular profiles, peripheral rim of heterochromatin in the nucleus). By morphological criteria, the final cell suspension usually contained 90–95% Leydig cells.

3.2. *Biochemical integrity*

Retention of biochemical integrity was verified by a comparative analysis of steroidogenesis in dependence of the hCG concentration. In crude and in the purified Leydig cell suspensions, the same concentration of 40 pg hCG/ml (0.8 pM) was required to produce half-maximal stimulation of cAMP accumulation and of testosterone synthesis (not shown). These results were obtained without phosphodiesterase inhibitors, which usually increase the 'sensitivity' towards the trophic hormone as well as the maximal response (cf. [3,11]). Furthermore, crude and purified cell suspensions reacted to hCG with an induction of ornithine decarboxylase to comparable degrees (not documented; cf. [12]). One of the most convincing criteria for the integrity of the purified cells was found, when the kinetics of the hCG-induced cAMP accumulation, the concomitant activation of protein kinase and the rate of testosterone synthesis were analyzed (fig.4). The isolated mouse Leydig cells responded to hCG in the absence of any phosphodiesterase inhibitor with an immediate and excessive accumulation of intracellular cAMP reaching values 70-times above controls already

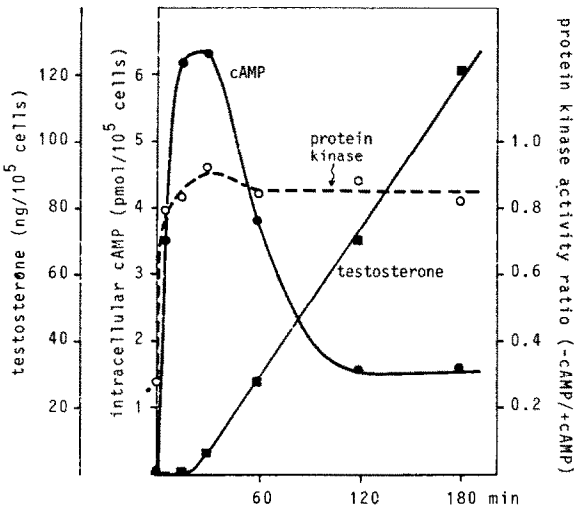


Fig.4. Kinetic analyses of hCG-induced cAMP accumulation, protein kinase activation and testosterone synthesis. Purified Leydig cells (1 × 10^5) were incubated in 400 μl medium in the presence of 20 ng hCG/ml for various times and analyzed as described in section 2. Control values (no hCG) at 60 (180) min were: 0.039 (0.035) pmol intracellular cAMP; 0.29 (0.35) protein kinase activity ratio; 0.47 (2.0) ng testosterone production. Each point represents the mean from triplicate incubations.

Fig.3. Electron microscopy of Leydig cells of the mouse. (a) Leydig cells in the interstitial space between seminiferous tubules; × 3500. (b) At higher magnification the nucleus (N), mitochondria (M), membranes and vesicles of the endoplasmic reticulum (ER) and lipid droplets (L) are seen; × 11 300. (c) Leydig cells isolated by the Percoll procedure. Note the well-preserved shape of cells, extensions of the plasmalemm and subcellular structures; × 3500. (d) Part of an isolated Leydig cell showing well preserved organelles; × 11 300.

at 5 min. The production of cAMP was overshooting being reduced soon to 25% maximal level. Such kinetics differ completely from those obtained with enzymically dispersed rat Leydig cells which require fortification with phosphodiesterase inhibitors to respond maximally to trophic hormones (cf. [3]). In spite of the marked changes in total cAMP, protein kinase activation was nearly maximal already at 5 min and remained at this level for the rest of the incubation period (fig.4). Since binding of cAMP to the regulatory subunits of protein kinase in vivo and the concomitant activation of protein kinase required only very small elevations of total cAMP [7,13,14], the persistence of protein kinase activation, and the persistence of a linear rate of steroidogenesis throughout 3 h incubation in the face of drastic changes in total cAMP are indicative of unimpaired responses. Similar overshooting cAMP accumulation and persistence of protein kinase activation, and of high levels of bound cAMP in spite of decreasing total cAMP concentration were seen in the livers of glucagon-treated rats [13] and in other systems (cf. [15]). The sensitivity and the kinetics of the hormone response indicated, together with the excellent preservation of the morphological appearance, that the isolated Leydig cells obtained by non-enzymic tissue dispersion and Percoll gradient centrifugation represent a fully responsive system for the long-term analysis of hormone action, not requiring the 'support' of unphysiological inhibitors of phosphodiesterase.

Acknowledgements

We would like to thank Mrs E. Spikofski for assistance in animal handling. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, SFB 34.

References

[1] Janszen, F. H. A., Cooke, B. A., Van Driel, M. J. A. and Van der Molen, H. J. (1975) J. Endocr. 70, 345–359.
[2] Conn, P. M., Tsuruhara, T., Dufau, M. and Catt, K. J. (1977) Endocrinology 101, 639–642.
[3] Mendelson, C., Dufau, M. and Catt, K. J. (1975) J. Biol. Chem. 250, 8818–8823.
[4] Van Damme, M.-P., Robertson, D. M. and Diczfalusy,E. (1974) Acta Endocrinol. 77, 655–671.
[5] Lichtenberg, V. and Pahnke, V. G. (1976) Acta Endocrinol. 82, suppl. 202, 54–57.
[6] Steiner, A. L., Parker, C. W. and Kipnis, D. M. (1972) J. Biol. Chem. 247, 1106–1113.
[7] Schumacher, M. and Hilz, H. (1978) Biochem. Biophys. Res. Commun. 80, 511–518.
[8] Russell, D. H. and Snyder, S. H. (1968) Biochemistry 60, 1420–1427.
[9] Holstein, A. F. and Wulfhekel, U. (1971) Andrologie 3, 65 69.
[10] Pertoft, H., Rubin, K., Kjellen, L., Laurent, T. C. and Klingeborn, B. (1977) Exp. Cell Res. 110, 449–457.
[11] Cooke, B. A., Lindh, M. L. and Janszen, H. A. (1976) Biochem. J. 160, 439–446.
[12] Möbius, G., Schumacher, M., Fanick, W., Lichtenberg, V. and Hilz, H. (1977) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robinson, G. A. eds) vol. 9, Raven Press, New York, in press.
[13] Schwoch, G. and Hilz, H. (1977) Eur. J. Biochem. 76, 269–276.
[14] Dufau, M. L., Tsuruhara, T., Horner, K. A., Podesta, E. and Catt, K. J. (1977) Proc. Natl. Acad. Sci. USA 74, 3419–3423.
[15] Costa, E., Kurosawa, A. and Guidotti, A. (1976) Proc. Natl. Acad. Sci. USA 73, 1058–1062.