Biochimica et Biophysica Acta, 603 (1980) 27—35

BBA 79009

STEROID-INDUCED INHIBITION OF NUCLEOSIDE UPTAKE IN ISOLATED MOUSE THYMOCYTES

D. GAGNE, F. HOMO and D. DUVAL

INSERM U7, Department of Nephrology, Hôpital Necker, 161 rue de Sèvres, 75015 Paris (France)

(Received May 12th, 1980)

Key words: Nucleoside uptake; Steroid inhibition; Uridine; Immunosuppression; (Mouse thymocyte)

Summary

In view of the evidence suggesting a possible effect of high concentrations of steroids on membrane properties, we have investigated the effect of several steroid molecules on the uptake and incorporation of [^{3}H]uridine in isolated mouse thymocytes. Our results demonstrate that the sex steroids, the estrogenic compound, diethylstilbestrol, and several non-hormonal steroid molecules induce a marked inhibition of nucleoside uptake. This effect, which occurs only at concentrations above 10^{-6} M, is almost instantaneous but transient and does not therefore appear to be mediated through specific receptor occupancy. Since sex steroids have been shown to inhibit mitogen-induced blast transformation at concentrations close to 10^{-5} M, we suggest that this membrane effect of sex steroids may partly explain their immunosuppressive effects.

Introduction

An increasing amount of evidence suggests that sex steroids can exert marked immunosuppressive effects in vivo [1—3]. Treatment with estradiol, testosterone and some of the placental metabolites of progresterone and testosterone induces thymic atrophy and this also occurs during gestation [4—7]. In addition, several alterations in the immune system have been observed during pregnancy or hormonal therapy [8—13]. In contrast, with the immunosuppressive action of glucocorticoids which has been extensively studied and is thought to be mediated partly through an interaction of the steroid hormones with stereospecific cytoplasmic receptors [14], the mechanisms of the sex steroid effects have been investigated little in vitro. Several

authors, however, have reported the ability of androgens, estrogens and progestins to block lymphocyte transformation in vitro but at concentrations much higher than those required for glucocorticoids [15—18].

Binding studies performed in isolated thymocytes have failed to demonstrate specific receptors for sex steroids [19,22], whereas such receptors have been recently described within epithelial cells of the thymus [21,22] and Bursa of Fabricius [23]. Several indications suggest that steroids at high concentrations can induce rapid alterations of cell membrane properties. In addition to the well documented effects of steroids on the stabilization-lysis of lysosomal and erythrocyte membranes [24] and the action of these drugs on the electron-transport pathway in isolated mitochondria [25], steroids have also been shown to inhibit the transport of glucose in human erythrocytes and adipocytes [26,27] and to decrease the uptake of glucose and amino acids in rat hepatoma cells [28]. In addition, Batra [29] and Pietras and Szego [30] presented evidence for the modulation of calcium uptake in uterine cells by estrogens. Recently, Baulieu and coworkers [31] have demonstrated that progesterone and other related compounds can promote the maturation of oocytes from *Xenopus laevis* by a direct action at the level of the cell membrane. Similarly, Dufy et al. [32] have shown that estrogens exert a rapid effect of the electrical activity of pituitary cells in culture, and the membrane anaesthetic properties of several steroid molecules are well documented [33—35]. In order to explore the possibility that the inhibitory effect of sex steroids on lymphocyte transformation can be related to a direct membrane action, we have studied the uptake of [^{3}H]uridine in isolated thymocytes in the presence of various steroids. The transport of this nucleoside has been extensively studied by Plagemann and Richey [36]. Following its entry into the cell by facilitated diffusion, uridine is rapidly phosphorylated, accumulates in the acid-soluble pool mainly as UTP and is then incorporated into acid-insoluble material.

Materials and Methods

Animals and reagents

Female C_{57} BL/6 mice, 6—8-week old (15—25 g body wt.) were supplied by CSEAL (Orleans, France). Adrenalectomy has been shown to decrease the proportion of thymocytes which die spontaneously during in vitro incubation [37,38]. Therefore, the animals used in our experiments were routinely adrenalectomized 4—5 days before each experiment and kept on a standard diet with free access to 1% saline.

Steroids (kindly provided by J.P. Raynaud, Roussel Uclaf, France) were first dissolved in ethanol and then diluted to the appropriate concentrations with the incubation medium. The final concentration of ethanol was always less than 0.5% and therefore did not modify cell viability or nucleoside transport. All reagents used were analytical grade. Alfaxolone was a gift of Dr. Alexandre (Glaxo laboratories, Paris, France).

Isolation of thymocytes

The procedure used for the isolation of thymocytes has been described

in detail elsewhere [38]. Cells were isolated at room temperature and adjusted to 10^7 cells/ml in minimum essential medium (Gibco Ref. 138) supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), 100 U/ml penicillin and 100 μg/ml streptomycin, and 1% (v/v) non-essential amino acid solution. Cell viability was determined at the end of the isolation procedure using the trypan blue exclusion test and was always over 95%. This cell suspension, as judged by light-microscopic observation, contains more than 95% of small lymphocytes.

Analysis of nucleoside uptake and incorporation

The kinetics of [^{3}H]uridine uptake and incorporation were measured at 37°C in the absence (control) or presence of various steroids. At the beginning of the experiment, each sample ($3 \cdot 10^6$ cells in a final volume of 0.6 ml) received simultaneously 1 μCi of [^{3}H]uridine (25 Ci/mmol, CEN Saclay, France) and steroid. Then, at various intervals after tracer addition, both the whole cell uptake and the incorporation into acid-precipitable material were determined.

Determination of whole cell uptake. 0.2 ml aliquots of cell suspension were centrifuged for 15 s at 11 000 × *g*. Supernatants were discarded and the cells resuspended at room temperature in 0.9% saline. After two additional washes, the cell pellets were digested overnight in 1 ml of Soluene 350 (Packard) and counted by liquid scintillation spectrometry.

Determination of incorporation. The residual 0.4 ml aliquots were precipitated by addition of ice-cold 5% trichloroacetic acid. The precipitates were filtered on Whatmann GF/A filters, and washed three times with 10 ml of ice-cold 5% trichloroacetic acid. The radioactivity collected on the filters was counted by liquid scintillation spectrometry. Makman et al. [39] have demonstrated that more than 85% of the radioactivity recovered after trichloroacetic acid precipitation represents RNA-associated material.

Pulse chase experiments. The experimental procedure permitting the dissociation between [^{3}H]uridine uptake and its subsequent incorporation into macromolecules has been described by Plagemann and Richey [36]. In these experiments, the cells were first incubated with the radioactive precursor at 20°C. At this temperature, macromolecule synthesis is almost completely blocked, whereas transport and phosphorylation continue at a significant rate. After centrifugation of the cells (800 × *g*, 5 min, 20°C), further incubation at 37°C in a medium devoid of uridine then allows the analysis of the utilisation of the acid-soluble pool for RNA synthesis. In these experiments, the steroid was added either at the beginning of the incubation at 20°C and then washed together with the radioactive precursor, or at the beginning of the incubation at 37°C. At various intervals during the incubation, both precursor uptake and incorporation were determined as described above. [^{3}H]Thymidine- (CEA, France, 27 Ci/mmol) was used instead of [^{3}H]uridine as the radioactive precursor in some experiments.

Results

Kinetics of [^{3}H]uridine uptake and incorporation in isolated mouse thymocytes

The time course of uridine uptake and incorporation at 37°C, in the absence

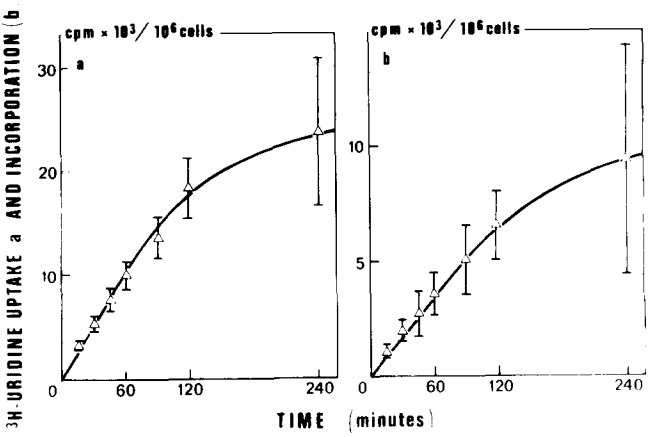

Fig. 1. Kinetics of [^{3}H]uridine uptake (a) and incorporation (b) at 37°C. At the beginning of the experiment, each sample received 1 μCi of [^{3}H]uridine and was incubated at 37°C for various periods of time. At the times indicated on the figure, the whole cell uptake and the incorporation into trichloroacetic-precipitable material were determined stimultaneously. Each value is the mean (±S.D) of triplicate determinations from four experiments.

of steroid, are presented in Fig. 1. Both uptake and incorporation proceed almost linearly during 60—90 min and then tend to plateau. Similar results were obtained by Plageman and Richey [36] in Novikoff rat hepatoma cells.

Effect of steroids on uridine uptake and incorporation: dose-response curves

Preliminary experiments (not shown) demonstrated that in contrast to glucocorticoids which only elicit inhibition of uridine uptake and incorporation after 2—3 h of incubation, most of the steroids tested, when active, inhibited the uptake and the incorporation within the first hour of incubation. The dose-response curves for the various steroids tested were therefore established after a 45 min incubation. Fig. 2 shows the results obtained for diethylstilbestrol,

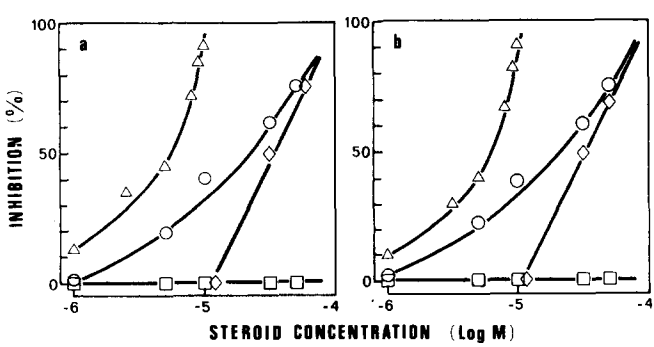

Fig. 2. Dose-response curves of the effects of steroids on the uptake (a) and incorporation (b) of [^{3}H]-uridine at 37°C. At the beginning of the experiment, each sample received 1 μCi of [^{3}H]uridine and various concentrations of steroids. After 45 min of incubation, whole cell uptake and incorporation were determined. Results are expressed as percentage inhibition of control samples, incubated in the absence of steroids. Each value is the mean of triplicate determinations from three experiments. (△) Diethylstilbestrol; (○) alfaxolone; (◇) testosterone; (□) dexamethasone.

alfaxolone, testosterone and dexamethasone. After this period of incubation, dexamethasone, whatever the concentration used, up to 10^{-4} M, did not induce any modification of precursor uptake and incorporation. Similarly, aldosterone and ouabain (not shown) were inactive under our experimental conditions.

In contrast, at concentrations above 10^{-6} M, diethylstilbestrol, alfaxolone and testosterone as well as progesterone, deoxycorticosterone and 25-hydroxycholesterol (not shown) all caused a decrease in both uridine uptake and incorporation. The slope of these dose-response curves differs markedly from one compound to another. Testosterone, for example, has no effect at 10^{-5} M but induces a 50% inhibition at $3 \cdot 10^{-5}$ M, whereas alfaxolone and diethylstilbestrol induce a progressive inhibition of uridine uptake and incorporation with increasing concentrations.

Kinetics of steroid action

The time course of the inhibition of [^{3}H]uridine incorporation by the various steroids at 37°C is presented in Fig. 3. Dexamethasone and aldosterone (10^{-5} M) elicit a progressive inhibition of precursor incorporation which becomes significant after 2 h of incubation and increases further with prolonged incubation. On the other hand, most of the other steroids tested, at concentrations comparable to those used for dexamethasone (10^{-5} M), induce a transient inhibition of uridine incorporation. Under these experimental conditions, the inhibitory effect reaches a maximum within 15—30 min after steroid addition and then decreases with uridine incorporation slowly returning to control values.

The most potent inhibitor of uridine incorporation is diethylstilbestrol, a non-steroidal estrogenic compound, which induces about 80—90% inhibition at 10^{-5} M, followed in order by progesterone, deoxycorticosterone and testosterone ($5 \cdot 10^{-5}$ M). Under our experimental conditions, the uptake of nucleo-

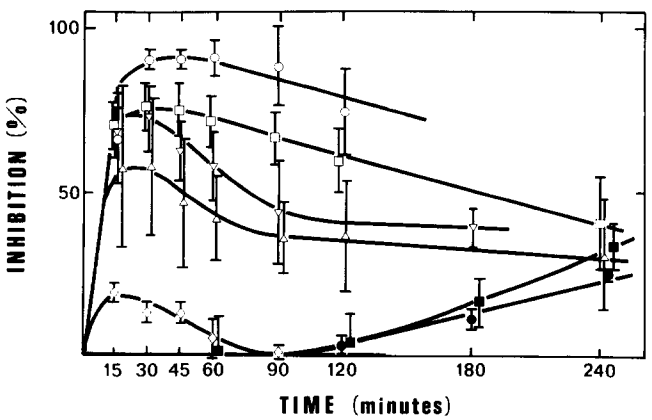

Fig. 3. Kinetics of inhibition of [^{3}H]uridine incorporation in the presence of steroid at 37°C. Steroid and [^{3}H]uridine were added at the beginning of the incubation period and the incorporation into acid-insoluble material was determined after various periods of incubation. Each value is the mean (±S.D.) of triplicate determinations from three to five experiments. (○) Diethylstilbesterol, 10^{-5} M; (◇) 25-hydroxycholesterol, 10^{-5} M; (●) aldosterone, 10^{-5} M; (■) dexamethasone, 10^{-5} M; (▽) deoxycorticosterone, $5 \cdot 10^{-5}$ M; (□) progesterone, $5 \cdot 10^{-5}$ M; (△) testosterone, $5 \cdot 10^{-5}$ M.

TABLE I

EFFECT OF STEROIDS (10^{-5} M) ON THE UPTAKE AND INCORPORATION OF [^{3}H]URIDINE AFTER 45 MIN INCUBATION AT 37°C

Each value is the mean (±S.D.) of triplicate determinations from three experiments.

	Inhibition	
	Uptake	Incorporation
Diethylstilbestrol	90.6 ± 2.8	91 ± 3
Progesterone	19.7 ± 7	24.5 ± 9.7
Deoxycorticosterone	19.6 ± 9.6	21 ± 11.7
Alfaxolone	21 ± 17	25 ± 13
25-Hydroxycholesterol	15 ± 14	14 ± 4
Testosterone	0	0
Dexamethasone	0	0
Aldosterone	0	0
Ouabain	0	0

side proceeds in parallel with the incorporation and appears to be equally inhibited by the steroids (Table I).

Pulse chase experiments

Fig. 4 shows the results obtained in experiments performed according to the procedure defined by Plageman and Richey [36] with a preliminary incubation at 20°C followed by an incubation at 37°C. This procedure allows one to study specifically the effect of the various steroids tested on either nucleoside uptake and/or incorporation. Addition of 10^{-5} M diethylstilbestrol at the beginning of the experiment induces a marked inhibition of precursor uptake at 20°C, and also a marked inhibition of incorporation during the following

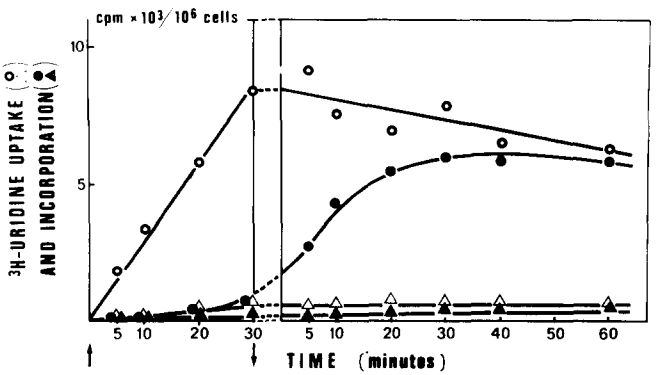

Fig. 4. Effect of diethylstilbesterol on [^{3}H]uridine uptake and incorporation. Cells were first incubated for 30 min at 20°C in the presence of [^{3}H]uridine ± 10^{-5} M diethylstilbestrol. At the end of this incubation period, cells were washed and resuspended at the same concentration in a medium devoid of steroid and nucleoside at 37°C. Whole cell nucleoside and incorporation into acid-insoluble material were determined at various intervals during these incubation periods. (○) [^{3}H]Uridine uptake in the absence of diethylstilbestrol; (●) [^{3}H]uridine incorporation in the absence of diethylstilbestrol; (△) [^{3}H]uridine uptake in the presence of diethylstilbestrol; (▲) [^{3}H]uridine incorporation in the presence of diethylstilbestrol. Each value is the mean of triplicate determinations from a typical experiment.

TABLE II

The cells were preincubated for 30 min at 20°C in the absence of drug, with 1 μCi of [^{3}H]uridine, then washed and resuspended at 37°C in a nucleoside-free medium ±10^{-5} M dexamethasone or diethylstilbesterol. The whole cell content of nucleoside was then determined at various intervals after steroid addition. Each value is the mean (±S.D.) of triplicate determinations in three experiments.

	10 min	30 min	180 min
[^{3}H]Uridine uptake (cpm/10^6 cells) (control)	6483 ± 3299	5847 ± 3536	4005 ± 1768
Dexamethasone (10^{-5} M) (% of control)	101.8 ± 2.9	91.6 ± 8	108.8 ± 36.4
Diethylstilbestetrol (10^{-5} M) (% of control)	83.8 ± 1.1	72.4 ± 10.1	75.7 ± 22.3

incubation at 37°C in drug-free medium (Fig. 4). Under similar experimental conditions, dexamethasone (10^{-5} M) does not significantly alter either the uptake at 20°C or the subsequent incorporation at 37°C (not shown). Addition of 10^{-5} M dexamethasone at the time of resuspension of cells in a medium free of radioactivity at 37°C did not lead to any significant change in the pattern of uptake and incorporation, whereas under similar conditions diethylstilbestrol (10^{-5} M) induced a slight decrease (20%) in the total amount of radioactivity associated with the cells (Table II).

Studies on thymidine incorporation

Wohlhueter et al. [43] have shown that thymidine transport in mammalian cells is a carrier-mediated system distinct from that ensuring the entry of uridine. The high velocity of this transport system makes it relatively difficult to measure using procedures currently applied for the determination of uridine uptake. Therefore, in this study, we have only measured the effect of steroids on [^{3}H]thymidine incorporation. As shown in Table III, most of the compounds tested are also able to decrease thymidine incorporation after 45 min incorporation at 37°C. It appears, however, that the inhibitory action of a given steroid on uridine and thymidine incorporation is not equivalent. Progesterone, for example, is more potent in decreasing thymidine incorporation than that of uridine whereas the opposite is true for diethylstilbestrol. In addition, 25-hydroxycholesterol, which slightly decreases uridine uptake

TABLE III

EFFECT OF STEROIDS (10^{-5} M) ON [^{3}H]THYMIDINE INCORPORATION AFTER 45 MIN AT 37°C

Each value is the mean (±S.D.) of triplicate determinations from three experiments.

	% inhibition
Progesterone	90 ± 4.3
Diethylstilbestrol	67.3 ± 18.5
Deoxycorticosterone	42.8 ± 2
Testosterone	29.9 ± 14.5
Alfaxolone	15.3 ± 7.3
Dexamethasone	12.1 ± 4.2
25-Hydroxycholesterol	−17.6 ± 9.5

and incorporation enhances thymidine incorporation. Kinetic experiments demonstrated that this effect of steroids on thymidine incorporation also appears transient (not shown).

Discussion

The catabolic effects of glucocorticoids on lymphoid cells have been extensively studied [14]. These compounds induce in particular an inhibition of the incorporation of radioactive precursors into RNA. This action is thought to be mediated through an interaction with cytosolic glucocorticoid receptors on the basis of the following criteria: the effect takes place at concentrations close to that achieving receptor saturation ($5 \cdot 10^{-8}$ M), is only measurable after a lag period of 1—2 h and can be blocked by inhibitors of RNA and protein synthesis. In the present work, we have shown that sex steroids as well as some other steroids and non-steroidal compounds are also able to modulate the uptake and incorporation of this nucleoside in isolated mouse thymocytes. However, the characteristics of this inhibitory effect are markedly different from those measured in the presence of dexamethasone. Firstly, the onset of inhibition is very rapid and almost instantaneous (Fig. 4) with a progressive decrease during prolonged incubation (Fig. 3). Secondly, the inhibitory effect of these steroids occurs only at very high concentrations, 10^{-5} M or more. It should be noted that the majority of active compounds do not bind with appreciable affinity to glucocorticoid receptors, with the exception of deoxycorticosterone and progesterone which present a moderate affinity for dexamethasone-binding sites [14,18]. Given the fact that isolated thymocytes do not contain binding sites for sex hormones [19—21], it is possible that these steroids act by a non-receptor-mediated process. Moreover, in experiments performed at 20°C, we have shown that this effect primarily affects the uptake.

Steroids have been shown to interact with membranes either at the level of the plasma membrane [40—42] or at the level of lysosomal and mitochondrial membranes [44,45]. Therefore, we suggest that the inhibitory action of sex steroids on nucleoside uptake is mediated by an effect of the drug at the level of cell membrane. As shown in our experiments, a given compound may exert different actions on uridine and thymidine uptake. This variability could thus reflect more specific interactions with the different membrane components.

Several authors have previously shown that sex steroids are able to block mitogen-induced lymphoblast transformation as appreciated by following [^{3}H]thymidine incorporation at concentrations higher than 10^{-5} M. We suggest that the inhibition of nucleoside incorporation demonstrated at these concentrations could in part explain this effect. Steroid concentrations of this order of magnitude can be achieved locally at the level of the placenta during pregnancy [46] or systemically during steroid treatment and therefore could be responsible for some of the immunosuppressive effects observed under these conditions.

References

1 Ahlqvist, J. (1976) in Endrocrine Influences on Lymphatic Organs, Immune Responses, Inflammation and Autoimmunity (Ahlqvist, J., ed.), pp. 18—57, Almqvist and Wiksell International, Stockholm
2 Claman, H.N. (1972) New. Engl. J. Med. 287, 388—397
3 Fauci, A.S. (1978-9) J. Immunopharmacol. 1, 1—25
4 Gregoire, C. (1945) Arch. Int. Pharmacodyn. Ther. LXX, 45—77
5 Sobhon, P. and Jirasattham, C. (1974) Acta Anat. 89, 211—225
6 Nelson, J.H., Hall, J.E., Manuel-Limson, G., Freidberg, H. and O'Brien, F.J. (1967) Am. J. Obstet. Gynecol. 98, 895—899
7 Millar, K.G., Mills, P. and Baines, M.G. (1973) Am. J. Obstet. Gynecol. 117, 913—918
8 Purtilo, D.T., Hallgren, H.M. and Yunis, E.J. (1972) Lancet 1, 769—771
9 Finn, R., Hill, C.A.S., Govan, A.J., Ralfs, I.G., Gurney, F.J. and Denye, V. (1972) Br. Med. J. 3, 150—152
10 Fabris, N., Piantanelli, L. and Muzzioli, M. (1977) Clin. Exp. Immunol. 28, 306—314
11 Bulmer, R. and Hancock, K.W. (1977) Clin. Exp. Immunol. 28, 302—305
12 Strelkauskas, A.J., Davies, I.J. and Dray, S. (1978) Clin. Exp. Immunol. 32, 531—539
13 Ablin, R.J., Bruns, G.R., Guinan, P.D., Al Sheik, H. and Bush, I.M. (1976) J. Lab. Clin. Med. 87, 227—231
14 Munck, A. and Leung, K. (1977) in Receptors and Mechanism of Action of Steroid Hormones, Part II (Pasqualini, J.R., ed.), pp. 311—397, Marcel Dekker, Inc., New York
15 Schiff, R.I., Mercier, D. and Buckley, R.H. (1975) Cell Immunol. 20, 69—80
16 Mendelsohn, J., Multer, M.M. and Bernheim, J.L. (1977) Clin. Exp. Immunol. 27, 127—134
17 Wyle, F.A. and Kent, J.R. (1977) Clin. Exp. Immunol. 27, 407—415
18 Homo, F., Dardenne, M. and Duval, D. (1980) Cell. Immunol., in the press
19 Homo, F., Picard, F., Durant, S., Gagne, D., Simon, J., Dardenne, m. and Duval, D. (1980) J. Steroid Biochem. 12, 433—443
20 Grossman, C.J., Sholiton, L.J. and Nathan, P. (1979) J. Steroid Biochem. 11, 1233—1240
21 Grossman, C.J., Sholiton, L.J., Blaha, G.C. and Nathan, P. (1979) J. Steroid Biochem. 11, 1241—1246
22 Stumpf, W.E. and Madhabananda, S. (1976) in Receptors and Mechanism of Action of Steroid Hormones, Part I (Pasqualini, J., ed.), pp. 41—84, Marcel Dekker, Inc., New York
23 Sullivan, D.A. and Wira, C.R. (1979) J. Immunol. 122, 2617—2623
24 Weissman, G. (1969) in Lysosomes in Biology and Pathology (Dingle, J.T. and Fell, H.B., eds.), Vol. II, pp. 276—295, North-Holland, Amsterdam
25 Tomkins, G.M. and Maxwell, E.S. (1963) Annu. Rev. Biochem. 12, 677—708
26 Lacko, L., Wittke, B. and Geck, P. (1975) J. Cell. Physiol. 86, 673—680
27 Livingston, K.J. and Lockwood, D.H. (1975) J. Biol. Chem. 250, 8353—8360
28 Plagemann, P.G.W. and Renner, E.D. (1972) Biochem. Biophys. Res. Commun. 2, 816—823
29 Batra, S.C. (1973) Biochem. Pharmacol. 22, 803—809
30 Pietras, R.J. and Szego, C. (1975) Nature 253, 357—359
31 Baulieu, E.E., Godeau, F., Schorderet, M. and Schorderet-Slatkine, S. (1978) Nature 275, 593—598
32 Dufy, B., Partouche, C., Poulain, D., Dufy-Barbe, L. and Vincent, J.D. (1976) Neuroendocrinology 22, 38—47
33 Selye, H. (1941) Proc. Soc. Exp. Biol. Med. 46, 116—121
34 Atkinson, R.M., Davis, B., Pratt, M.A., Sharpe, H.M. and Tomich, E.G. (1965) J. Med. Chem. 8, 426—432
35 Holzbauer, M. (1976) Med. Biol. 54, 227—242
36 Plagemann, P.G.W. and Richey, D.P. (1974) Biochim. Biophys. Acta 344, 263—305
37 Shortman, K. and Jackson, H. (1974) Cell. Immunol. 12, 230—246
38 Homo, F., Duval, D., Hatzfeld, J. and Evrard, C. (1980) J. Steroid Biochem. 13, 135—143
39 Mackman, M.H., Nakagawa, S. and White, A. (1967) Rec. Prog. Horm. Res. 23, 195—227
40 Munck, A. (1957) Biochim. Biophys. Acta 24, 507—514
41 Snart, R.S. and Wilson, M.J. (1967) Nature 215, 964
42 Heap, R.B., Symons, A.M. and Watkins, J.C. (1970) Biochim. Biophys. Acta 218, 482—495
43 Wohlhueter, R.M., Marz, R. and Plagemann, G.W. (1979) Biochim. Biophys. Acta 553, 262—283
44 Wade, R. and Jones, H.W. (1956) J. Biol. Chem. 220, 547—551
45 Gomez-Puyou, A., Feder, W., Tuena, M. and Pena-Diaz, A. (1964) Arch. Biochem. Biophys. 106, 455—460
46 Siiteri, P.K., Febres, F., Clemens, L.E., Chang, R.J., Gondos, B. and Stites, D. (1977) Ann. N.Y. Acad. Sci. 286, 384—387