BIOCHEMICAL ACTIONS OF HORMONES

Edited by Gerald Litwack

Volume III

Biochemical Actions of Hormones

Edited by GERALD LITWACK

Fels Research Institute and Department of Biochemistry
Temple University, School of Medicine
Philadelphia, Pennsylvania

VOLUME III

ACADEMIC PRESS New York San Francisco London 1975

A Subsidiary of Harcourt Brace Jovanovich, Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

Library of Congress Cataloging in Publication Data
Main entry under title:

Biochemical actions of hormones.

Includes bibliographies.
1. Hormones. I. Litwack, Gerald, ed. II. Axelrod, Julius, (date) [DNLM: 1. Hormones. 2. Physiology. WK102 B615]
QP571.B56 574.1'927 70-107567
ISBN 0-12-452803-1 (v.3)

PRINTED IN THE UNITED STATES OF AMERICA

Preface

Research directed toward an understanding of the mechanisms of hormone action has been concentrated at the biochemical and molecular levels using individual hormones in mature or developing cells. Volumes I and II of "Biochemical Actions of Hormones" summarized and evaluated the results of this research, covering virtually every area of the field in which significant advances were made through 1971. Volume III evolved from the realization of further advances in established areas and new approaches not previously developed.

A wealth of new information on steroid hormone action is summarized in four chapters. Although a certain amount of overlapping information on the mode of action of estrogens could not be avoided, it comes from some of the foremost laboratories involved with this problem, and, if anything, will make the description of current work on this hormone all the more complete. The recent and previously unreviewed areas of action of hypothalamic-regulating hormones and plasma membrane receptors are presented. These new areas promise to have wide repercussions on endocrine research. Three contributions deal with the approaches using genetics and cell culture to advance our knowledge of hormone action and of systems by which previously undescribed hormones are being discovered. Two other chapters summarize recent advances in the mode of action of thyroid hormone and of hormones acting on the synthesis of proteins in liver perfusion systems.

Originally, only a two-volume treatise had been planned. However, as mentioned above, new advances signaled the need for a third volume. Additional volumes will be published if new knowledge warrants them.

I wish to commend the staff of Academic Press for their excellent cooperation in the publication of these volumes.

GERALD LITWACK

Contents

CHAPTER 1

Hormones and Regulation of Cell Division: Mammalian Cell Cultures as an Experimental Approach

Hugo A. Armelin

I. INTRODUCTION

Studies by classical endocrinologists developed the concept that tropic hormones (proteins, such as the gonadotropins, or steroids, such as estrogens) are endocrine regulators involved in the control of mammalian cell proliferation *in vivo*. The experimental conclusions of these studies were derived from observations of the effects of endocrine organ ablation (hypophysectomy, ovariectomy, adrenalectomy, etc.) and hormone replacement therapy (administration of

crude tissue extracts or pure hormones) in experimental animals. However, many practical difficulties inherent in *in vivo* experiments have limited our understanding of tropic hormone-dependent tissue growth. It seems a reasonable assumption that the isolation of the target cells in culture would circumvent most of the difficulties of *in vivo* experiments, opening a new avenue of experimental investigation. In this chapter, we will examine the present status of mammalian cell culture as a viable approach to the study of physiological growth regulatory mechanisms, considering in particular the role of hormones in these processes. It is not our intention to provide a comprehensive review of the subject. Rather, we will present selected data from the recent literature in order to convey the main viewpoints that have guided our research efforts in the last few years. We shall see that (a) presently, a general procedure to establish "tropic hormones' target cells" in culture is not available; (b) a few cell lines displaying "physiologically significant growth response" are available; (c) classical hormones and hormonelike substances, recently discovered, are key extracellular regulators of cell proliferation in culture. A discussion of formal models also will be presented.

II. ESTABLISHMENT OF "GROWTH RESPONSIVE" CELL LINES: ITS EMPIRICISM AND PITFALLS

Practically any kind of mammalian tissue can be put in culture after dispersion of the cells by enzymatic (trypsin, collagenase, hyaluronidase, etc.) or mechanical means. These primary cell cultures can display properties of the tissue of origin for days or weeks, and in some cases, they can be subcultured to give viable secondary cultures. Such short-term cultures (cell cultures or organ cultures) have been fundamental instruments for basic studies in endocrinology; for instance, the discovery and isolation of hypothalamic releasing factors (Burgus *et al.*, 1969) and sulfation factor or somatomedin (Salmon and Daughaday, 1957; Hall and Van Wyk, 1973; Uthne, 1973). However, for purposes of growth-control studies, short-term cultures are of limited value, and the future of an *in vitro* experimental approach will depend on the establishment of permanent lines displaying, in culture, the growth regulatory mechanisms operative *in vivo*.

In the primary and secondary cultures, clonal selection occurs

since only particular cell types divide. As a general rule, connective tissue fibroblasts divide in culture and usually overgrow other cell types, so that they take over the culture (Sato *et al.*, 1960). Nonetheless, it is possible to isolate functional clones of other cell types provided an adequate selection procedure is used. Starting with functional tumors, and using a specially designed selection technique (Buonassisi *et al.*, 1962), a large number of stable differentiated clones has been isolated in the last 10 years: ACTH-secreting pituitary cells (Buonassisi *et al.*, 1962), ACTH-sensitive adrenocortical cells (Yasumura *et al.*, 1966a), steroid-secreting rat and mouse interstitial cells (Yasumura *et al.*, 1966a; Shin *et al.*, 1968), rat glial cells (Benda *et al.*, 1968), growth hormone- and prolactin-secreting pituitary cells (Yasumura *et al.*, 1966b; Tashjian *et al.*, 1968), and neuroblastoma cells (Augusti-Tocco and Sato, 1969). The isolation and subsequent investigation of these differentiated cell lines demonstrated that mechanisms of hormonal action can be studied using permanently established cell clones as experimental models.

In principle, there are no theoretical limitations to the isolation of clonal cell lines that retain physiological growth regulatory mechanisms in culture. In practice, however, the situation is not that simple. Under the usual condition of primary cultures, factors (unknown humoral factors, classical hormones, etc.) that regulate growth and cell division are limiting, and thus a strong selection exists for cells that escape physiological growth regulation. Consequently, established cells are likely to be independent of physiological growth factors unless systematic precautions are taken to minimize or eliminate this unwitting selection. The approach to these precautions must be cautious because we are dealing with circular arguments; hence, we might easily trap ourselves in a fallacy: first, we want to isolate cells that retain in culture the growth regulatory mechanisms operative *in vivo* (in order to make the study of these regulatory systems possible); second, we know that under culture conditions, a strong selection occurs for cells that escape physiological growth regulation (perhaps all established cell lines presently available are of this type); and third, none of the growth regulatory mechanism operative *in vivo* can be concretely defined at present (we simply assume they must exist because tissue growth in the animal is highly regulated). Necessarily, therefore, "physiologically significant growth response" in culture has had to be operationally defined, and the isolation of cell lines that retain physiologically significant growth response in culture has had to be approached empirically, using common sense as a guide.

The first utilization of a systematic procedure to successfully es-

tablish a cell line with physiologically significant growth response was done by Todaro and Green (1963) when they developed contact inhibited mouse embryo fibroblast lines. In this case, the responsive cells (3T3 lines) grow in culture under strict control of serum factors (Todaro *et al.*, 1965; Holley and Kiernan, 1968) and they do not develop tumors in appropriate hosts (Aaronson and Todaro, 1968). Transformation of these cells by a small DNA virus, such as SV40 or polyoma, abolishes the growth response in culture, so that the cells behave like tumor cells. These mouse fibroblast lines have been an important experimental model in the last 10 years. We will consider them from the viewpoint of recent studies in another section of this chapter.

In the last few years, workers in Gordon Sato's laboratory have taken a second approach to the development of growth-responsive cell lines. The procedure adopted here was to use hormone-dependent tumors of experimental animals as a source of target cells whose growth is dependent on tropic hormones active on the parental tissue. The final goal of this project was to obtain stable cultured clonal lines of cells that develop hormone-dependent tumors in appropriate hosts. It has long been known that ovaries implanted in the spleens of ovariectomized rats or mice develop tumors. Such tumors eventually become pituitary-dependent transplantable tumors (Biskind and Biskind, 1944; Furth, 1968). A possible explanation for the development of these ovarian tumors has been suggested: the spleen is drained by the hepatic portal system; thus, steroids secreted by the ovarian cells are inactivated in the liver before they reach the general circulation. The lack of sex steroids induces the pituitary to hypersecrete gonadotropins, which stimulate the growth of the ovarian transplant. Clark *et al.* (1972) took such ovarian growths, developed in the spleens of Fisher rats, and established a cell line in culture. Surprisingly this ovarian cell line does not respond to FSH or LH in culture, but it does respond to a previously unknown pituitary protein factor (Armelin and Sato, 1972; Gospodarowicz *et al.*, 1974; Jones *et al.*, 1974). When injected in spleens of Fisher rats, the same ovarian cell line seems to develop tumors only in ovariectomized animals (J. Clark, personal communication, 1973).

We followed a similar procedure with a transplantable hormone-dependent mammary carcinoma of AC rats provided by Dr. R. Iglesias (see Iglesias, 1971) as starting material. This adenocarcinoma grows in estrogen-treated normal animals, but fails to develop in hypophysectomized animals even with high estrogen levels. This tumor behavior was stable through serial animal trans-

plantations. The procedure of alternate animal culture passages to select and establish differentiated tumor cell lines (Buonassisi *et al.*, 1962) was used: (a) primary cultures were grown from the mammary tumor tissue in a medium containing horse serum and fetal calf serum supplemented with the hormones presumably active during mammary gland development (Lyons *et al.*, 1958), i.e., steroids (estradiol, hydrocortisone, and progesterone), insulin, and a crude preparation of prolactin; (b) after 15–30 days in culture, primary or secondary cultures were reinjected subcutaneously into male animals (some animals received an implant of estradiol pellets under the skin, other animals served as controls) and tumor development was followed by periodic inspection of the animals. This culture–animal cycle was repeated several times. Short-term, uncloned cultures regularly gave hormone-dependent tumors in animals. Epithelial cells present in these cultures possessed two interesting characteristics: (a) their presence in culture was required to obtain hormone-dependent tumors when cultures were reinjected into animals, and (b) they required prolactin supplementation to grow in a media containing serum from hypophysectomized dogs. Interesting clones have been isolated from these epithelial cells of the short-term cultures. For instance, a series of clones showed a remarkable growth response to glucocorticoids, even after more than 500 generations in culture. They grow very slowly in charcoal-extracted calf serum medium (population doubling time is 60 hours or more), but the addition of hydrocortisone at 10^{-9} *M* decreases the doubling time to 18 hours. The maximum response (doubling time of 13 hours) is achieved with a hydrocortisone concentration of 6×10^{-8} *M*. Progesterone, estradiol, and testosterone cannot replace glucocorticoids. However, we have been unable to isolate clones with a significant growth response to protein hormones like prolactin or insulin (H. Armelin, unpublished results).

Three preliminary conclusions can be drawn at this point: (a) Cultured cell clones exhibiting the identical dependencies in culture as in the animal have yet to be established. In the case of an ovarian cell clone, the cells grow independently of gonadotropins *in vitro*, but require hormonal conditioning of the host (ovariectomy) for growth *in vivo*. In another case, mammary cells seem to require estrogen for growth in the animal but require glucocorticoids *in vitro*. (b) Nonetheless, a number of clones have been established whose behavior in culture suggests novel physiological relationships but whose behavior in the animal is variable, ranging from nontumorogenicity to apparent hormone dependency to autonomy. (c)

The origin of passaged clones is unclear, since the tremendous selective pressures operating *in vivo* and *in vitro* promote the strong possibility that the cell lines ultimately established are variants of the original tumor cells.

III. CONTROL OF CELL PROLIFERATION AND "CELL CYCLE": PROSPECTIVE MODELS

Currently it is popular to divide cell life in a series of cyclically repeated phases: G_1, S, G_2, and mitosis (Mitchison and Creanor, 1971a,b; Mazia, 1974). Initially, these periods were defined by the morphological events that characterize mitotic cells and the biochemical evidences of DNA synthesis which delimit the S phase. G_1 and G_2 are the gaps between these two phases. However, experimental evidence accumulated over the last 10 years indicates that the so-called cell cycle phases are composed of concerted sequences of complex biochemical reactions (Baserga, 1968; Mitchison and Creanor, 1971a,b). Nonetheless, data derived from kinetic analysis of the "cell cycle" (Nachtwey and Cameron, 1968) are difficult to reconcile with the simplistic idea that the proliferating cell goes repetitiously through a series of deterministic events leading to division. These data include: (a) measurements of intermitotic times by time-lapse microcinematography, which have given figures whose variations are too broad to be explained by biological variability; (b) estimates of the length of the cell cycle phases by the ^{3}H-TdR pulsing of mitotic figures technique of Quastler and Sherman (1959), which have suggested that the cell population is not homogeneously traversing the cell cycle; (c) verifications by many laboratories that selected populations of synchronous growing cells (obtained, for example, by the procedure of mitotic cell collection of Terasima and Tolmach, 1963) do not remain stable; the synchrony is lost in the first cycle. This variability and/or instability demands an explanation if one is to preserve the simple idea of cell cycle; usually arguments involve biological variability, the vagaries of complicated experimental manipulations, etc. At this point, it is interesting to note that all these conflicting data are readily observed in rapidly growing cells like culture cell lines and tumor cells in experimental animals (Steel, 1972), which are the kind of cells whose behavior should correspond best to the formal model of a "cycling cell." One hypothesis that can account for all these observations is that the cell population contains

two subpopulations: a proliferating pool (cycling cells) and a resting pool (noncycling cells) and that cells can transfer between the two pools.

The situation with slowly proliferating cells (a rule for *in vivo* tissues) is more complicated than with rapidly dividing populations, because these cells show a rather complex behavior: they present a G_1 whose duration is extremely variable. To reconcile the behavior of cells in normal tissues with the concept of cell cycle, a cell phase called G_0 has been postulated (Lajtha, 1963). Cells in G_0 are thought to be out of cell cycle (resting cells); they are cells that "stand still" but are capable of dividing or entering the cell cycle if properly stimulated. This G_0 behavior is typified by liver cells, which normally seldom divide, but which can readily enter the cycle during liver regeneration. The conventional concept of cell cycle as depicted here can be properly summarized by the scheme presented in Fig. 1 (which will be called Model I).

Although the idea of G_0 has intuitive appeal, the concept has suffered from the lack of a precise definition. This lack, which has brought considerable confusion to the literature (Brown, 1968; Temin, 1971; Novi and Baserga, 1972), is highly undesirable because an unambiguous definition is essential for a wise experimental approach to the problem of cell division control and meaningful analysis of accumulated data.

The preceding discussion suggests the inadequacy of the formal model of cell cycle summarized in Fig. 1 for explaining all the experimental observations to date. Smith and Martin (1973) suggest abandoning the conventional concept of cell cycle in favor of adopting a formal model of cell life which contains an element of randomness.

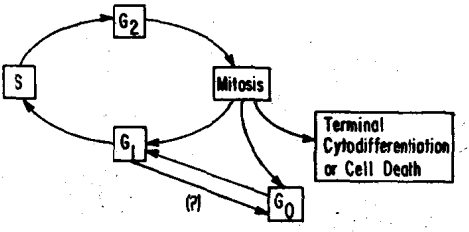

Fig. 1. Compartments of a population of proliferating cells according to Model I. The boxes represent the compartments of the several cell phases; the arrows indicate the direction of transitions that the cells can traverse among the compartments of the cycle, or in or out the cycle. The transitions to leave the cycle can be reversible (to G_0) or irreversible (to terminal cytodifferentiation or cell death). G_1, S, G_2, and M compartments comprise the pool of proliferating cells; G_0 consists of the pool of resting cells that retain the ability to divide.

Thus, the following is suggested as a more realistic model for reconciling experimental data with the concept of cell cycle: it is assumed that the fundamental state of a cell is as a "resting cell" (the state to be designated the R state), and that a resting cell is a cell whose program for cell division is shut off. The program for cell division consists of a precisely determined sequence of biochemical reactions and cytological events, which ultimately leads the cell to a final division into two daughter cells; that is, this program is a sum of those reactions that characterize G_1, S, G_2, and mitosis. The cells in the R state comprise the R compartment of the cell population; cells can exit from the R compartment and enter the P compartment (i.e., the proliferative cells or cells committed to the cell division program) by initiating the program for cell division. The exit from the R state is completely random; thus, any particular cell remains in the R state for an indeterminate time period. There is only one entry to the P compartment: the beginning of G_1 (one must realize that G_1 here has been redefined; in the literature the definition is imprecise and often includes what we are calling R state). After mitosis, only one exit exists; the cell necessarily returns to the R state. The time a particular cell spends in P is a completely determinate period of time (T), where T is the time required for a cell to traverse G_1, S, G_2, and M and finally to return to the R state. The scheme in Fig. 2 represents the proposed model, which for simplicity of description will be called Model II. One must notice that the element of randomness introduced by the random exit of cells from the R compartment (transition R to P is indicated by arrow 1 in Fig. 2) explains the variability of the data obtained by kinetic analysis of the cell cycle mentioned above. On the other hand, the regular cell flow through sequential

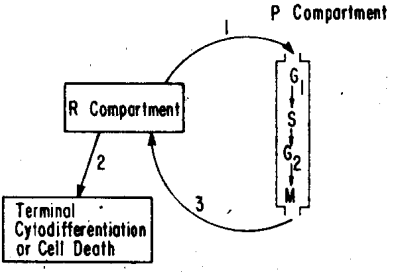

Fig. 2. Compartments of a population of proliferating cells according to Model II. As in Fig. 1, the boxes represent the compartments of the several "cell phases." G_1, S, G_2, and M are subcompartments of the P compartment. The arrows 1, 2, and 3 indicate the transitions that are allowed among compartments; only transitions 1 and 2 are random.

events in the P compartment is in accordance with the highly organized sequence of biochemical reactions that characterizes G_1, S, G_2, and M.

The R state described in the model above has general application; for instance, it applies to (a) stem cells like those of the erythrocyte line, which can exit from R toward P (Fig. 2, arrow 1) for replication, or can take the route of terminal cytodifferentiation (Fig. 2, arrow 2) to the final stage of erythrocyte; (b) liver cells, which retain their ability to replicate even though completely functional, and thus can leave R for proliferation during liver regeneration; (c) nonfunctional tumor cells, in culture or in animal, which exit from R for proliferation or death.

For the investigator preoccupied with the mechanisms of cell proliferation control, Model II (Fig. 2) implies that the proliferation of a cell population is dependent on three parameters: (a) the probability P_1 that a cell leaves "R state" for replication (transition to P compartment, arrow 1); (b) the probability P_2 which determines the exit of cells towards cytodifferentiation or death; and (c) the time T, which is the period of time required for a cell to traverse G_1, S, G_2, M, and return to the R state. If one considers, first, that experimental results obtained by analysis of labeled mitosis fraction (the procedure of Quastler and Sherman, 1959) indicated that S and G_2 durations do not vary for a particular cell type (Nachtwey and Cameron, 1968) and, second, that the duration of mitosis shows a narrow variation even among different cell types, one necessarily will conclude that T probably does not vary for a particular cell type, so that the control of growth in a cell population is exercised exclusively through variations in P_1 and P_2. In the particular situation of the established lines of mammalian cell cultures, which are unable to undergo terminal cytodifferentiation and which are under conditions of negligible cell death, population growth will be a function solely of P_1, the transition from the R state to the "P phase." Assuming that the enormous simplification obtained by this analysis is realistic, one can use cell cultures as experimental models to study how environmental conditions affect P_1 and, therefore, what controls cell proliferation. A number of predictions can be derived from the proposed Model II which are easily tested by simple experiments. One corollary is that through manipulation of environmental conditions, we should be able to induce cell rest (R state) in culture. The minimal characteristics of the resting state must include (a) a population which is not increasing in number, (b) the maintenance of a high level of cell viability, and (c) absent or negligible DNA synthesis. A second