Short-term Toxicity Tests for Non-genotoxic Effects
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CHAPTER 14

# *Methods for Assessing the Effects of Chemicals on the Endocrine System*

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## 14.1 INTRODUCTION

The endocrine system is an important integrating system of the body. The various endocrine glands influence the following major physiological functions: (1) maintenance of homeostatis (involving enzymes, substrates and co-factors) which provides for an optimum environment for the basic biochemical reactions of the body; (2) regulation of growth maturation; (3) reactions to exogenous stimuli such as stress, starvation and infection; (4) regulation of reproductive processes.*

Finely-regulated hormonal mechanisms make the endocrine system particularly sensitive to toxic effects of exogenous compounds. These effects can interfere with the action, biosynthesis and release of hormones, and result in alterations in hormonal target cells. In this review, methods are discussed that are useful for the short-term testing of chemicals that affect the endocrine system.

### 14.1.1 Important human and other mammalian health problems

Interaction of chemical compounds with endocrine structures leading to dysfunction of the endocrine glands may result in a broad variety of metabolic or neoplastic alterations (De Bruin, 1976; IARC, 1979). For example, the therapeutic use of lithium salts for the treatment of manic-depressive disorders has provided much information about the toxic action of relatively high doses of lithium on the endocrine system of man and animals (Cooper *et al.*, 1979; Fauerholdt and Vendsborg, 1981; Bagchi *et al.*, 1982). On the other hand, relatively little information is available about the effects of low doses of lithium from routine toxicity tests.

Barsano (1981) reviewed the occurrence of thyroid dysfunction as a consequence of exposure to polyhalogenated biphenyls. Rats exposed to polychlorinated

*A chapter dealing specifically with reproductive processes is included in this volume. This chapter deals with reproduction only in so far as this is influenced by endocrine organs other than the gonads (e.g. pituitary, thyroid, adrenal gland and the endocrine pancreas).

biphenyls (PCBs) and polybrominated biphenyls (PBBs) exhibited decreased serum thyroxine (T4) levels and goitrogenesis. From epidemiological evaluations, it appeared that PBBs might induce primary hypothyroidism although it has also been suggested that this effect was a PBB-induced exacerbation of pre-existing, but subclinical, thyroid disease. Exposure to antithyroid agents (e.g. thiourea or ethylenethiourea) impaired the biosynthesis of thyroid-hormones with a consequential decrease in thyroid hormone release (Graham and Hansen, 1972; Graham *et al.*, 1973). This, in turn, caused an enhanced secretion of pituitary thyrotropin (TSH) to promote proliferation of tumour cells in the target organ. In this way, chemical compounds may act, via the endocrine system, as tumour promotors.

These examples serve to show that the study of endocrine systems in routine toxicity tests is important, not only from a mechanistic point of view, but also to ensure reliable evaluation of the safety of chemicals.

### 14.1.2 General comments on successes and failures using routine *in vivo* toxicity tests

In the past, the endocrine system was not a common subject for study in toxicological investigations. Few effects have been described in either short-term or long-term experiments except for those influencing the thyroid. Thus, increased thyroid weight and induced hyperplastic alterations consisting of nodular or microfollicular goitre in rat have been observed following the administration of tetrasul (Verschuuren *et al.*, 1973a,b), and enlargement of the thyroid and activation of thyroid tissue after exposure to sodium bromide (Van Logten *et al.*, 1974, 1976), strontium chloride (Kroes *et al.*, 1977) and zinc phosphide (Muktha Bai *et al.*, 1980).

Recent developments in immunochemistry have resulted in the availability of a number of species-specific antibodies which have greatly expanded the possibilities to determine circulating hormone levels using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) techniques. These allow the detection of specific hormone-producing cells in tissue sections and enable one to carry out organ specific function tests.

The following procedures are suggested to detect effects on the endocrine systems in routine *in vivo* toxicity experiments: (1) determination of the weight of the endocrine organs and histology (haematoxylin/eosin) as screening parameters; (2) determination of circulating hormones or tropic hormones in combination with morphological or immunocytochemical methods; and (3) specific function tests and biochemical methods to determine dysfunction of the endocrine organ under study.

The use of such an approach in toxicity experiments has provided insight into the mechnisms of action and the toxicity of chemical compounds. In the case of sodium bromide, a decreased concentration of thyroxin and corticosterone in the serum of rats was found by radioimmunoassay. Using immunocytochemical techniques (peroxidase–anti-peroxidase antibody method or indirect peroxidase-labelled anti-

body method), a decrease was observed in the amount of thyroxin in the thyroid while the immunoreactivity of thyroid stimulating hormone and adrenocorticotropic hormone-producing cells in the pituitary gland was increased. Concomitantly, the serum TSH concentration was increased and a decrease in vacuolization of the zona fasciculata of the adrenals was observed (van Leeuwen *et al.*, 1983; Loeber *et al.*, 1983). From these observed changes, it was possible to conclude that bromide directly disturbs thyroid and adrenal function while changes in the pituitary are due to feedback regulation.

This combined biochemical and morphological approach has also been applied in toxicity studies in organotin compounds (Funahashi *et al.*, 1980; Manabe and Wada, 1981; Krajnc *et al.*, 1984). After a single oral dose of triphenyltin fluoride (TPTF) to rabbits, Manabe and Wada (1981) described a decrease in fasting glucose, inhibition of insulin release in response to glucose, glucagon and arginine, and a normal pancreatic islet morphology. They concluded that the diabetogenic activity of TPTF is due to the inhibition of insulin release. However, hypoinsulinaemia observed in rats treated with Bis(tri-*n*-butyltin) oxide (TBTO), was not accompanied by changes in immunochemical reactivity of insulin and glucagon in the pancreas (Krajnc *et al.*, 1984). In the latter case, it was suggested that the decreased insulin levels may reflect a decreased metabolic rate, possibly as a consequence of the decreased thyroxin concentration that was observed. This effect was accompanied by a marked increase in immunoreactivity for TSH in the pituitary, with a consequently reduced serum TSH concentration. No effect was seen on the ACTH (adrenocorticotropic hormone) producing cells in the anterior pituitary. Furthermore, increased stainability of LH (luteinizing hormone) cells and the absence of effects on FSH (follicle stimulating hormone) cells and the absence of effects on FSH (follicle stimulating hormone) cells was accompanied by decreased serum LH and normal FSH levels. These striking correlations between radioimmunochemical and cytochemical findings indicate that a combination of radioimmunoassay and immunocytochemistry can be a sensitive and reliable method to detect chemically induced dysfunction of the endocrine system.

### 14.1.3 Consideration of particular problems

The rat is the species most frequently used in routine toxicity tests. The rat has also served as an excellent model for the study of endocrine mechanisms. Thus, it would appear reasonable to study the endocrine system as part of toxicity experiments as they are presently performed. This offers the advantage of studying effects of chemicals on the system at the same time that other facets of endocrine toxicity are studied in the same species. However, stress, housing conditions, diet, age and sampling procedures can all influence the results obtained and lead to conflicting results. Therefore, special attention should be given to the experimental conditions. It has been known for a long time that animals housed in groups are particularly

sensitive to non-specific stimuli such as environmental change (transport stress), noise, weighing and injections (Barrett and Stockham, 1963). Handling the animals daily over a period of some weeks in order to get them accustomed to the experimental manipulations can improve the reliability of the results obtained.

The choice of a suitable blood sampling method is of great importance if reliable measurements of hormones are to be obtained. Decapitation without anaesthesia has been shown to result in less stress compared with cardiac or retro-orbital sinus puncture under ether anaesthesia, abdominal aorta puncture under chloroform anaesthesia, or decapitation after treatment with Nembutal ® (Dohler *et al.*, 1977). Nevertheless, daily handling of the animals (during which the decapitation procedure is simulated) remains a prerequisite.

In order to properly evaluate endocrine effects, it is necessary to establish baseline levels of circulating hormones. Systematic determinations of the circadian rhythm in the selected species and strain have to be performed. By selection of the appropriate time for sampling, the variance of the endocrine parameters within the test groups can be reduced, with improved resolution of the endocrine tests.

Little is known about the influence of perinatal exposure or exposure *in utero* to toxic compounds on the endocrine system. There is evidence to suggest that the developing endocrine system is more susceptible than the mature one.

Changes in dietary composition can affect the functioning of the endocrine system and hence influence the effects of toxic compounds. The purity, type and amount of nutrients and minerals in diets should be carefully controlled to enable toxic effects on the endocrine system to be properly interpreted, and to ensure comparability between different investigations. For example, if the amount of iodide in the diet is too high, tests to determine thyroid dysfunction will lose sensitivity due to the presence of excess substrate for thyroid hormone synthesis. Furthermore, depending on the protein source of the diet, goitrogens can be present (Liener, 1979). The total amount of protein is also of importance (Singh *et al.*, 1971). Carbohydrate and lipid composition of the diet should be established, since both influence pancreatic (Maji *et al.*, 1980) and adrenal function (Lawson *et al.*, 1981). Adequate calcium, magnesium and phosphate content of the diet is important because of the influence of these compounds on calcitonine-parathyroid hormone mechanisms, particularly in relation to the occurrence of a sex-linked nephro- calcinosis in female Wistar rats (Harwood, 1982). The same is true for other minerals, particularly potassium and sodium for which high dietary concentrations might mask and low dietary concentrations might enhance the effects of toxic compounds on mineral corticoid secretions.

Although most toxicological experiments involve exposure by the oral route (intubation or ingestion), impairment of pituitary and thyroid function has been described after short-term inhalatory exposure (Clemons and Garcia, 1980; Atwal and Pemsingh, 1984). It is, therefore, likely that systemic effects of chemical compounds on the endocrine system can be determined in short-term toxicity experiments regardless of the route of exposure.

## 14.2 *IN VIVO* STUDIES

### 14.2.1 Clinical observations

In general, the effects of chemicals on the non-reproductive endocrine organs are not easily observed clinically. In contrast to gonadal dysfunction, which is easily detected in reproductive studies, growth depression or changes in protein or carbohydrate metabolism ascribable to impaired pituitary, thyroid or pancreatic function can also result from other, non-endocrinal effects.

### 14.2.2 Morphology

Weight changes and histopathological findings are frequently indications of impairment of the endocrine system. Routine histological staining procedures for tissue sections (e.g. haematoxylin and eosin staining) do not reveal alterations in the number of the specific hormone-producing cells, or the amount of hormone in the endocrine organs following exposure to toxic agents. Immunocytochemical (and especially immunoperoxidase) techniques can be used to localize specific hormone-producing cells. In general, the use of these methods has the great advantage that many antigens can be demonstrated in paraffin sections of formalin fixed tissue. Sometimes, pre-treatment with proteolytic enzymes such as trypsin is necessary to unmask antigenic sites (Mepham *et al.*, 1979). In particular, fixation with formaldehyde solution containing mercuric chloride (sublimate) offers good preservation of antigens; the sublimate must be removed before the staining procedure. Apart from application on paraffin sections, however, recent advances in immunocytochemistry allow the localization of antigens in plastic-embedded material both in light-microscopic and immunoelectron-microscopic techniques (Dell'orto *et al.*, 1982; Casanova *et al.*, 1983; Figueroa *et al.*, 1984). A critical review of the applications of immunocytochemistry to the endocrine system has been given at El Etreby (1981).

Different immunoperoxidase techniques are used to achieve localization of antigents in tissue sections. These include the peroxidase-labelled antibody method (which can be divided into direct and indirect methods according to Nakane and Pierce, 1966), the unlabelled antibody method of peroxidase–anti-peroxidase (PAP) (Sternberger, 1979) and the avidin–biotin–peroxidase complex (ABC) method (Hsu *et al.*, 1981). In each of these techniques, peroxidase is localized in areas of tissue antigen through an antigen–antibody reaction. The sites of peroxidase localization are made visible through addition of a substrate that reacts with the peroxidase label to result in an insoluble staining product. Substrates commonly used are 3,3'-diaminobenzidine or 3-amino-9-ethylcarbazole dissolved in *N,N'*-dimethyl- formamide in combination with hydrogen peroxide. These methods achieve intense staining with minimal non-specific background staining; they have sufficient sensitivity to stain the cells with high dilutions of antiserum. The addition of compounds such as imidazole can enhance the cytochemical reaction for peroxidase

(Straus, 1982). For a comparison of the sensitivity and efficiency of the indirect peroxidase labelled antibody method with the peroxidase–antiperoxidase technique, the reader is referred to Bosman *et al.* (1983).

The techniques mentioned above were applied in the experiments described in Section 14.1.2. For the investigation of toxic effects on the endocrine system, information about pituitary function is of main importance. For the determination of the different tropic hormones, species-specific antisera are needed. Many of these antisera were obtained from the Rat Pituitary Hormone Distribution Programme, National Institute of Arthritis, Metabolism and Digestive Disease (NIAMDD), Bethesda, Maryland, USA.

A recent and very interesting development in the fields of immunochemistry involves the use of monoclonal antibodies. Their defined specificity for tissue compounds enables them to be used in double staining procedures for the simultaneous localization of antigens on membrane structures or intracellular components (Boorsma, 1984).

### 14.2.3 Biochemistry of tissue and biological fluids

Radioimmunoassay appears to be the most appropriate technique for the determination of hormones in body fluids and tissue homogenates. In this review, methods are not discussed in detail, but the reader is referred to recent reviews or handbooks (Abraham, 1977; Lorraine and Bell, 1981; Hunter and Corrie, 1982). Radioimmunoassay can also be applied as a post-column detection method after chromatographic separation of various hormones (Loeber, 1984).

The availability of many commercial clinical radioimmunassay kits for steroids and thyroid hormones has undoubtedly stimulated the attention of toxicologists to these types of hormones. However, the use of these kits in routine animal toxicity tests has some disadvantages. Most kits require a relatively large sample size so that the method must be modified to a microscale for use in tests involving small animals. Furthermore, differences in the metabolism of steroids between humans and rats may lead to unexpected cross-reactions with metabolites that cause interference.

In general, serum or plasma can be used in RIA methods; in practice, serum is preferred because of coagulation that occurs during thawing of frozen plasma samples. In principle, radioimmunoassays can also be performed with urine although one should realize that steroids are present in urine as conjugated glucuronides or sulphates.

Hormones may be present in two physical forms in blood; as free compounds or bound to serum proteins. Because of its high concentration, albumin is a major absorbent for circulating hormones although specific binding proteins also exist. In the rat, transcortin is the binding protein for corticosterone, whereas (in contrast to the human) the rat does not possess the specific thyroxin-binding globulin (TBG). Therefore, the determination of free-T4 used in clinical chemistry is of no additional

value in rat toxicity experiments because the effect of toxic compounds on T4 levels will parallel changes in free-T4.

Nevertheless, the concept of bound versus free hormones is of importance for the evaluation of changes in circulating hormone levels. A decrease in total concentration as measured with RIA methods does not indicate, *per se*, a physiologically important effect because the concentration of the biologically active free form might be unchanged while the total amount might be reduced due to a decrease in binding protein.

No kits are commercially available for the determination of tropic hormones in animal toxicity investigations because it is necessary to use specific antisera for the species used. For method development, antisera for tropic hormones are provided by NIAMDD in the Rat Pituitary Hormone Distribution Programme. Iodination is generally carried out using the Chloramine-T technique according to Hunter and Greenwood (1962), whereas counting data can be evaluated using the computer program developed by Rodbard and Lewald (1970).

New methods have been developed recently as an alternative to the use of radioisotopically labelled materials. However, the sensitivity of luminescent immunoassays as compared with RIAs has not yet been fully evaluated. Schall and Tenoso (1981) reviewed the possibilities of various labels as substitutes for radioisotopes; they concluded that there was no single, all-round best label to replace the radioisotopes. But any new label might have its own advantages over radioimmunoassays. Improvements in luminescence-measuring equipment are likely; a promising development in this respect might be the introduction of fibre-optics (Whitehead *et al.*, 1979).

Biochemical analysis of endocrine tissue from animals exposed *in vivo* is an important tool for elucidating the mechanisms of action of toxic compounds. A recent development, for instance, is the application of lipid-free homogenates for study by two-dimensional chromatography or high-performance liquid chromatography in order to determine the various thyroid hormones and their precursors (Gordon *et al.*, 1982). The determination of thyroid-peroxidase activity can also be important, since changes in iodide-dependent or guaiacol-dependent activity might reflect alterations in the incorporation of iodine in tyrosine residues or in the coupling of iodinated tyrosine residues in thyroglobulin, respectively.

### 14.2.4 Function tests

Function tests are a valuable tool in assessing the endocrine toxicity of chemical compounds. Due to the complexity of the endocrine system, there is no single overall function test, but the various endocrine organs have their own specific tests based upon the uptake of radioisotopes or their reaction on 'externally' added hormonal stimuli. The need for undertaking function tests of the endocrine system is indicated by changes in organ weights or morphology, or by changes in circulating hormone levels observed in screening studies.

Release tests are very suitable for the study of endocrine organ function. For the anterior pituitary, the function of various tropic-hormone producing cells can be examined by stimulation with hypothalamic-releasing factors. Following intravenous injection of 1 $\mu g/kg^{-1}$ body weight of luteinizing hormone-releasing hormone (LHRH), also called luliberin, the release of luteinizing hormone (LH) and follicle-stimulating-hormone (FSH) can be determined in serum using RIA. In a similar manner, thyrotopin-releasing hormone (TRH or thyroliberin) induces the secretion of thyroid-stimulating hormone (TSH) and prolactin into the serum, and corticotropic-releasing homone (CRH or corticoliberin) affects the adrenocorticotropic hormone (ACTH)-producing cells.

Following the administration of ACTH, the concentration of corticosterone can be determined in the serum as a measure of adrenal cortex function.

For the study of pancreatic function, administration of glucose by the measurement of immuno-reactive insulin (IRI) is most appropriate. It should be noted that oral administration of glucose produces higher insulin levels than parenteral administration, leading to equal hyperglycaemia (McIntyre *et al.*, 1964).

At the National Institute of Public Health and Environmental Hygiene (NIPHEH) in Bilthoven, the Netherlands, various release tests are performed consecutively in the same animals. This necessitates the collection of blood samples without killing the animal and under conditions of minimal stress. This can be achieved by inserting a silicon rubber cannula into the jugular vein under Nembutal ® anaesthesia. Two to three days after surgery, the animals are fasted overnight and blood samples taken (without anaesthesia) before and 3, 5, 10, 15 and 20 minutes after intravenous injection of glucose (4 $mol/kg^{-1}$ body weight) for IRI determination. Pituitary function is tested in the same animals 48 hours later with a 48–72 hour interval between the administration of TRH and LHRH (1 $\mu g/kg^{-1}$ body weight). Blood samples are taken before, and 8, 20 and 60 minutes after injection. The various hormone concentrations in the serum of individual animals are plotted and the area above the zero-time value is calculated and statistically evaluated. This method offers the advantage of requiring the use of a small number of animals while providing reliable results.

Another type of function test is the uptake of radiolabel. For the assessment of thyroid function, for instance, a well-known technique is the determination of the uptake of parenterally administered 125 or 131-iodine (2–2.5 $\mu C/kg^{-1}$ body weight) by the thyroid. Radioactivity can be determined in excised thyroid glands, but it is also possible to anaesthetize the animals and put them between the detectors of a gamma counter. In this way, individual rats can be screened for the uptake as well as release function, by determining the radioactivity 6, 24 and 48 or 72 hours after administration of the radioisotope.

Function studies can also be performed with non-invasive techniques. The determination of 17-hydroxy-corticosteroids in 24 hour urine samples is an effective way to determine adrenocortical activity. An increase in 24-hour levels, whether or not after ACTH administration, regularly reflects hyperadrenocorticism. For a

differential diagnosis, determining the suppression of corticosteroid production by metyrapone might be useful.

Although there are some ethical drawbacks, function tests can also be applied in toxicity experiments in humans. This facilitates the comparison with animal models as a predictive model for human risk assessment.

### 14.2.5 Surgically modified animals

As an alternative to studies of endocrine function by hormone determinations and function tests, surgically-modified animals (in which particular organs have been removed) can be used to establish the effects of impairment of the endocrine system, or to rule out the involvement of particular endocrine organs in the toxic action of chemicals. This approach is not only valuable in endocrine toxicity studies, but also in experiments to determine hormone mediated immune alterations (Vos and Dean, this volume). By using adrenalectomized or hypophysectomized rats, for example, it can be shown that alterations in glucocorticoid or growth hormone concentrations in the circulation are not the cause of thymic involution observed in animals exposed to TCDD (Van Logten *et al.*, 1980).

## 14.3 *IN VITRO* TESTING

In theory, every known aspect of cell regulation can be tested *in vitro* and the effects of toxic substances on these control mechanisms can be examined. However, *in vitro* techniques have not been used extensively in practice; consequently, little is known concerning the toxic effects of chemicals in such systems. For this reason, much of the following discussion relates to test systems that may prove useful in the future. The methods proposed do not cover all systems or all possible endocrine interactions; rather, examples have been provided for selected systems which may be broadly applicable to others.

### 14.3.1 Thyroid

The synthesis and secretion of thyroxin ($T_4$) and triiodothyronine ($T_3$) involves several steps that can be influenced by exposure to toxins. Dietary iodine is taken up as iodide by the thyroid in response to the binding of thyrotrophin (TSH) to cell membranes. Once inside the thyroid cell, iodide is oxidized and combined with tyrosine residues of thyroglobulin to form monoiodotyrosyl or diodotyrosyl residues. $T_4$ and $T_3$ are formed by the coupling of iodotyrosyl residues within the thyroglobulin molecule and secretion of $T_4$ and $T_3$ occurs following proteolytic cleavage of these hormones from thyroglobulin. Methods for the analysis of these various steps in the biosynthetic pathway are considered in Sections 14.2.1.1 to 14.3.1.3.

#### *14.3.1.1 Uptake of iodide*

The inhibition of the uptake $^{125}I$ can be readily measured in thyroid tissue or cell culture (Weiss *et al.*, 1984). Several substances, such as perchlorate, nitrate and thiocyanate, are known to act as competitive inhibitors of the TSH-regulated, Na/K-ATPase mediated transport. Such inhibitors can be used as reference standards in toxicological studies. Thyroid glands or cell cultures can be used to examine the effects of toxins on iodide transport (Weiss *et al.*, 1984).

#### *14.3.1.2 TSH receptor binding*

The binding of TSH to plasma membrane fractions which contain the receptor for TSH can also be assessed (Tate *et al.*, 1975; Pekonen and Weintraub, 1979). Potential inhibitors of TSH binding can be examined by competitive inhibition analysis to determine the effects on the number of receptors and relative binding affinities. Such inhibition would be expected to correlate with decreased synthesis and secretion of $T_4$ and $T_3$. The binding of TSH to membrane receptors is associated with the stimulation of adenylate cyclase and increased production of cyclic AMP (Lefort *et al.*, 1984; Carayon *et al.*, 1978). Therefore, any interference of TSH binding would likely be involved in inhibiting adenylate cyclase activity. However, TSH binding and adenylate cyclase activity should be examined in conjunction in order that the validity of any assumptions made about physiological effects which might result from toxic inhibition of TSH receptor interactions may be determined. An example of discrepancies that might occur is found in the work of Marshall *et al.* (1977) who showed that D-propanalol increased the binding of TSH to thyroid membranes but this increase was not associated with an increase stimulation of adenylate cyclase.

#### *14.3.1.3 Oxidation and coupling reaction*

The oxidation and coupling reactions that lead to formation of $T_3$ and $T_4$ are inhibited by substances such as propylthiouracil and methylmercaptoimidazole. These compounds inhibit thyroid peroxidase, a membrane-bound haemoprotein which catalyses both oxidation and coupling reactions in thyroid tissue (Taurog, 1970). Toxic substances could be tested in thyroid membrane preparations for their ability to inhibit thyroid peroxidase, and thiourelylene drugs could be used as model inhibitors to establish relative potencies (Engler *et al.*, 1982). Such inhibition could be examined further by measuring the amount of monoiodothyronine and diiodothyronine in thyroid glands grown in culture.

### 14.3.2 Adrenal gland

The adrenal cortex is responsible for the synthesis and secretion of glucocorticoid steroid hormones which regulate many important functions of the body. The pitui-

tary hormone adrenocorticotropin (ACTH) binds to membrane receptors on adreanl cortical cells which activate the adenylate cyclase systems and ultimately stimulate the synthesis of glucocorticoid hormones. The biosynthetic pathway for these hormones involves many enzymatic steps and utilizes cholesterol as the primary precursor. Cholesterol is accumulated in the adrenal cortex by the endocytosis of lipoproteins from the blood; this process is under the control of ACTH. The mineralocorticoid hormone, aldosterone, is also synthesized by the adrenal cortex. However, its synthesis is primarily controlled by plasma sodium levels and angiotensin II. The various control functions (such as receptors, enzyme activation and synthesis, pituitary secretion of ACTH, angiotensin II production and action of adrenal steroids on target cells) present a complex array of possible points at which toxins could interfere with proper adrenal function. Some of the possible *in vitro* methods that may be useful to determine effects on adrenal function are reviewed in Sections 14.3.2.1 to 14.3.2.3.

#### *14.3.2.1 ACTH receptor interactions*

Radiolabelled ACTH can be used to establish saturation curves for the assessment of binding parameters of adrenal cells or plasma membrane preparations (Simonian *et al.*, 1982). The effects of chemicals on either the dissociation constant or binding capacity (number of receptors) in these systems may provide an indication of toxicity. Should specific alterations in these receptor characteristics be found, it would be important to establish whether they were associated with alterations in adenylate cyclase activity or alterations in the biosynthesis of adrenal steroids. Such a system has been described for bovine adrenal cells in serum-free media which respond to ACTH by synthesizing the appropriate corticoids (Simonian *et al.*, 1982).

#### *14.3.2.2 Lipoprotein uptake by adrenal cells*

Although the adrenal gland synthesizes cholesterol from acetate, it also acquires cholesterol by endocytosis of low density lipoprotein (LDL) from the blood (Brown *et al.*, 1979). It is conceivable that lipophilic toxins could not only be delivered to cells by this route, but also interfere with the uptake process. $^{125}$I-LDL could be examined and the effects of toxic substances on binding parameters assessed. The influence of toxicants transferred by means of LDL-endocytosis on the activity of HMG CoA reductase (the rate limiting enzyme in cholesterol synthesis), acyl-CoA : cholesterol acyltransferase (the enzyme which reesterifies excess cholesterol for storage as cholesterylesters) and synthesis of LDL receptors could be examined in adrenal cells or in cultured fibroblasts (see Brown *et al.*, 1981).

#### *14.3.2.3 Biosynthesis of adrenal steroids and enzyme inhibition*

The biosynthesis of adrenal steroids involves a complex pathway consisting of

many enzymatic steps. The overall effect of a toxicant could be examined by adding $^{14}$C-cholesterol to adrenal cells or to glands in culture and measuring the production of $^{14}$C-corticosterone and/or $^{14}$C-aldosterone. The individual enzymatic steps could also be examined. One of the most important of these would be the conversion of cholesterol to pregnenolone which is performed by the desmolase enzyme complex; this is the rate-limiting step in steroid biosynthesis. Other important enzymes in the pathway, such as 3B-hydroxysteroid dehydrogenase, 17 ∝-hydroxylase, 21-hydroxylase and 11B-hydroxylase, could also be examined by precursor-product analysis.

### 14.3.3 Adrenal hormone action at the target cell

#### *14.3.3.1 Glucocorticoids*

As indicated previously, the glucocorticoid hormones secreted by the adrenal cortex influence many important physiological functions. The concentration and activity of several liver enzymes are increased by glucocorticoid treatment (Rousseau and Baxter, 1979). These increases appear to result from the binding of glucocorticoids to their respective receptor which stimulates the transcription of specific mRNAs that code for the synthesis of these enzymes. The binding of glucocorticoid receptor complexes to nuclear sites is closely correlated with the level of enzymatic stimulation. Thus occupancy of the receptor by hormone is closely coupled with response.

Although there are clear-cut examples of the stimulator (anabolic) effects of glucocorticoids, these hormones also have an inhibitory or catabolic effect in many systems. These include: the suppression of DNA synthesis, the promotion of protein breakdown in muscle, the suppression of immunological and inflammatory responses, and the inhibition of cell proliferation in lymphoid, fibroblastic, epithelial and bone cells. Although the mechanism of action of glucocorticoids in these inhibitory responses is not known, it appears to involve hormone–receptor interactions. Therefore, regardless of the stimulatory or inhibitory actions, the binding of glucocorticoids to cellular receptors is important for their action. Since a great deal is known about hormone–receptor interactions in these systems, and since such interactions are likely to be involved in toxic interference of hormonal regulation, a study of these receptor systems may provide valuable insight about the effects of toxic substances.

Glucocorticoid receptors are present in many tissues and several cell lines are available (Gasson and Bourgeois, 1983). Receptors are present in the cytosol and nuclei of homogenized cells or tissues and the binding parameters (numbers of sites and dissociation constant) can be determined by saturation analysis (for a discussion of the principles and methods involved, see Chapter 2, Clark and Peck, 1979). Toxic agents can be added in these systems. Classical competitive inhibition curves are easily performed and provide a rapid method for the determination of relative binding affinity. If substances are found that inhibit the binding of [$^{3}$H]-

glucocorticoids to the cytosol receptor, it is possible that such a substance may interfere with glucocorticoid action. That is, the binding of a chemical to the glucocorticoid receptor may interfere with nuclear accumulation of the hormone receptor complex. This possibility can be tested by measuring the nuclear accumulation of receptor following exposure of cells or tissues to both toxin and [$^3$H]-glucocorticoid. Although a blockade or reduction in the binding of [$^3$H]-glucocorticoid receptor complexes in the nucleus would be suggestive of an inhibitory action, it is possible that such substances might form stable receptor–toxin complexes in the nucleus and act as glucocorticoid agonists. However, it would require analysis of the response parameters (such as enzyme synthesis) in order to establish whether the chemical is acting in an agonistic or an antagonistic manner.

#### *14.3.3.2 Mineralocorticoids*

Mineralocorticoids, such as aldosterone, regulate electrolyte balance in the kidney, salivary glands, sweat glands and the gastrointestinal tract. Aldosterone augments the transport of sodium across epithelia by stimulating the synthesis of proteins that are involved in increasing the permeability of the apical membrane to sodium and the energy metabolism of the cell.

Aldosterone receptors are present in target organs for mineralocorticoids, such as the kidney and toad bladder (Marver *et al.*, 1978). These receptors form activated nuclear bound complexes in a fashion similar to those of other steroids. The mechanism by which aldosterone controls sodium transport probably involves the synthesis of proteins involved in the function of the sodium channel and energy production (ATP). Aldosterone stimulates an increase in the number of sodium-specific apical membrane channels and increases the activity of at least four mitochondrial enzymes. Therefore, the major effect of aldosterone is to increase the activity of enzymes involved in the generation of ATP. The increased ATP acts as an energy source for the sodium pump and also may increase the number of sodium pumps. In addition to these effects, aldosterone also stimulates phospholipase activity, fatty acid synthesis and acyltransferase activity. All of these actions are probably involved in altering the membrane functions in the renal cell.

The assessment of toxic effects of mineralocorticoid actions could involve any one of the above steps; however, most of these would involve difficult methodology that would be unsuitable for use in routine testing. The most easily accessible parameter for testing would be receptor binding interactions analogous to those discussed for the glucocorticoid system.

### 14.3.4 Hypothalamic–pituitary system

The hypothalamus produces releasing hormones that stimulate the secretion of pituitary hormones which control many other endocrine gland functions. Although

toxic effects could interfere with many aspects of these complex pathways (some of these have been discussed in previous sections) *in vitro* analysis would best be done at the pituitary level. For this reason, interactions with pituitary cells or glands in culture are the focus of potential methods discussed here.

The plasma membrane of anterior pituitary cells contains specific receptors for the various releasing hormones produced by hypothalamic neurones. These hormones include: corticotropin releasing hormone (CRF), which stimulates secretion of ACTH; thyrotropin releasing hormone (TRF), which stimulates secretion of TSH; and growth hormone releasing hormone (GRH), which stimulates the secretion of somatotropin (STH). Not included in this list are the gonadotropin releasing hormone and prolactin inhibitory factor since these are discussed in relation to reproduction by Nadolney *et al.* (Chapter 16, this volume).

The binding of each of the releasing hormones is important for the production of pituitary hormones, and thus could be an important point at which toxic substances could interfere with pituitary function. This interference may be tested by measuring the ability of toxins to inhibit or enhance the binding of radiolabelled releasing hormones to pituitary glands *in vitro*, pituitary cells in culture or to pituitary membrane preparations. Any observed effects on the binding parameters of hormone receptor interactions could be coupled to the release of specific pituitary hormones from cells or glands in culture. Radioimmunoassays are available for each of the pituitary hormones so that effects on stimulated release into the culture medium could be checked readily.

The effects of opiate drugs on TSH secretion by superfused anterior pituitaries can serve as an example of this type of analysis (Judd and Hedge, 1983). Beta-endorphin, and endogenous opioid, and other opiate drugs, stimulate TSH secretion from the anterior pituitary *in vitro*. Thus, model compounds are available to serve as reference standards. Nothing is known about the mechanisms involved; however, it is possible that stimulation may be due to enhanced TRF binding. Toxic substances with similar activity would be potential inducers of hyperthyroidism. It should be noted, however, that opioid peptides decrease plasma TSH, *in vivo*, rather than enhance its secretion. This action is presumed to be at the level of the hypothalamus where opioids cause a decrease in TRF. Therefore, it would be important to verify *in vitro* observations and predictions using *in vivo* tests.

In addition to the effects of releasing hormones on the secretion of pituitary hormones, chemicals could also interfere with the normal steroid homrone feedback mechanisms. For example, glucocorticoids suppress the ability of CRF to stimulate ACTH secretion. This feedback inhibition is necessary for the maintenance of normal stimulation and secretion of adrenal steroids. Cultured anterior pituitary cells respond to CRF by increasing the production of cyclic AMP which results in the stimulation of secretion of ACTH (Bilezikjian and Vale, 1983). These responses are inhibited by the addition of glucocorticoid hormones to the medium. Such a system could be established to identify toxic substances which interfere with feedback mechanisms.

## 14.4 STRATEGY AND FUTURE DEVLEOPMENTS

Due to the complex nature of the endocrine system, it is obvious that no single test exists that can provide all the data that are necessary to assess the potency of compounds that may disturb endocrine functions. *In vivo* function tests of the various endocrine organs are indicated when the potential for chemicals to affect the endocrine system is established by:

(1) changes in weight of the endocrine organs;
(2) morphological alterations, detected with conventional as well as immunocytochemical staining;
(3) changes in circulating hormone concentrations.

*In vitro* tests are potentially useful in order to ascertain:

(1) mechanisms of action (e.g. receptor binding studies, determination of enzymatic activity, release tests), although it should also be emphasized that *in vitro* metabolic activation of the compound should be taken into account;
(2) the relative potency of chemical compounds (or their metabolites) that interfere with endocrine function.

Most of the *in vitro* test systems need validation; in particular, the correlation between *in vivo* and *in vitro* tests remains to be clarified.

In the near future, techniques already in use in clinical medicine should be explored for their suitability for use in animal studies. In general, the use of non-invasive, *in vitro* techniques should be encouraged since these offer the possibility to obtain sample material under conditions of minimal stress and may reduce the number of animals required in toxicity studies.

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