Short-term Toxicity Tests for Non-genotoxic Effects
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CHAPTER 10

The Developing Kidney in Toxicity Tests

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10.1 INTRODUCTION

The developing kidney offers several advantages for testing the harmful effects of genetic and non-genetic factors at the molecular, cellular, tissue and organism levels. The developmental stages of this organ are well understood, making toxic effects relatively easy to monitor. *In vivo*, the developing kidney is a sensitive target for harmful effects of biological, physical and chemical factors (see Monie, 1977). Recently, *in vitro* techniques have been elaborated to study the development and differentiation of the kidney and its components. These techniques have been devised primarily to explore normal kidney development, but they also provide a good basis for toxicological studies.

10.2 DEVELOPMENT OF THE METANEPHRIC KIDNEY

10.2.1 Early morphogenesis

Excellent accounts describing the histogenesis of the metanephric kidney have been published since the turn of the century by a number of scientists including Huber (1905) and Rienhoff (1922). These have been supplemented by observations using microdissection (Potter, 1965; Osthanondh and Potter, 1966), histochemistry (Vetter *et al.*, 1966; Kazimierczak, 1970), immunohistochemistry (Croisille *et al.*, 1971; Ekblom *et al.*, 1981a,b), and electronmicroscopy (Wartiovaara, 1966; Reeves *et al.*, 1978, 1980; Larsson and Maunsbach, 1980).

Three originally separate cell types contribute to the metanephric kidney: (1) the epithelium of the Wolffian duct; (2) the mesenchymal cells of the nephric blastema; and (3) the endothelial cells derived from an outside vasculature. The epithelial bud, the prospective collective system, invades the mesenchymal blastema where it branches repeatedly to form the ureteric tree. Mesenchymal cells around the tips of the ureteric tree differentiate to ultimately form the secretory portion of the nephron. In most mammalian species, the development of the nephron proceeds throughout the intra-uterine period. Therefore, it is possible to observe a whole repertoire of

developing nephric tubules at various stages of development, an advantage in comparative studies.

The origin of the vascular component of the kidney, which includes the glomerular endothelium, has remained controversial until recent years. Some authors have regarded the mesenchymal cells of the nephric blastema as the progenitors of this important component of the kidney (Emura and Tanaka, 1972; Reeves *et al.*, 1980). Others have postulated that vascularization may have originated from outside vessels (Osthanondh and Potter, 1966; Kazimierczak, 1970).

Data from recent grafting experiments appears to support the hypothesis that the kidney's vascular component originates from outside vessels. The specific evidence for this hypothesis was acquired from an experiment which involved the grafting of mouse metanephric rudiments (prior to vascularization) onto the chorioallantoic membrane of the Japanese quail (Sariola *et al.*, 1983). These grafts regularly showed rich vascularization. The quail origin of these vessels could be verified by the quail-type nuclear structure of the endothelial cells and by monoclonal antibodies against quail endothelial (and haematopoietic) elements. The grafted kidneys developed rather advanced glomeruli showing a hybrid origin with mouse podocytes and avian endothelial cells. The same dual origin could be demonstrated in the glomerular basement membrane to which both mouse and quail cells had contributed (Sariola *et al.*, 1984a).

The initially separate mesenchyme also undergoes dramatic changes (Figure 10.1). In the early stages, it condenses around the epithelium where it forms a comma-shaped body. The mesenchyme then develops into an S-shaped structure which vascular elements invade. Growth continues centrifugally while the epithelium grows into deeper layers of the mesenchyme. New condensates are added and the more advanced tubules become established in the central portion of the anlage. As a result of this process, an arcade arrangement is created (Figure 10.2).

The early development of the kidney is preceded and accompanied by a variety of molecular changes. These changes can be detected by immunohistochemical staining using antibodies against the various components of the cells and their extracellular matrix (ECM).

Prior to any visible morphogenesis, the composition of the ECM of the tubule mesenchyme (around the epithelial tips) undergoes a change. The interstitial proteins (collagen type I and type III, fibronectin) are replaced by compounds which contribute to the basement membrane (collagen type IV, laminin, and heparin-sulphate proteoglycan) (Ekblom *et al.* 1981a).

The molecular changes in the ECM are accompanied by changes in the cytoskeleton indicating a transformation from a mesenchymal cell into an epithelial cell. The vimentin filaments of the mesenchymal cells are replaced by cytokeratin (Lehtonen *et al.*, 1984).

The subsequent segmentation of the nephron into a glomerular, proximal and

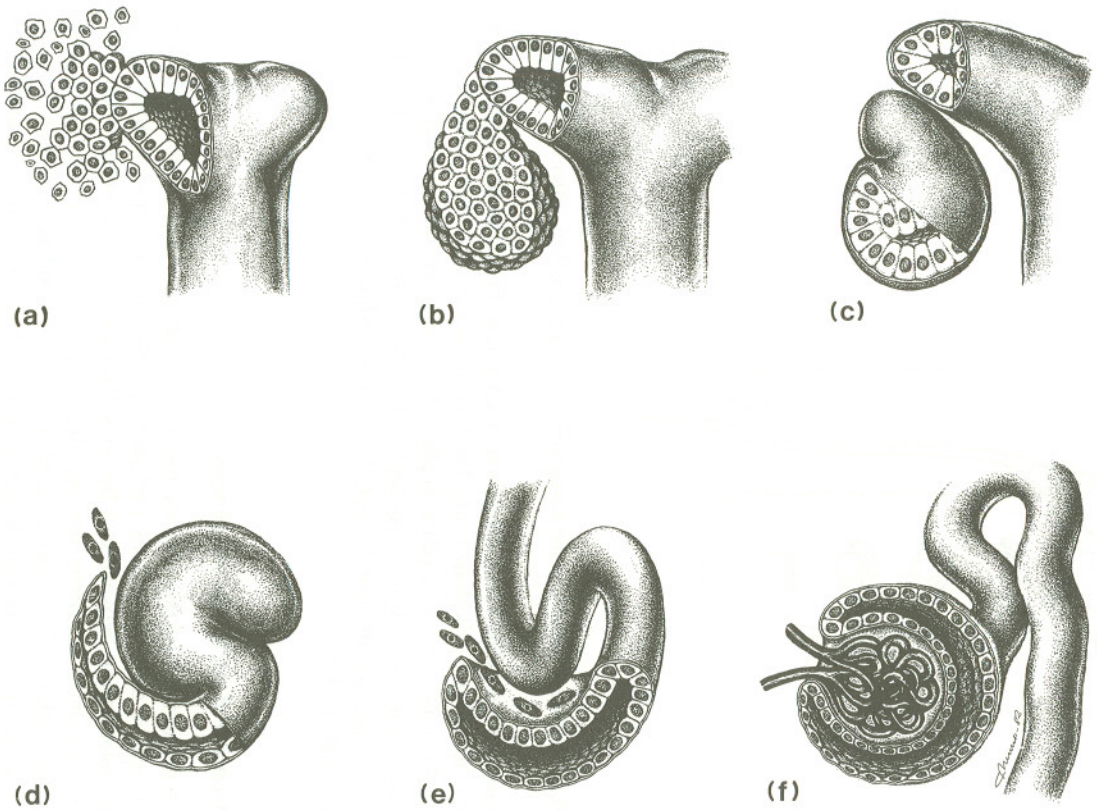

Figure 10.1 Early stages of morphogenesis of the metanephric nephron (Saxén, 1984).

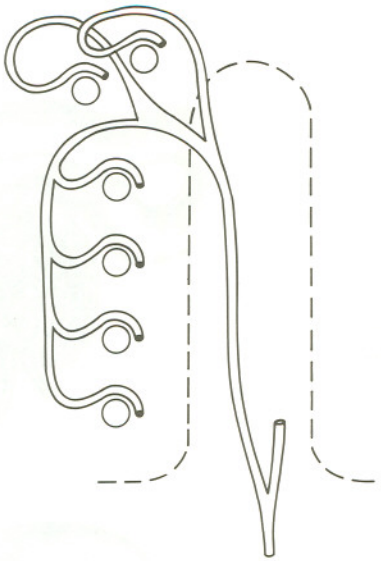

Figure 10.2 Arcade formation in the developing metanephric kidney (after Neiss, 1982).

distal portion can be studied with immunohistochemical, histochemical and electron microscopic techniques (Ekblom *et al.*, 1980; Lehtonen *et al.*, 1983). Also, important details of the ultra-structure of the nephron have been obtained by transmission, scanning and immunoelectron microscopy. These latter methods have focused on the glomerulus and its filtration barrier (Larsson and Maunsbach, 1980; Reeves *et al.*, 1980; Farquhar, 1981).

10.2.2 Control of early morphogenesis

Direct observations of certain urinary tract abnormalities (Gruenwald, 1939) and related studies of different kidney cell lineages demonstrates a significant morphogenetic relationship between cell lineages. This property has been observed in experiments involving the ureter bud and the mesenchyme. If separated by microdissection and cultured in isolation, neither the ureter bud nor the mesenchyme undergoes further differentiation. However, if recombined soon after separation, an almost normal morphogenesis is observed (Grobstein, 1953, 1955).

The inductive or morphogenetic tissue interaction which is frequently associated with embryonic organ formation has been analysed by the transfilter technique developed by Grobstein (1956).

The sequence of events during the epithelial transformation can be triggered by

various heterotypic tissues through a filter membrane. This technique opens up various possibilities for the detailed analyses of morphogenetic tissue interaction. It also permits the use of different filter types and the separate analysis of two interacting tissues. More importantly, contact between the interacting cells can be broken at any time and the mesenchyme cultured separately for further study. This technique can also be used to study the temporal correlation of events at the molecular and structural levels.

The reader is referred to reviews by Grobstein (1967), Saxén *et al.* (1968, 1985), Lehtonen (1976) and Saxén (1987) for further information on morphogenesis. Some central conclusions about kidney morphogenesis are:

(1) (i) The nephrogenic mesenchyme is predetermined prior to the action of the ureter inductor;
   (ii) various heterotypic tissues can trigger its differentiation (Grobstein, 1955);
   (iii) no other embryonic mesenchyme will respond to the triggers by forming tubules (Saxén, 1970).

(2) Induction requires actual cell-to-cell contact between the interacting tissues. Induction can be prevented by filters that do not allow close cell-to-cell appositions (Wartiovaara *et al.*, 1974; Lehtonen, 1976; Saxén *et al.*, 1976).

(3) (i) Induction is a time-related event. After 12 hours of contact, the first mesenchymal cells become irreversibly committed to become epithelial cells;
   (ii) The process of induction is completed after some 24 to 28 hours of contact (Saxén and Lehtonen, 1978).

(4) (i) Immediately after the initial 24 hours of induction, no morphogenesis is detectable, but the mesenchymal cells have been programmed for differentiation;
   (ii) After 24 hours, the cells begin to differentiate into specialized cell types such as glomerular podocytes, proximal tubule cells, and distal tubule cells;
   (iii) These new phenotypes are expressed three to five days after the initial induction (Ekblom *et al.*, 1981b; Lehtonen *et al.*, 1983).

(5) (i) The first detectable change in the state of the cells is a decreased cell generation time (detected by increased uptake of tritiated thymidine);
   (ii) This change coincides with the 'induction time' and occurs within 12 to 24 hours after the transfilter contact (Figure 10.3). Only an inductive situation leads to this change (Saxén *et al.*, 1983).

(6) The changes in the protein composition of the extracellular matrix and in the cytoskeleton (described above) are regularly detected in the transfilter explants during early stages of differentiation but not in mesenchymes that are not exposed to an inductor (Ekblom *et al.*, 1981a; Lehtonen *et al.*, 1984).

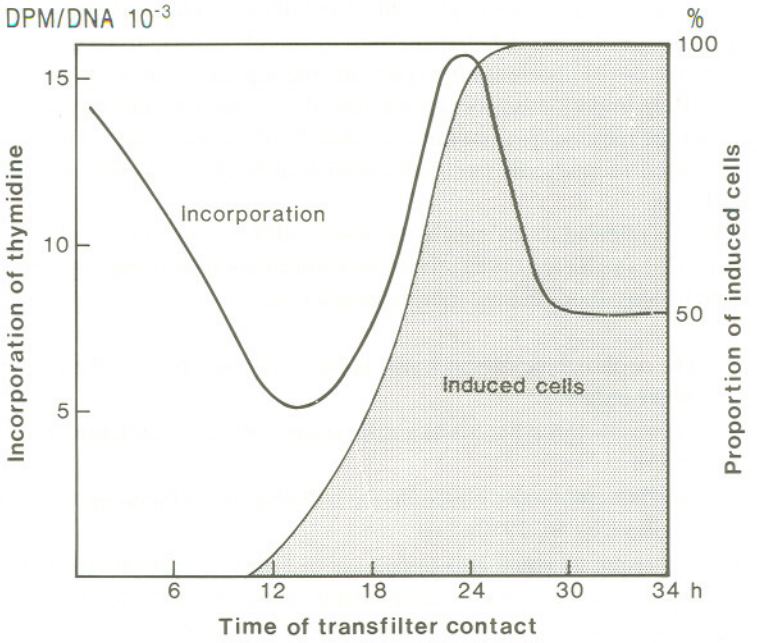

Figure 10.3 Correlation of the 'induction time' (shaded area) to the incorporation of thymidine into the induced mesenchyme after the onset of the transfilter contact (Saxén *et al.*, 1985).

Due to the technique used, the various events under (3) to (6) can be temporally correlated as in Figure 10.4. Speculations on their causal correlations have been presented (Lehtonen and Saxén, 1986), but final conclusions cannot yet be drawn about the mechanism of this complicated chain of events. The sequence of events and the kinetics described do, however, provide a useful background when the application of this model-system is considered for toxicological studies.

10.3 *IN VIVO* TOXICITY STUDIES

The urinary tract of murine embryos is a sensitive target for various exogenous factors. Wilson and Warkany (1948) found kidney malformations in the offspring of rats that were fed a diet which was deficient in vitamin A. Monie *et al.* (1954) reported malformations in rats following maternal pteroylglutamic acid deficiency (Monie *et al.*, 1954). Numerous other studies have been published (see Monie, 1977).

Sodium arsenate injected intraperitoneally into mice on days nine and ten of pregnancy caused various malformations and fetal death, but almost no effects if

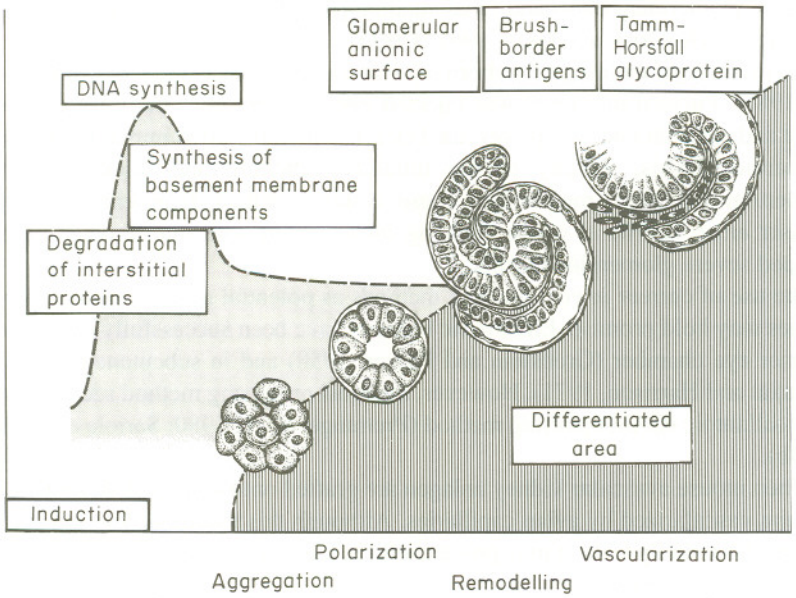

Figure 10.4 Summary of the differentiative events following the onset of transfilter contact between the metanephrogenic mesenchyme and its inductor (Lehtonen and Saxén, 1986).

injected after this time (Burk and Beaudoin, 1978). The primary defect caused by this exogenous factor was impaired growth of the ureteric bud (the inductor). This impaired growth prevented the ureteric bud from reaching the metanephric blastema, which subsequently failed to differentiate. A similar mechanism has been suggested for the genetically-determined renal agenesis in Sd-mutant mice (Gluecksohn-Waelsch and Rota, 1963).

Fetterman *et al.* (1974) provided an example of malformations caused by *in vivo* micromanipulation. Ligation of the ureter in fetal rabbits between days 19 and 24 of gestation proved successful in that a high percentage of fetuses developed cystic dilations in various portions of the nephron. The authors felt that this might be a good model for certain types of obstructive kidney cysts that occur in human subjects (see also Crocker and Vernier, 1970b; Crocker *et al.*, 1971).

Kavlock *et al.* (1982) have reported that the liver and kidney appear to be the most sensitive organs studied in tests involving the use of biochemical endpoints of organ differentiation as criteria for fetotoxicity. Various biochemical measurements were made to follow the growth and maturation of different organs in rat embryos on days 19 to 22 following maternal exposure to known fetotoxic chemicals. Some of the results reflected impaired kidney development (Figure 10.5). These authors have used a diuresis test (with and without anti-diuretic hormone on the third

postnatal day) and a hydropenia test on the sixth postnatal day in addition to kidney weights, glomerular counts and renal alkaline phosphatase tests to assess the effects of toxicants (Kavlock and Gray, 1982, 1983).

An example of the use of newborn mice in toxicity tests is the work of Gresser *et al.* (1981). Purified interferon was injected into suckling mice daily for eight days. Light and electron microscopy revealed effects which included immature glomeruli, glomerulosclerosis, atrophy of the tubular epithelium and thickening of the glomerular basement membrane. These results confirmed an earlier study by Gresser *et al.* (1976) in which suckling mice treated with interferon at birth developed severe glomerulonephritis.

The use of current *in vivo* grafting methods as potential models for toxicological studies may hold promise. Embryonic kidneys have been successfully grafted in the anterior eye chamber (Grobstein and Parker, 1958) and in subcutaneous locations (Barakat and Harrison, 1971). However, the most promising method seems to be the chorioallantoic (CAM) grafting method (Preminger *et al.*, 1980; Sariola *et al.*, 1983, 1984b).

When mouse avascular kidney anlagen are grafted onto avian CAM, they quickly become vascularized by avian capillaries. Although this technique has not yet been adapted for toxicity tests, it may prove useful in the assessment of cell migration and circulation development.

This technique has been used to study abnormal kidney development in CAM-grafts with chick kidneys (Maizels and Simpson, 1983). These experiments succeeded in simulating human kidney dysplasia; however, it is not yet certain whether or not this is a true model-system for the human kidney defect.

10.4 *IN VITRO* METHODS AND TOXICITY STUDIES

Several *in vitro* techniques for the cultivation of kidney anlagen and the various cell types derived from it are now available. A number of these techniques have been developed for the study of normal kidney development, but they also have potential value for toxicity tests.

10.4.1 Cell cultures

In an experiment conducted by Taub *et al.* (1979), Nadin–Darby canine kidney cells were introduced to a chemically-defined medium supplemented with insulin, transferrin, prostaglandin E_1, hydrocortisone and tri-iodothyronine. Cell growth and survival were as good as in a serum-supplemented medium. Cells could be maintained in culture for a month. This technique allows detailed analysis of the effect and mode of action of various medium constituents on the growth and hemicyst formation of kidney epithelial cells.

Primary cultures can be prepared from mesenchyme explants derived by the transfilter technique (see Section 10.2.2). The mesenchyme, prior to overt

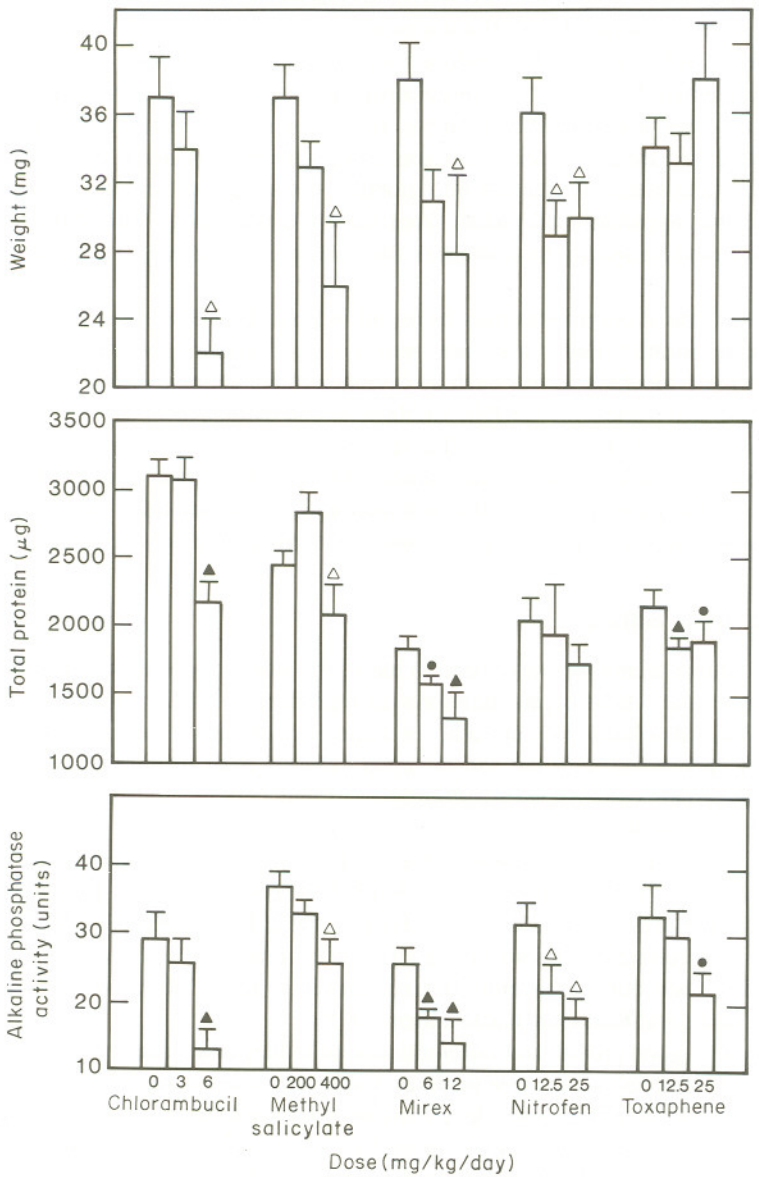

Figure 10.5 'Biochemical endpoint' measurement reflecting an impaired development of the kidney rudiment after treatment with various toxic compounds in different concentrations (after Kavlock *et al.*, 1982).

differentiation, can be cut into fragments which demonstrate good cell outgrowth in subculture. These outgrowths are known to contain both stroma, fibroblast-type and differentiating epithelial cells (Lehtonen *et al.*, 1985).

Minuth and Kriz (1982) developed an explanation technique for the study of kidney epithelial cells in their more advanced stages. Small cortical pieces were dissected from newborn rabbit kidneys. These pieces consisted of a fibrous capsule, collective duct fragments and immature, S-shaped tubule anlagen. Within 24 hours, these cortical pieces developed into globular bodies demonstrating epithelial outgrowth that apparently originated from the collecting ducts. The outgrowth also demonstrated a capacity to synthesize the renal glycoprotein $gp_{CD}I$ (Minuth *et al.*, 1984).

These cortical outgrowths may be useful in toxicological studies. Minuth (1983) has demonstrated that these are sensitive to various protein-inhibitors and cytoskeleton-blocking agents such as cycloheximide, actinomycin C, tunicamycin, 6-diazo-5-oxo-norleucine (DON), vinblastine, and cytochalasin B.

Minuth (1983) expressed the belief that cultures of this type could be employed for the direct testing of drug teratogenicity. Of special interest would be the effects of agents that interfere with cell attachment and cell locomotion. The method may have potential as a direct, rapid and convenient means of assessing toxicity.

10.4.2 Organ culture

Organ culture techniques developed in the 1920s make it possible to examine tissue fragments and whole organ rudiments in reproducible *in vitro* conditions. These techniques also have proven value in some toxicological studies (Saxén, 1983). Studies undertaken with the use of organ cultures offer distinct advantages over monolayer cell cultures since differentiation and certain physiological functions persist rather well.

Organ cultures of both murine and avian kidneys have been investigated *in vitro* since the early 1950s (Grobstein, 1953, 1955; Saxén *et al.* 1968; Bernstein *et al.*, 1981; Avner *et al.*, 1983a; Wolff and Haffen, 1952; Lash, 1963; Strudel and Pinot, 1965). The culture conditions of the different laboratories vary somewhat, and we have described ours in several contexts (Saxén *et al.*, 1968; Saxén and Saksela, 1971; Saxén and Karkinen-Jaaskelainen, 1975).

Recently, good growth and differentiation of kidney anlage has been reported in a chemically defined, serum-free medium. Using a transferrin-supplemented medium, it was possible to obtain good development of mouse kidney rudiments (Ekblom *et al.*, 1981b). Without transferrin, or in media with transferrin-depleted serum, culture survival was poor and differentiation did not proceed.

Avner *et al.* (1982) used a more complex medium where proteins were replaced with insulin, prostaglandin E_1, transferrin and hydrocortisone. This medium resulted in good differentiation of mouse embryonic kidneys with advanced proximal tubules, brush-border and avascular glomeruli and highly differentiated podocytes.

Although supplements added to synthetic media cannot fully compensate for the proteins present in serum-based organ culture media, the elimination of various serum factors from the culture medium (and their possible effects) is advantageous for experiments where additional factors are to be tested.

As *in vitro* microperfusion method to study the physiology and functional development of the nephron has been developed (Horster, 1978; Horster and Schmidt, 1978; Horster and Zink, 1982). Microperfusion of isolated tubular fragments, dissected from kidneys of embryonic and newborn rabbits, permitted the evaluation of fluid and ion transport by the epithelium during embryogenesis and postnatal development. Also, enzyme activities could be monitored in these nephron fragments. Horster and Zink (1982) used this method to study the effect of vasopressin on the functional maturation of the nephric epithelium. These experiments indicate that this system might be applicable to toxicological studies.

The use of human embryonic kidney tissues for organ culture analysis remains largely unexploited at this time. According to Lash and Saxén (1972), human embryonic kidneys grow well in organotypic culture. Studies by Crocker and Vernier (1970a) and Crocker (1973) provide further information on related experiments.

10.4.3 Effects of xenobiotics on *in vitro* systems

Some examples serve to illustrate the use of metanephric cultures in toxicological studies. Shabad *et al.* (1972) used kidney fragments obtained from mouse embryos in the third trimester. The embryos had been exposed to various carcinogens or their non-carcinogenic analogues as a result of maternal dosage and transplacental transport. In culture, the epithelium of kidneys taken from the embryos of mice exposed to carcinogens showed hyperplastic growth that was nodular, diffuse or that formed solid, compact zones. Occasional papillary outgrowth was also observed more frequently in the specimens from embryos exposed to carcinogens than in specimens from embryos in the control groups. Even though the assessment criteria were solely morphological and difficult to evaluate, the technique, nevertheless, merits further consideration for studies on transplacental exposure to chemical carcinogens.

By the addition of glycocorticoids to the culture medium, it has been possible to induce cystic maldevelopment in murine metanephric cultures (Avner *et al.*, 1983b, 1984). This defect is similar to that observed in the polycystic kidney disease, a condition well-known to both experimental teratologists and pediatric pathologists (Bernstein, 1968). Although the exact mechanisms involved in the occurrence of these maldevelopments remain unknown, this method does seem to allow new means for experimental studies involving the aetiology and pathogenesis of multicystic kidney disease and, possibly, a method to test certain drugs for side-effects.

The effects of interferon on development and differentiation in embryonic kidneys was recently tested in two types of metanephric cultures (Saxén, 1985). One culture type involved complete 11-day-old embryonic kidneys, while the other type consisted of a culture prepared by the transfilter technique. Kidneys were carefully

dissected from pairs of mouse embryos, which were selected at random, and were cultured *in vitro*. Mouse interferon was added to the culture medium in final concentrations of 10^3 and 10^5 units/ml. The kidneys were harvested 24 or 48 hours later to measure growth and histogenesis, and five days later for immunohistology. The results were later compared with those obtained for control kidneys which were similarly cultivated. Proliferation was measured by incorporation of tritiated thymidine and by total DNA content (Table 10.1).

The results in Table 10.1 and other similar experiments suggest that interferon has no definite effect on DNA synthesis of the kidneys after 24 hours of cultivation but seems to interfere with thymidine incorporation following a prolonged cultivation period of 48 hours. The lower concentrations were without effect.

Histologically, the kidneys treated by the higher concentration of interferon showed a slightly delayed development. Similar symptoms appeared in transfilter cultures, subjected to the same treatment. Cultivation of these transfilter cultures for a period of five days, however, resulted in well differentiated tubules which expressed the various markers for the three main segments of the nephron: the glomerulus, the proximal tubule and the distal tubule (Saxén *et al.*, unpublished results).

Although some of the results on whole kidney rudiments are preliminary, they demonstrate the advantage of using paired organs. Even the most skilfully dissected kidney samples tend to show variations in size and growth rate. Therefore, pooled samples of such target tissues may render erroneous results. For this reason, the use of randomized pairs of kidney rudiments is favoured.

Induction and early differentiation of the metanephric nephron can be analysed in a simplified model-system by using transfilter induction. This model-system might prove useful in toxicity tests since the kinetics of this process are known and many of the first steps of differentiation have been characterized. Previous experience with this method can be found in Saxén and Ekblom (1981) and Saxén (1983). Figure 10.6 shows how various stages (and components) of the developing kidney can be

Table 10.1 The effect of interferon on the incorporation of thymidine into embryonic kidneys cultivated *in vitro* for 24 and 48 hours

	Experiment			
	Control		Interferon	
Number of kidneys	7	11	7	11
Time of cultivation (h)	24	48	24	48
Incorporation of thymidin (DPM/DNA)	5.900	10.200	4.500	6.400
Total DNA (ng)	294	320	283	337

Concentration of interferon was 10^5 u/ml in a medium supplemented with 10 per cent serum (Saxén 1985).

exposed to the compound in question. The whole inductor/mesenchyme explant can be exposed, or the inductor and the target mesenchyme might be pre-exposed and brought together after treatment. Exposure to exogenous compounds can be continuous, only during induction, or after induction. Criteria to be monitored in such experiments include survival of tissue(s), proliferation of the mesenchymal cells, incorporation of various radioactive precursors, as well as histochemical, immunohistochemical, and ultrastructural measures of differentiation.

Tests with compounds interfering with the synthesis of DNA, RNA, and proteins have shown that nephrogenesis is most sensitive during the initial induction period. At later stages, the mesenchyme is resistant to such chemicals (Nordling *et al.*, 1978).

To determine if nephrogenesis might be mediated by glycosylated surface-associated compounds, some inhibitors of glycosamine synthesis and protein glycosylation were tested. 6-diazo-5-oxo-norleucine (DON) is a glutamine analogue

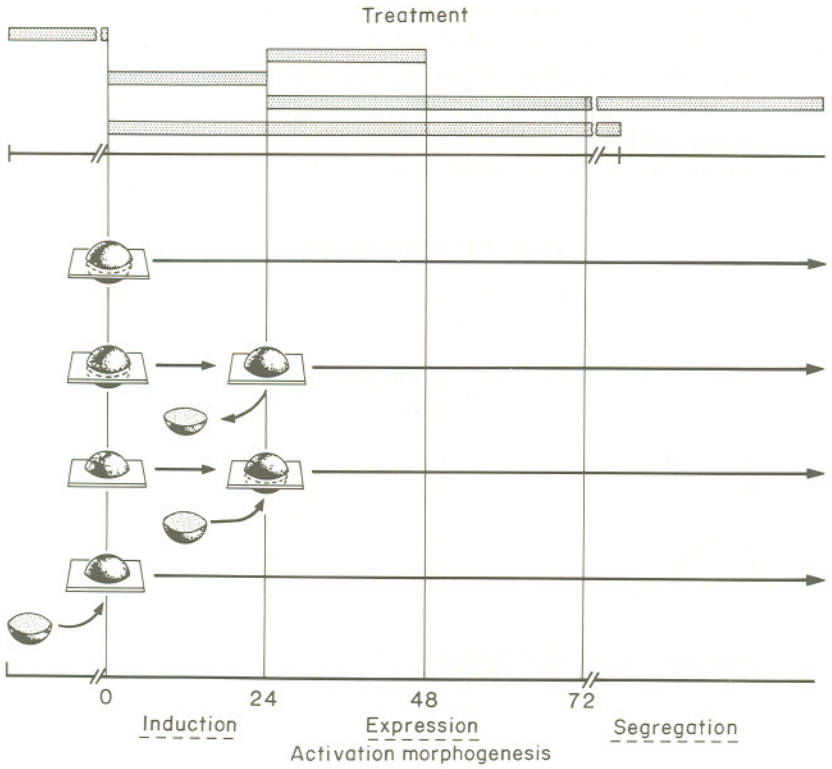

Figure 10.6 Experimental possibilities to expose the metanephrogenic mesenchyme and its inductor at various stages of determination and differentiation (Saxén, 1983).

which affects the synthesis of glycosaminoglycans as well as protein glycosylation. Tunicamycin is considered a rather selective inhibitor of protein glycosylation. When tested in transfilter explants of the metanephric mesenchyme, these compounds caused a dose-dependent inhibition of tubule formation during the early induction phase of kidney development (Figure 10.7) (Ekblom *et al.*, 1979a,b). However, if mesenchyme was induced for 24 hours before being exposed to DON or tunicamycin, no effect on morphogenesis was evident.

The mode of action of these two compounds is still open to speculation. If the inhibitors are removed after 24 hours of exposure, and the inductor kept in place, tubule formation will resume.

The transfilter model-system, although valuable, cannot fully simulate the kidney *in vivo* and must be used with caution. The procedures needed to prepare the system—enzymatic and microsurgical separation of components, transfer onto filters and into *in vitro* conditions, etc.—result in an adaptive phase of low metabolic activity (Vainio *et al.*, 1965; Saxén, 1983), and may cause sensitivity to some exogenous agents. The use of radioactive tracers, for instance, may cause direct toxicity instead of providing a method of monitoring the effects of other agents on kidney development. This high sensitivity of cultured organ rudiments to low levels of certain radiolabelled compounds (aminoacids) has been demonstrated by Minor (1982). Therefore, such experiments must be conducted and interpreted with care. This sensitivity might also be used to advantage for toxicity tests.

10.5 CONCLUSIONS

Analytical work over the past thirty years has elucidated many of the complex

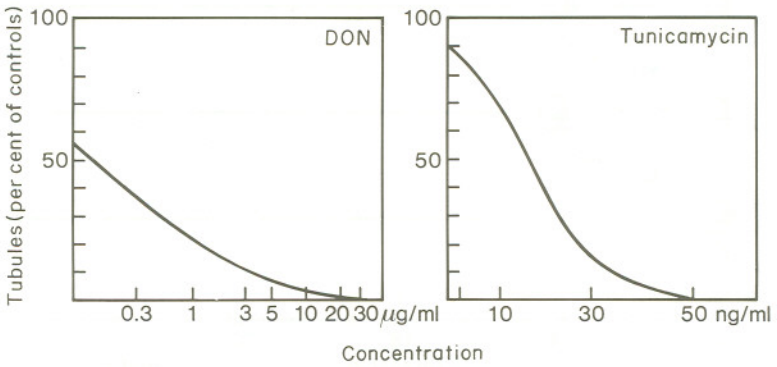

Figure 10.7 Graphs demonstrating the dose-dependent inhibitory action of DON and tunicamycin during the 24-hour induction period. The number of nephric tubules is given as percentage of that in untreated controls. The low number of DON-treated explants is partially due to the omission of glutamine from the medium (Ekblom *et al.*, 1979a, 1979b).

events and principles of kidney development. In addition to the classic *in vivo* tests, the kidney has proven to be a good object for various simplified grafting and *in vitro* methods. These techniques may be useful to toxicologists as they search for rapid and convenient tests for examining the effects of environmental chemicals. Effects that can be tested with these systems include direct toxic (lethal) effects on cells, interference with morphogenetic tissue interactions, inhibition of cell migration, aggregation and organization, and interference with known metabolic pathways (such as synthesis of macromolecules, protein glycosylation, energy metabolism and ion transfer).

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