A CELL CULTURE INVESTIGATION OF THE MEDIATION OF COMPENSATORY RENAL GROWTH

PENELOPE ANN HANSEN

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©Penelope Ann Hansen, B.S., M.S.

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ABSTRACT

Although the phenomenon of compensatory renal growth has been under investigation for more than a century, its mediation has not been elucidated. The participation of a circulating, kidney-specific growth factor, either stimulatory or inhibitory, has been hypothesized, but its existence has not been proved. Theoretical considerations predict that *in vitro* methods could be profitably used in these circumstances, since these methods would allow dissociation of the effects of the postulated factor from the complex physiological changes which accompany compensatory renal growth *in vivo*.

The work of this thesis consists of the development of a cell culture system suitable for testing serum from previously uninephrectomized or sham-operated animals for the presence of such a growth factor. A method of primary culture of adult Fischer rat kidney epithelium is described. The morphology of these cultured cells is compatible with that of proximal tubule epithelium *in situ*, these being the cells stimulated to divide *in vivo* by uninephrectomy. The chief finding reported in this thesis is that treatment with serum from rats uninephrectomized 48 hours previously results in consistent, significant increase in uptake of tritiated thymidine by cultures, when compared with control sera from sham-operated rats. Serum from rats uninephrectomized 18-36 hours previously is not consistently stimulatory. Preliminary investigation with this culture system indicates that: (a) the differential effect of control and uninephrectomy sera is due to the presence of a stimulatory factor in the latter

rather than an inhibitor in the former; (b) soluble fractions of homogenates of both kidney and liver tissue exhibit inhibitory dose-effects in cultures; (c) xanthopterin, a compound that stimulates kidney tubule epithelial cell division *in vivo*, has no stimulatory effect *in vitro*; (d) serum obtained from human kidney transplant donors 24-72 hours after uninephrectomy stimulates increasing thymidine uptake in cultures.

Use of this culture system has confirmed the existence of a serum factor involved in compensatory renal growth, and should prove to be a valuable tool for its characterization.

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ABBREVIATIONS

BiNx	bilateral nephrectomy
BSS	basal salt solution
C.P.M.	counts per minute
cyclic AMP	adenosine 3',5'-monophosphate
cyclic GMP	guanosine 3',5'-monophosphate
D.P.M.	disintegrations per minute
ECFV	extracellular fluid volume
EDTA	ethylene diamine tetra-acetate
G_2	second gap phase of mitotic cycle
GFR	glomerular filtration rate
HnRNA	heterogeneous nuclear RNA
^{3}HTdR	tritiated thymidine
JG	juxta-glomerular
M	mitotic phase of mitotic cycle
mRNA	messenger RNA
RBF	renal blood flow
REF	renal erythropoietic factor
rRNA	ribosomal RNA
RPF	renal plasma flow
S	DNA synthetic phase of mitotic cycle
S.E.M.	standard error of the mean
UNx	unilateral nephrectomy
UUC	unilateral ureterocaval anastomosis
UUD	unilateral ureteroduodenostomy
Xn	xanthopterin

REVIEW AND DISCUSSION OF LITERATURE

A. Introduction

The phenomenon of compensatory renal growth has been under investigation for more than a century; the literature comprises well over a thousand citations. Most of this effort has been directed towards describing the morphological, biochemical and functional events which follow unilateral nephrectomy. Relatively few investigators have addressed themselves to the question of the control or mediation of this growth. Although events occurring as soon as five minutes after unilateral nephrectomy have been described (Lowenstein and Toback, 1978), the nature of the primary stimulus is unknown.

In this chapter I shall consider first the phenomenon of compensatory renal growth, and secondly, the stimulus to growth. I have been critical in both the selection and discussion of papers, paying particular attention to such variables as characteristics of the animal used, sample size, assay method, variance of data and use of appropriate controls. The most recent publications were not necessarily selected in preference to their predecessors. Some information was derived from papers in which it was presented incidentally, or in the course of experiments pertaining to a different subject. This review was designed to provide a summary of soundly-based information concerning the phenomenon of compensatory renal growth, especially as seen in rats and humans. I have also attempted to present an unbiased but critical survey of the more controversial aspects of the subject, which relate to control of the growth.

B. Description of the phenomenon

I Organ and cellular hypertrophy

When one kidney is removed, the remaining kidney increases in weight during the succeeding days; this is not due merely to an increase in contained blood volume or to accumulation of fluid, but is caused by: (a) an increase in the size of some of the cells (hypertrophy) and (b) an increase, by cell division, in the number of the cells (hyperplasia).

This mass increase has been found in all mammalian species studied, with the possible exceptions of the baboon (Dicker and Morris, 1972) and the cat (R. Janicki, in the general discussion included in Nowinski and Goss, 1969).

A statistically significant increase in rat kidney mass occurs by 24 hours after unilateral nephrectomy (UNx) (Halliburton and Thomson, 1966). At the end of a week, the mass increase is in the range of from 30 to 40 per cent when compared with sham-operated controls (Katz and Epstein, 1967; Kurnick and Lindsay, 1968a). The time course of kidney mass increase in the Fischer rats used in this study is described in the Results section.

The proportion of dry mass to wet remains constant at about 24 per cent during the course of compensatory growth (Halliburton and Thomson, 1965b; Threlfall et al., 1967; Kurnick and Lindsay, 1968a). Therefore, measurement of either wet or dry weight is an equally reliable index of growth.

When more than half of the renal mass is excised, the compensatory growth is greater: Kaufman et al. (1974) report that removal of about 70 per cent of kidney mass from adult rats results in restoration of about 75 per cent of the original mass by four weeks, or about 65 per cent of the kidney mass in sham animals at four weeks. This is an increase of about 2.7 times the weight of the tissue which remained after surgery. UNx rats restored 90 per cent of the original mass and 76 per cent of sham mass, or an increase of about 1.8 times the residual mass.

The observation that kidney mass increase after UNx shows an inverse correlation with age raises several questions: (a) what is the normal course of kidney growth in unoperated animals - i.e., what is the background of growth upon which compensatory growth is superimposed; and (b) do the relative proportions of the hypertrophic and hyperplastic components of the compensatory growth change with age? Consideration of these questions will be limited to investigations on rats and humans.

Rats continue to increase in body and kidney weight throughout their lives, the rates of gain decreasing with age. Using data on unoperated Sprague-Dawley rats obtained from Potter et al. (1969), plots of body weight, kidney weight, and kidney weight expressed as a percentage of body weight vs. age (Figure 1.) show that the rate of body weight gain decreases with age, and kidney weight gain does not keep pace with that of body weight. Kaufman et al. (1974) report that in male Sprague-Dawley rats, kidney weight shows a linear relationship with body weight throughout life, but that the

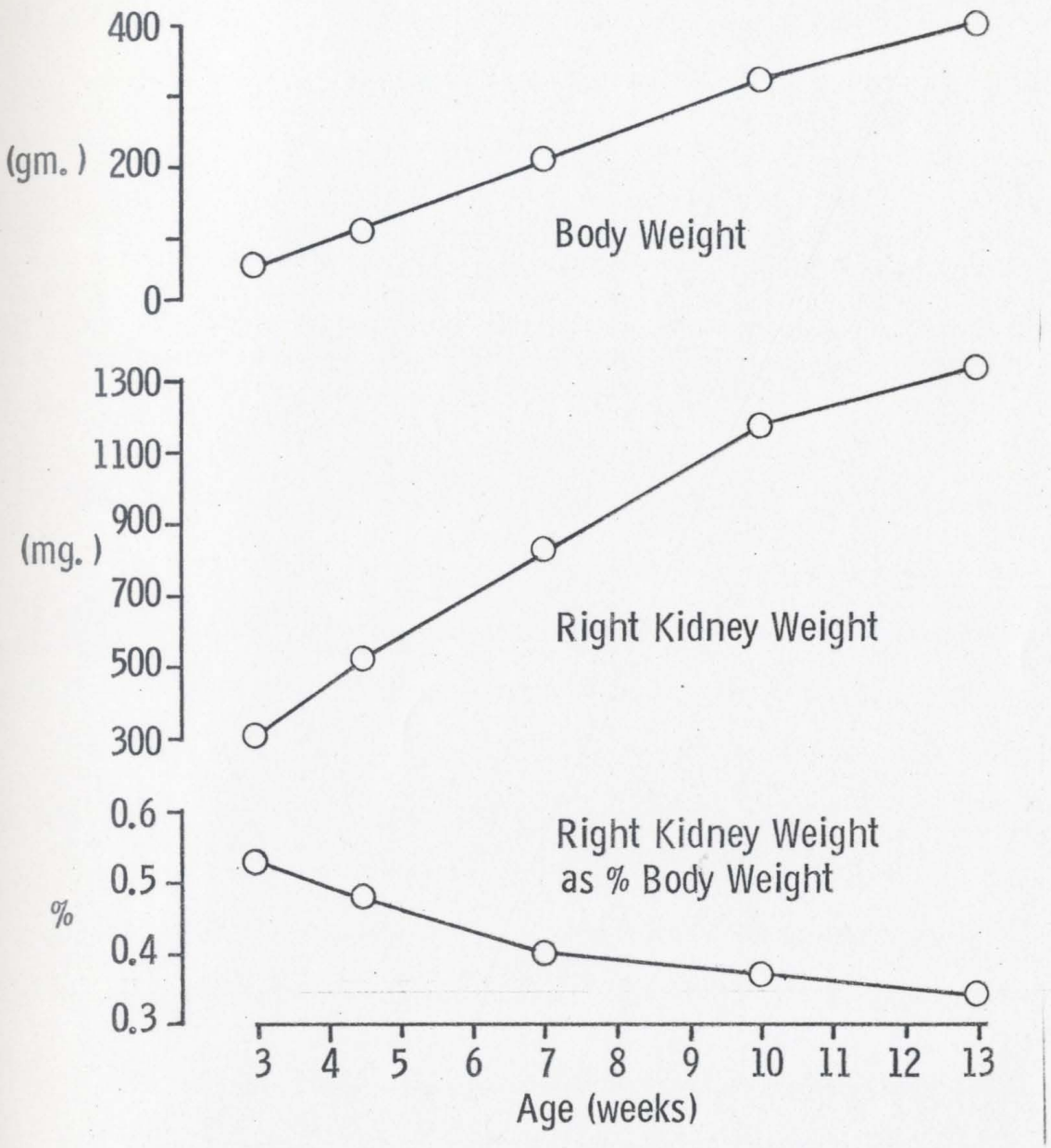

Figure 1. Influence of age on kidney and body weights in Sprague-Dawley rats. Kidney weight increases at a slower rate than body weight in young male rats. These data are from Potter et al. 1969.

regression line has a decreased slope for animals over 150 gm., compared with that for smaller animals. Potter et al. (1969), report that Fischer strain rats gain body weight at a slower rate than Sprague-Dawleys, but that their kidneys bear the same proportion to body weight throughout life. Allowance must be made for these age correlations when kidney weight data after UNx in rats of different ages and strains is analyzed.

Five authors who report data on rats undergoing UNx conclude that compensatory renal growth is greater in young animals than in old, but these data must be looked at in the light of the consideration just discussed.

Dicker and Shirley (1973) used male albino rats of unspecified strain, divided into two groups by age, each including sham-operated and UNx rats: one group consisted of rats five days old at operation, and the other of "adult" rats - their age not stated by the authors. The data were expressed as per cent difference between UNx and sham kidney weight expressed as per cent body weight. There was no difference between age groups until ten days post-operation, after which the younger group showed an increasingly greater compensatory growth than the older group until the last data point at 70 days.

MacKay et al. (1932) used male albino rats of unspecified strain, of ages 5 to 720 days. They measured the difference between UNx and sham kidney weights expressed as per cent of body surface area 40 days after sham operation or UNx. The authors state, with reference to their own published work, that the ratio of kidney weight to body surface area remains constant at all ages (unlike the ratio of kidney

weight to body weight). They found a rapid decrease in compensatory growth from age five days to sixty days, and then a slow decline until their last data point at 540 days.

The other three papers report conclusions, based on absolute weight gain of young and adult Sprague-Dawley kidneys, that compensatory growth is greater in young animals. However, as we have seen, absolute kidney weight data from different ages of rats may be misleading, since there is not a direct correlation of kidney weight with age. From data included in two papers (Kaufman et al., 1975 and Galla et al., 1974), I have calculated kidney weight gains as a percentage of body weight. Both authors performed sham and UNx operations, and collected data four weeks later. My calculations show that the UNx kidney is 154 per cent of sham in 50 to 80 gm. rats, and 143 per cent in 155 to 210 gm. rats using Galla's data; 150 per cent in 55 gm. rats and 140 per cent in 175 gm. rats using Kaufman's data. In the third paper (Barrows, 1962) no body weight data are given, so no interpretation may be made. Since the raw data are not given, it is not possible to analyse these results statistically; it appears, however, that they are in line with those of MacKay et al., and Dicker and Shirley, in showing that after allowance is made for normal growth characteristics, there is indeed a decreased growth response to UNx as age advances in the rat. The decrease in response is probably not apparent until about 10 days after surgery, and the rate of decrease is much greater between birth and about 60 days of age than thereafter.

The change in response to UNx which occurs at puberty may result

from sex hormone changes. The direct effects of sex hormones on kidney size, in both normal and compensatory growth, have been investigated, primarily in male rats using testosterone. Several approaches to the problem have been made: (a) administration of testosterone to normal, unoperated rats; (b) castration; (c) castration with replacement testosterone treatment.

Most authors have found that administration of testosterone to normal male rats results in an increase in kidney weight (Selye, 1940; Lattimer, 1942; Ludden et al., 1949). MacKay (1940), however, found no increase due to testosterone treatment, which may be because he gave less frequent injections than the other investigators (only five injections in 29 days as opposed to daily injections by others).

This increase in kidney weight has been found to be due to hypertrophy, and not hyperplasia, of the tubular epithelium (Selye, 1939; Ludden et al., 1949; Kassenaar et al., 1962).

Castration of male rats has been reported to decrease kidney weight (Korenchevsky and Ross, 1940; MacKay, 1940). Korenchevsky and Ross (1940) also report that the decrease is reversed by testosterone injection. The atrophy occurs without any cell loss; the existing cells become smaller (Kochakian, 1948; Jelinek et al., 1964).

Testosterone therefore appears to have a generalized protein anabolic effect; it also causes, secondarily, sodium, potassium and

water retention (Ganong, 1977b). The testosterone stimulus to increase in kidney size may be either the direct protein anabolic effect, or the mechanisms involved with salt and water retention, or both. Testosterone influences kidney size by means of tubular cell hypertrophy or atrophy, without effect on cell number; in fact, there is evidence that testosterone is a mitotic inhibitor in the kidney epithelium: as discussed below puberty and the associated increase in testosterone production inhibits tubule epithelial cell hyperplasia, and yet, the kidneys do not decrease their growth rate during this same time. An increase in cellular hypertrophy concomitant with the decrease in hyperplasia must be occurring.

What then, is the influence of testosterone on compensatory renal growth? Zumoff and Pachter's (1964) results are discussed below: they found an inhibition of hyperplasia after UNx during puberty in rats, with resumption of mitosis in adulthood. Castration prevented the inhibition effect of puberty, and testosterone administration reversed the effect of castration.

MacKay (1940) examined kidney weight in adult male rats, but used only two animals per experimental group. He concluded that castration had no effect on, and that testosterone administration increased, kidney mass after UNx. However, there are difficulties with this report: inadequate sample sizes, and a lower body weight gain in testosterone-treated rats than in controls.

More recently, there have been reports that testosterone has no effect on compensatory renal growth in rats. Basinger and Gittes

(1974) measured kidney weight seven and thirty days after UNx in intact and castrated adult male Sprague-Dawley rats. The authors found no increase in kidney weight caused by testosterone in sham-operated or UNx rats as compared to controls. Castration alone decreased kidney weight at seven and thirty days; testosterone administration eliminated the effect. Castrated rats undergoing UNx had smaller kidneys than controls at seven days, but not at thirty days; the deficit was reversed by testosterone.

Schlondorff et al. (1977) measured kidney weight in adult male Sprague-Dawley rats one or two weeks after UNx. The authors found no differences in kidney weight gain among control, castrated and testosterone-treated animals. Testosterone was administered by pellet implantation; evidence is given that this method is more effective than injection for increasing body weight in female rats.

Thus, the best-controlled experiments with adequate sample sizes indicate that testosterone has no effect on compensatory growth (kidney mass) after UNx. Perhaps the opposing effects of testosterone - inhibition of hyperplasia and stimulation of hypertrophy - are balanced in this situation, or perhaps testosterone simply has no effect on the kidney after sexual maturity. The question is far from being resolved.

The human capability for compensatory renal growth, and its possible correlation with age, has been studied by radiographic measurement of kidney size in transplant donors. Measurement of length, or planimetry of kidney area, was done by intravenous

urography before and after UNx in these healthy adults. This technique is an inaccurate index of growth compared with weighing the kidney; the following sources of error are recognized (Dossetor, 1975): (a) a one- or two-dimensional measurement only approximates the volume or mass of the organ; (b) the experimental error in measuring kidney length is about 0.5 cm. (about four per cent error); (c) there are physiological changes in kidney size; (d) there is sometimes an increase in kidney size at the beginning of I.V. urography.

The results of three data collections on healthy human kidney donors are shown in Table 1. No correlation of kidney size was found with age, sex or interval after UNx, except by Edgren et al. (1976), who found an inverse correlation with age, with all patients in this series showing some compensatory growth.

Table 1. Increase in contralateral kidney size in human kidney donors.

# of Patients	Age Range (yrs.)	Interval After UNx	Parameter Measured	Mean Per Cent Increase	Reference
22	22-63	2 wks-4 yrs.	length	8	Boner et al. (1972)
15	30-60	6 mos.-4 yrs.	length	1	Heideman and Rosenbaum (1970)
46	20-74	2 mos.-5 yrs.	area	23	Edgren et al. (1976)

There have also been studies of adult patients (Dossetor, 1975) and children (Laufer and Griscom, 1971) who underwent UNx consequent to unilateral kidney disease (neoplasm, hydronephrosis or pyonephrosis).

Of 19 adult patients 60 per cent showed no evidence of compensatory growth after intervals of one month to eight years, and 40 per cent showed a 3 per cent mean increase in kidney length. It is quite probable that some of these patients had undergone compensatory growth before UNx due to destruction of kidney tissue in the disease process.

In a series of 24 children born with one multicystic dysplastic kidney and studied by Laufer and Griscom (1971), the contralateral kidney was of normal size at birth. After UNx was performed during the first month of life, compensatory growth occurred over the next 18 months, resulting in a total renal volume (approximated by the kidney length) of 100 per cent of normal. This length increase, from which volume was calculated, is about 120 per cent of the expected normal. The observation that the normal kidney in these children was not hypertrophied at birth will be discussed next.

These admittedly imperfect measurements lead one to the conclusion that the capability for compensatory kidney growth in humans exists throughout life, with a possible inverse correlation with age. Certainly, in the neonatal period, the growth response after UNx is large enough to compensate fully for the loss in renal mass.

We have seen that neonatal rats and humans respond to UNx with vigorous compensatory growth. Can we extrapolate to fetal animals? The presence of kidney tissue in the mammalian fetus is not essential for its survival: renal homeostatic functions are carried out via the placenta by the mother's kidneys. The fetal kidneys do excrete

a dilute urine, contributing to amniotic fluid; oligohydramnios is associated with renal agenesis (Laufer and Griscom, 1971; Goss and Walker, 1971).

Two experiments have been reported on the effect of UNx on fetal rat kidneys and there is a paper reporting observations at birth of humans with unilateral non-functioning kidney. Rollason (1969) uninephrectomized inbred albino rats on day 18½ of gestation. All incisions were sutured, although the fetuses were not replaced in the uterus. Umbilical cords remained patent. Sham operations were done on some fetuses, and others were untouched and used as controls. Fetal body weights and contralateral kidney weights were determined 24 and 48 hours after surgery. The authors plotted kidney vs. body weight for each animal, resulting in a linear distribution with all points falling closely about the mean. Neither kidney nor body weight is decreased due to deleterious effects of surgery in UNx or sham animals compared with unoperated controls. Although the number of fetuses per group is small - five to eight - there is a clear indication that no compensatory growth has occurred due to UNx.

Goss and Walker (1971) report a similar experiment using Sprague-Dawley rats. They did sham or UNx operations on the fetuses on the 19th day of gestation. The kidney and body weights of these and of unoperated control animals were determined 24, 48 or 72 hours later. (The last time was the final day of gestation - day 22.) Unlike the previous investigators, these did not suture the fetuses or uterus. The rate of survival was low (from 25 - 50 per cent with an inverse

relationship to time after surgery); but sufficient operations were done to yield at least 50 survivors in each group. Operations were also done on rats on their day of birth. These rats were killed one, two or three days later. The authors calculated (for each group) the mean values of kidney weight expressed relative to body weight. They did a statistical comparison of groups and plotted the relative weights vs. time. They found a significant increase in relative kidney weight after UNx only on day 21 of gestation, and on days one, two and three postnatally. They conclude that fetal rats can compensate after UNx, although the magnitude of the response is less than in the neonatal period. However, if these data are plotted (Figure 2) in a fashion similar to that employed by Rollason (1969), the evidence for fetal kidney hypertrophy after UNx seems to disappear. The rate of gain of both body and kidney weight after sham or UNx surgery is less than in unoperated controls, and there are two points of interest in this difference: (a) the relative decrease in weight gain must be a non-specific result of surgery since sham and UNx rats show similar weights; (b) kidney weight correlated with body weight in spite of rate changes, as shown by the points falling in a linear plot.

The loss of body weight and high rate of mortality, increasing with time, indicates that the fetuses were in distress as a result of the surgery. A parallel situation in older rats may be that of starvation: body weight and kidney weight decrease concomitantly both in 10 - 36 day old rats (Fraser and Alleyne, 1974) and in adult rats (Kurnick, 1955). Starvation has inhibited compensatory growth after

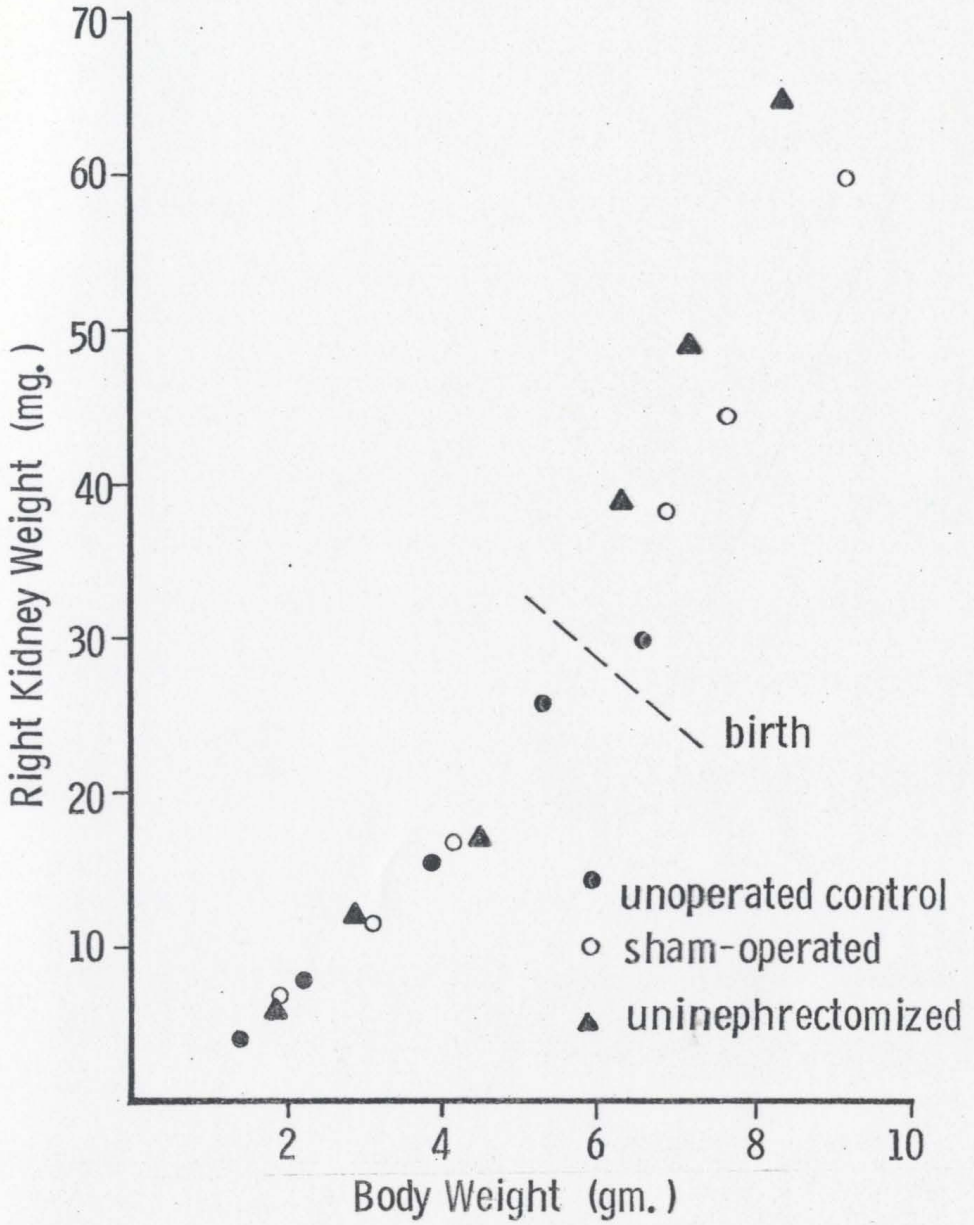

Figure 2. Compensatory renal growth in rats during the perinatal period. Sprague-Dawley fetal rats do not appear to undergo compensatory renal growth when uninephrectomized three days before term. When uninephrectomized one day after birth, their contralateral kidneys increase in mass more than those of sham-operated animals. These data are from Goss and Walker, 1971.

UNx either partially (Wachtel and Cole, 1965) or totally (Williams, 1962), or the weight of the contralateral kidney has even decreased (Goldman, 1971).

Neither experiment on fetal rats has shown good evidence of compensatory growth: the animals seemed to be in good condition in one (Rollason, 1969), and there was low variance in the data, even though the number of animals used was small; in the other (Goss and Walker, 1971) sufficient animals were used, but their poor condition might have precluded the capability to respond to UNx. What is needed for conclusive evidence is a large sample of animals, surgical techniques which result in low mortality, and an experimental design in which fetuses receive UNx and are killed at 24 hour intervals over the perinatal period up to three or four days after birth. Such an experiment would be difficult, but feasible, to carry out.

Microdissection preparations give a qualitative but clear illustration of the enlargement of rat kidney tubules that occurs after uninephrectomy. Figure 3. is a reproduction of camera lucida tracings of nephrons from normal and hypertrophied rat kidneys prepared by Jean Oliver (1944).

Quantitative measurements have been reported by Hayslett et al. (1968). Table 2. gives the percentage increases in size of adult male Sprague-Dawley rat nephrons two to four weeks after uninephrectomy. The lengths were determined from formalin-fixed, microdissected specimens, diameters were measured from sectioned, rapidly-frozen specimens, volumes were calculated from length and diameter

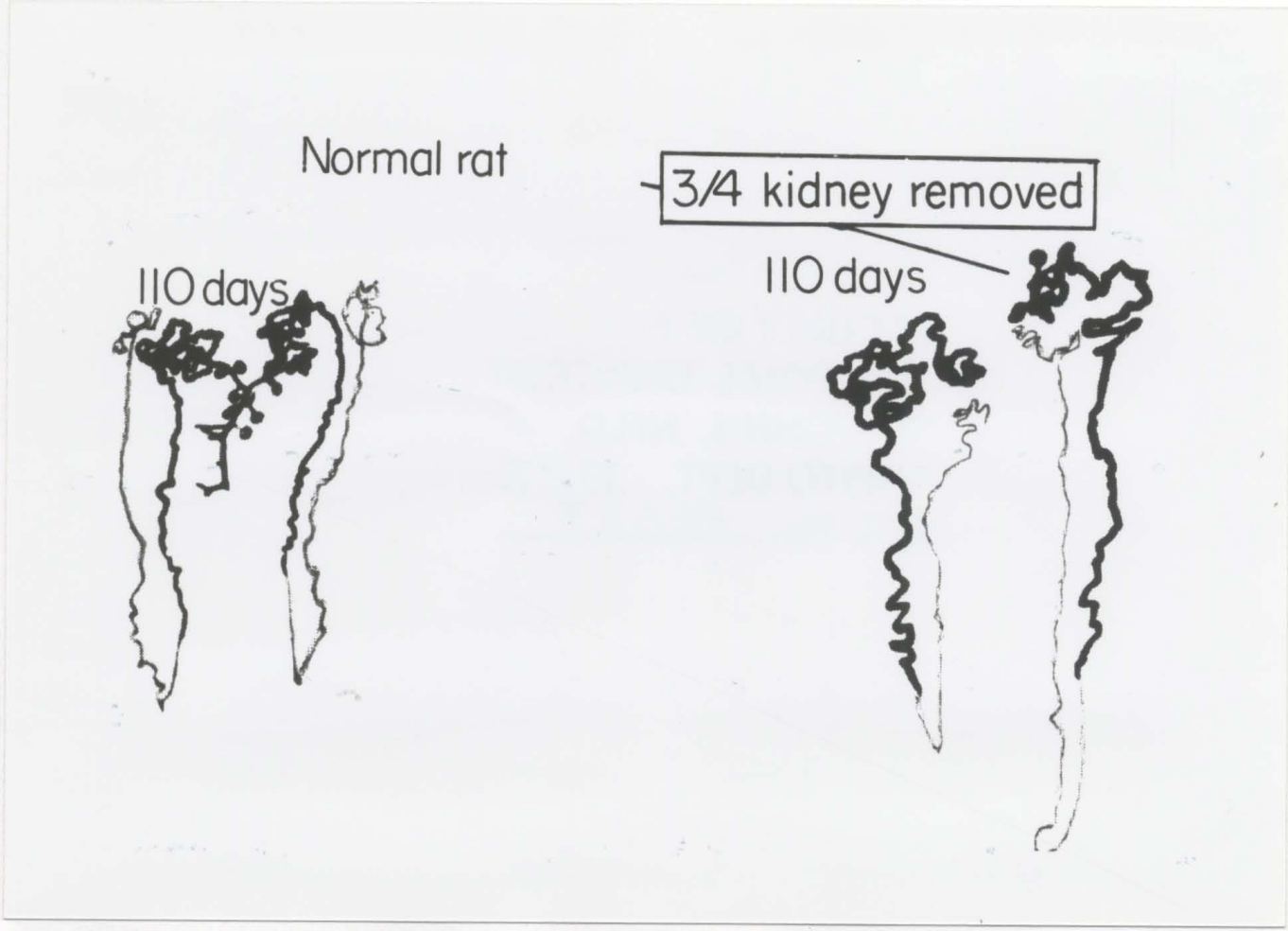

Figure 3. Camera lucida tracings of rat nephrons. These preparations, made by Jean Oliver (1944), show the enlargement of rat kidney tubules 110 days after removal of three-quarters of the renal mass (on the right), compared with tubules from an unoperated rat of the same age (on the left).

Table 2. Increase in nephron size after uninephrectomy in the rat*

	luminal diameter	outside diameter	length	volume
% increase in proximal convoluted tubule	17%	14%	35%	96%
% increase in distal convoluted tubule	12%	10%	17%	25%

* Data are from Hayslett et al. (1968), comparing mean tubule dimensions two to four weeks after UNx with those of control animals.

measurements. The near-doubling of proximal tubule volume is particularly interesting: this implies the ability of the nephron to handle twice the normal volume of glomerular filtrate. If this is so, then the modest mass increase of 30 to 40 per cent seen after uninephrectomy represents a doubling of functional capacity.

The large size increase in proximal relative to distal tubule correlates with the higher mitotic indices observed in proximal cells.

The volume of the glomeruli also increases after UNx. Shea (1978), using two-dimensional measurements of ultrathin sections of glomeruli, reports that mean glomerular volume has increased 2.75 times at 21 weeks after UNx in the adult rat. During the same interval, sham-operated rats' glomerular volume has doubled, due

to normal kidney growth, so that the effect of UNx is to cause a 38 per cent greater increase in volume.

In the 50 years around the turn of the century there were at least twenty published reports on the question of whether or not new nephrons are formed during compensatory renal growth. Various ages, sometimes unspecified, of four mammalian species (rats, rabbit, guinea pig and dog) were used for experiments. The authors arrived at different conclusions, probably partly because they used very small sample sizes. The four authors which have been most commonly quoted in discussions of this question (Arataki, 1926; Shiels, 1926-27; Jackson and Shiels, 1927; Saphir, 1927) all concluded that no new nephrons are formed after UNx. None of these investigations included sufficient animals (there were one to four per group), and the technique of serial sections which was used is not suitable because the investigators did not take into account changes in morphology in the growing cortex. Jackson and Shiels noted a "nephrogenic" zone at the perimeter of the kidney cortex. This undifferentiated tissue, thought to be the source of new nephrons, was of greatest mass in newborn rats, and had fully differentiated by 20 days. Jackson and Shiels' conclusion was based on the data from one seven-day-old rat using, as a control, the data from one other rat whose glomerulus count was done by another person. Arataki (1926), using the same strain of rat but starting his experiments at 20 days, also found no new nephrons. Saphir used eight littermate rabbits of unspecified age (but young ones, as evidenced by their body weights). He killed them from three days to one year after UNx. There were three control

rabbits. Glomerulus counts were made in every tenth serial section; the author states that sometimes glomeruli were counted twice using this method. Although the increase in number of glomeruli counted is proportional to the kidney mass increase, the author discounts the result, due to the probable errors in counting.

Even though the conclusion that no new nephrons were formed was based on such inadequate experimental data, further work was not reported until the 1960's. Hiramoto et al. (1962) treated nine rats of body weight range 300 - 400 gms. with rabbit, anti-rat kidney (glomerular membrane), antiserum. After 24 hours they performed uninephrectomies on six of the animals, keeping three as controls. Seven months later, they removed the remaining kidneys, sectioned them and stained with fluorescein-labeled anti-rabbit antiserum, and double-stained with rhodamine-labeled anti-rat kidney antiserum. Any glomeruli formed after the UNx surgery would have stained rhodamine red, and all pre-existing ones would have double-stained yellow (rhodamine red plus fluorescein green). They found no new nephrons. Their method was successful in showing the development of new nephrons in the outer zone of the cortex in very young rats; Hiramoto and his colleagues examined tissue from six-week old rats only; and thus did not make any observations about the time course of new nephron formation in the neonatal period.

Bonvalet et al. (1972) examined two groups of rats, one of ages four to seven weeks and the other greater than seven weeks at the time of UNx, and found that the younger group but not the older formed new nephrons. He obtained consistent data from two different

methods: (a) a count of glomeruli; and (b) calculation based on whole kidney and single nephron glomerular filtration rate data. Since the report is an abstract of a paper, there is no information available about sample size or methods. Bonvalet (1978) has reviewed this subject and stated that young mice, but not guinea pigs,are able to produce new nephrons,but no data is given in support of these findings.

There is an indication from these reports that the formation of new nephrons, or the capability to do so, might disappear at some point in the late neonatal period - an hypothesis which is compatible with the known ontogeny of the mammalian kidney. Canter and Goss (1975) tested this hypothesis. They used a method wherein a suspension of the glomeruli from kidney tissue was used to obtain an accurate count of nephrons. They found that in unoperated Sprague-Dawley rats the number of nephrons stops increasing by about 40 days of age. They showed an increase in nephron formation due to UNx between birth and 50 days, but not at 70 days. This effect of UNx had an inverse correlation with age.

Kunes et al. (1976) used 18 and 80 day-old rats for UNx, and killed them 27 days later. Using techniques similar to those of Canter and Goss they found seven per cent more nephrons than controls in the younger group, and no difference from controls in the older rats. Administration of a high salt diet produced no change in the younger controls or operated rats, but resulted in an increase in nephron number in all older rats. This work confirmed that of Canter and Goss with respect to the situation after UNx but presents

some provocative new questions about the formation of nephrons in the adult rat.

It seems reasonable to make the following statements regarding post-natal nephron formation in rats: (a) the production of nephrons continues for about six weeks after birth in the outer zone of the cortex; (b) UNx during this period, and probably for a short time after it, stimulates production of new nephrons.

II Hyperplasia

The renal growth following UNx is often referred to as compensatory renal hypertrophy - a correct term if organ hypertrophy is being referred to, but a misleading one with respect to cellular growth, since both cellular hypertrophy and hyperplasia occur.

Until 1949, when Rollason reported otherwise, it was the prevailing opinion that renal epithelium, like neurons, did not have the capacity for cell division. In 1966, Johnson and Vera Roman published experimental results and a discussion of the relative contributions of hyperplasia and hypertrophy after UNx in mice. They derived a calculus equation whose solution gives the percentage increase in number of kidney epithelial cells. The data required are fraction of epithelial cells labeled at various times after UNx or sham operation, and the estimated mean duration of the synthetic phase of the cell cycle. They found an increase in cells of 4.4 per cent at three days, and 7.0 per cent at five days. At five days, the kidney dry weight had increased by about 30 per cent; therefore about one-fourth of the mass increase was due to hyperplasia.

These authors used the data of Goss and Rankin (1960) to calculate the same parameters for the rat (young adult, male, Sprague-Dawley), and found a 5.6 per cent increase after three days, and 6.8 per cent after four days. Kidney weights were not reported, so the contribution of hyperplasia can not be calculated; however, it would be of the same order as that for mice.

The diploid DNA content of cells is constant (Vendrely, 1955), so there is a direct correlation between number of cells in the kidney and its total DNA content. Enesco and Leblond (1962) have quantitated this relationship for rat cells: number of nuclei in kidney = total kidney DNA (mg.)/6.2×10^{-9}, since each diploid nucleus contains 6.2×10^{-9} mg. DNA. A comparison of the per cent increase in total DNA with mass increase at various times after uninephrectomy would yield an estimate of the proportion of growth due to hyperplasia. The assumption would have to be made that all cells have the same mass, and that no multinucleated cells are present; in fact, recently-divided cells are smaller than others, so the amount of hyperplasia would be underestimated by this method. Multinucleated cells are rare in kidney tissue (unpublished personal observations).

Threlfall et al. (1967), using inbred male rats aged six to eight weeks and of body weight 90 to 120 gm., found an increase of 25 per cent in total DNA content and of 35 per cent in kidney wet or dry weight 14 days after UNx. The contribution of hyperplasia in these young adult animals is then 25(100)/35 = 71 per cent.

Uninephrectomy in the rat results in an elevation of the mitotic index of the contralateral kidney with a peak at 40 to 48 hours, and a second, smaller peak at three to four days (Williams, 1961; Goss, 1964; Saetren, 1970). There is no significant increase in mitotic index in male Sprague-Dawley rats 24 hours after UNx (Goss and Rankin, 1960). Dividing cells are most frequently seen in the proximal convoluted tubule epithelium, much less frequently in the distal convoluted tubule epithelium, and very rarely in the renal corpuscle,

blood vessels, connective tissue, loops of Henle and collecting ducts; therefore, the hyperplasia occurs almost entirely in the cortex (Williams, 1961).

DNA synthesis, as measured by tritiated thymidine uptake, shows a slight increase one and four days after UNx, and a five-fold increase at two and three days, according to Mayfield et al. (1967). Since only two rats per day were used, these results can be taken only as a general indication of DNA synthetic activity. Threlfall et al. (1967) reported a similar experiment, again using only two rats per group: they found a five-fold increase on day two, smaller increases on days three and four, and no difference from controls on days one, seven and fourteen.

One may conclude only that there is some correspondence between mitotic index and DNA synthesis after UNx in the rat. Although the time sequence of change in DNA synthesis is predictable from existing data on mitotic rates, correlation of the two phenomena has not been well investigated.

The normal rat kidney has a very low rate of cell division; Messier and Leblond (1960) classified the kidney as having an "expanding" cell population: i.e., one that, although normally nearly quiescent, has the capacity for proliferation in certain circumstances (and therefore might be better named "expandable"). There are no stem cells, but each tubule cell (presumably) has the capacity for division.

A daily mitotic activity in the range of 0.8 to 1.5 per cent in adult male rats is just sufficient for cell number to keep pace with body weight increase (Leblond and Walker, 1956). The parenchymatous cells of the kidney fall into this range. McCreight and Sulkin (1962) report a mitotic index (M.I.) of 0.10 per cent among proximal convoluted tubule cells. Goss and Rankin (1960) report 0.024 per cent in cortical cells. The latter dataare based on observation of about 20,000 cells per kidney, while the former on only about 2,000, so the figure of 0.024 per cent is more reliable. Since the duration of mitosis in the rat kidney tubule is about 45 minutes (Saëtren, 1970), the proportion of kidney cells dividing in 24 hours can be approximated from the index at any point in time: there are 16 intervals of 90 minutes each per day and an average of 0.024 per cent M.I. at any one time, so the daily index will be about 0.77 per cent, since any discrete observation will reveal half the number of cells dividing over a total range of 90 minutes.

The normal low level of cell division in the rat kidney exhibits a diurnal rhythm. This variation has not been studied in a very rigorous manner: only three intervals of two hours each (out of 24) have been compared. Williams (1961) found the mitotic index at 2 to 4 p.m. twice as great as at 6 to 8 a.m. Blumenfeld (1938) reported a higher index at 2 to 4 p.m. than at 10 - 12 p.m. This indication of a maximum in the afternoon may also help to explain the discrepancy between the mitotic indices given above: McCreight and Sulkin's rats were killed at 3 p.m., Goss and Rankin's at 10 a.m.

The use of live animals in experiments on compensatory renal growth introduces a large number of variables. Failure to appreciate or control these has contributed to variability in results and confusion in interpretation. The existence of diurnal variation in the mitotic rate of kidney cells is only one example of such a variable. The daily maximum mitotic index is at least twice as large as the minimum, and the wave of hyperplasia following UNx has been variously reported as only about a six-fold increase (Goss and Rankin, 1960), a seven-fold increase (McCreight and Sulkin, 1959) or an eight-fold increase (Argyris, 1961).

Saètren (1972) looked specifically at the influence of the endogenous diurnal rhythm on the wave of hyperplasia following UNx. He found and analyzed a large effect: the highest peaks were three times the lowest, and reached their maxima about eight hours earlier. The highest peaks occurred around noon and were due to nephrectomies performed two days earlier at three to nine p.m. This finding is illustrative of the large influence that biological variables can have on experimental data.

It is important to examine the influence of age on the relative proportions of hyperplasia and hypertrophy in both normal and compensatory kidney growth of the kidney. Recognition of these age-related changes is necessary for critical interpretation of experiments which use mitotic index or DNA synthesis as a measure of compensatory growth.

Five investigations of normal kidney growth in rats agree that the relative contribution of cell division, compared with increase in cell size, decreases at about six or seven weeks of age. Shirley (1971) found that the concentration of DNA in kidney tissue decreases from birth to six weeks, when it has reached adult values. Toback and Lowenstein (1974b) report that DNA concentration in rat kidney cortex decreases from birth to eight weeks, decreasing at a faster rate between about six and eight weeks, when it has reached its adult value. The total amount of DNA should parallel that of cell number. To interpret this information, one would have to calculate the total number of cells per kidney from DNA concentration and kidney weight; then the change in rate of cell division with age would be obvious. Therefore, the only conclusion that may be drawn from Shirley's experiment is that a decrease in cell division occurs at about six weeks, since the rate of kidney weight gain remains constant.

Potter et al. (1969) determined kidney weight and total DNA in Sprague-Dawley males, and from these data calculated cell number and mean cell size. They concluded that hyperplasia contributed to kidney growth up to about seven weeks; thereafter (until three months, when their experiment ended) growth was due to hypertrophy.

Winick and Noble (1965) reported their very carefully-obtained and complete data on the increases in weight and protein, DNA and RNA content of various organs, including the kidney, during growth of the Sprague-Dawley rat. Kidney DNA content increased linearly from birth to six weeks, when there was a one-week duration of rate decrease,

after which DNA increased only very slightly until the experiment ended at 14 weeks. Weight per nucleus, an indication of cell size, decreased slightly until about two weeks, and then increased steadily. These rates of change in DNA and weight may be interpreted to mean that only hyperplasia is occurring during the first two weeks after birth; then both hyperplasia and hypertrophy occur until about seven weeks, after which growth is almost pure hypertrophy.

Zumoff and Pachter (1964) measured total cell number directly in kidneys of growing Sherman rats. They prepared suspensions of nuclei isolated from whole kidney homogenates. When they plotted number of kidney cells vs. rat body weight, they found a plateau in the rate of increase - and therefore a cessation of hyperplasia - from about 60 to 120 gm. in males, and 75 to 140 gm. in females. These body weights probably correspond to age ranges of three to seven weeks in males and four to eight weeks in females of the Sherman strain, although the authors do not give age/weight correlations. This is the only report in which there is an indication that mitosis resumes after a period of cessation. Since the hiatus in cell division coincides with puberty, the authors suspected sex hormones might be involved. They castrated a series of animals of both sexes, and found that the inhibition of hyperplasia was abolished; hormone replacement restored the plateau, and extended it as long as the hormone was given.

There is a consensus that kidney cell division occurs until six or seven weeks after birth in the normal rat. There are indications that following puberty the rate of cell division remains low, the

decrease being causally related to the increase in blood sex hormone levels.

Knowing the background of normal kidney growth, it is possible to examine compensatory growth which is superimposed on it. As we have seen, there is agreement among published results that kidney cell hyperplasia after UNx decreases in rats after sexual maturity.

Two groups of researchers assayed protein, RNA and DNA in rat kidneys remaining after UNx at various ages. Paulini et al. (1970) used two to thirteen month-old males four weeks after UNx. Total DNA per kidney increased steadily with age in control rats; there was no change in the rate of increase at puberty. These rats were of a strain not used in other work (SIV-50, obtained in Germany), which may account for the differences between these results and those discussed above. The DNA content of contralateral kidney four weeks after UNx (when compensatory growth would have been accomplished), increased at the same rate as controls at all ages except in animals of ages four to seven months, when the DNA content decreased with age. These rats, three to six months old when surgery was performed, must have been post-pubescent.

Karp et al. (1971) measured total kidney protein, RNA and DNA in male Sprague-Dawley rats two weeks after UNx. Three groups of rats were used: four days, six weeks,or three to four months of age at surgery. Hyperplasia, as determined by relative amounts of DNA, RNA and protein, occurred to an equal extent in the two younger groups of rats, but to a far lesser extent in the adult group. The

authors state that Sprague-Dawley rats attain sexual maturity at 40 days of age. These results were from groups consisting of three, three and ten rats, respectively.

Two groups of investigators found that the mitotic index of kidney epithelium in control, unoperated rats decreased with age. Uninephrectomy caused a similar "degree" of increase in mitotic index in all ages of rat studied, but since the increase was superimposed on a decreasing baseline, the absolute index after UNx also decreased with age. McCreight and Sulkin (1959), using a small number of rats (a total of ten) of the C.F. Nelson strain, found a seven-fold increase in mitotic index 72 hours after UNx in four month-old rats, and a five-fold increase in senile rats aged 3.2 years (see Table 3.). The kidneys removed at uninephrectomy served as the controls.

Table 3. Influence of age on contralateral mitotic index after uninephrectomy in the rat

Reference	# of Animals	Age	Operation	Mitotic Index (percentage)
McCreight and Sulkin 1959	6	4 mos.	pre-UNx	0.10
			UNx	0.69
	4	3.2 yrs.	pre-UNx	0.03
			UNx	0.14
Phillips and Leong 1967	5	4 wks.	none	0.056
	5	4 wks.	UNx	0.30
	5	4 mos.	none	0.0062
	5	4 mos.	UNx	0.088

Phillips and Leong (1967) used four week and four month old Sprague-Dawley males, and found a five-fold increase in mitotic index 36 hours after UNx in the younger rats, and a nine-fold increase in the older ones (see Table 3). Rats receiving no operation were controls. There were five rats per group.

Both of these experiments can be criticized for the small sample sizes; adequate numbers of rats are especially necessary when measuring such small values - as low as 62 mitoses in 10,000 cells.

Zumoff and Pachter (1964) determined the total number of nuclei per kidney in Sherman rats. As described above, they found an increase with age except during the time of puberty (at three to seven weeks), during which there was a plateau. Castration abolished this cessation of hyperplasia. These authors also reported the effects of UNx on kidney nuclei counts. The Sherman rats used for this series of experiments showed a normal kidney-cell-number plateau between about 80 and 200 grams body weight. Rats were uninephrectomized at 160 gms. (ten to eleven weeks). Sixteen days later the remaining kidneys were assayed, and no increase in cell number was found. Other groups were castrated at the time of UNx: these animals showed a 27 per cent increase in cell number. Treatment with isosexual hormone (testosterone propionate in males) gave results similar to uncastrated animals.

It is not possible to draw any conclusions from these five reports because of variation among techniques and strains of rat, and inadequate sample sizes. However, there is indication that the capacity for a hyperplastic response to UNx is compromised at puberty due to the increase in sex hormones, an effect which probably continues throughout the remainder of life. Because the correlation among age, body weight, time of puberty and capacity for hyperplasia vary with strain of rat, information regarding strain is necessary for interpretation of mitotic index and DNA synthesis data.

Both hyperplasia and hypertrophy of the remaining kidney after UNx are modest compared with liver regeneration: more than 90 per cent of hepatocytes undergo mitosis, and the liver returns to its pre-partial hepatectomy mass (Bucher and Malt, 1971). There are several possible explanations for the difference in growth response seen in these two organs.

Compensatory liver growth after partial ablation is not true regeneration - the excised lobes do not regrow, but the residual tissue enlarges. The rate of cell division parallels that of mass increase, and all the cell types exhibit hyperplasia. New lobules are formed, so that normal micro-anatomical architecture is preserved.

In the rat kidney, no new nephrons are formed after about six weeks of age. The contribution of hyperplasia to the mass increase falls at puberty, so that compensatory renal growth in adult

rats is primarily a matter of cellular hypertrophy. Both hyperplasia and hypertrophy occur to the greatest extent in the proximal tubular epithelium. Thus the only possibility for kidney growth in adult animals lies in enlargement of existing nephrons, and even this growth is limited by the narrow range of surface-volume relationships permissable for the function of the nephron (Hayslett) et al., 1968). A teleological view is that the growth of the nephron is limited in order to preserve its functional efficiency. From another viewpoint, mechanisms for the limitation of growth have evolved since there may be a selective advantage for a limited nephron size. The mechanisms may involve a low mitotic capacity coupled with a maximum possible cell size, as discussed by Mitchison (1971).

Another possibility is that the tough, fibrous tissue capsule covering the kidney limits its growth. Anyone who performs uninephrectomies in rats has observed that the kidney capsule is not able to be stretched. It is composed of dense, collagenous connective tissue, the fibres of which are inelastic and randomly arranged. In the mongrel dog, the elastic modulus (force/unit area required to double the length of the specimen) of the kidney capsule was found to exceed that of the aorta (Hebert et al., 1976). These authors propose that the capsule is a major determinant of whole-kidney volume/pressure relationships.

In general, the amount of connective tissue collagen increases with age. Although there is no information available specifically

about the influence of age on the kidney capsule, it may be hypothesized that one factor responsible for the decrease in compensatory renal growth (mass increase) with age is an increase in density and decrease in elasticity of the capsule.

Choie and Richter (1973) have reported that renal decapsulation stimulates a six-fold increase in tritiated thymidine incorporation into proximal and distal tubule cells of adult female Sprague-Dawley rats. The maximum rate of DNA synthesis occurred at about 42 hours, and labeled cells were seen most frequently near the outer cortex. There was no effect on the contralateral kidney when compared with sham-operated controls. No trauma due to the decapsulation procedure was detected.

Uninephrectomy with simultaneous decapsulation of the contralateral kidney is an experiment that obviously should be done.

III Biochemical changes

One of the components of the phenomenon of compensatory growth following UNx is the biochemistry of the synthesis of new RNA, DNA and protein by the cells of the contralateral kidney. These changes, the necessary basis for cellular hypertrophy and hyperplasia, have been described in ever-increasing detail by biochemists and molecular biologists. It is frustrating that in spite of our knowledge of these events, which occur beginning just a few minutes after removal of one kidney, the stimulus which sets them in motion is not yet understood.

It has been established that adenosine 3',5'-monophosphate (cyclic AMP), functions in cells as a second messenger mediating a variety of non-steroid hormonal effects (Sutherland et al., 1968). With respect to the kidney, this compound has been implicated in the action of anti-diuretic hormone (vasopressin), parathyroid hormone, calcitonin, glucagon and catecholamines (Dousa and Barnes, 1977). Intracellular levels of cyclic AMP are raised by these hormones. Intracellular phosphodiesterase, which degrades cyclic AMP, is inhibited by methyl xanthines. Administration of these compounds, caffeine or theophylline for example, results in augmentation of hormonal effects mediated via cyclic AMP.

Cyclic AMP and cyclic GMP (guanosine 3',5'-monophosphate) also play a role in regulation of cellular growth. In general, an increase in cyclic AMP content of cells is associated with

inhibition of growth, and an increase in cyclic GMP content with cell growth and proliferation. The two compounds show a reciprocal relationship. A good review of the role of cyclic nucleotides in growth stimulation of cultured cells by various serum factors has been written by Schonhofer and Peters (1977).

Three groups have reported the effects of UNx on the cyclic nucleotide content of contralateral kidney tissue. Schlondorff and Weber (1976 and 1978) used adult male Sprague-Dawley rats. They found a decrease in cyclic GMP to 20 per cent of normal levels at 15 minutes, followed by an increase to 200 to 300 per cent of normal from 1 to 72 hours. Cyclic AMP decreased to about 75 per cent of normal during 1 to 8 hours after UNx, and returned to normal by 24 hours. The authors ascribe the increase in cyclic GMP to an increase in its synthesis rate.

Dicker (1977 and 1978) reported similar results using adult male Wistar rats, except he found a different temporal pattern to the changes in cyclic nucleotides: cyclic GMP increased by ten minutes, fell to less than normal levels by 20 minutes, and returned to baseline by 90 minutes. Cyclic AMP had decreased by ten minutes, and did not rise again to baseline values until three hours.

Solomon et al. (1978), also using adult male Wistar rats, found no significant change in either cyclic nucleotide between two hours and seven days after UNx.

These dissimilar results, using apparently similar experimental conditions, probably indicate that a variable is not being recognized and controlled.

Another phenomenon, possibly related to cyclic nucleotide induction of growth, has been reported by Lowenstein and Toback (1978): rate of synthesis of acid-insoluble phospholipids in kidney cortical cells is increased by five minutes after UNx in rats. This may represent increased synthesis of membranes, associated with a membrane-mediated second-messenger stimulation mechanism. Alternatively, it could, as the authors suggest, be a prelude to proliferation of membranes of mitochondria and endoplasmic reticulum.

A group of aliphatic amines - putrescine, spermidine and spermine (collectively named polyamines) - are normal cell constituents of kidney and various other tissues. By virtue of their basic nature, polyamines interact with the acidic phosphate groups of nucleic acids. These compounds exert a stabilizing and stimulatory effect on nucleic acid synthesis: (a) they effect changes in the secondary structure of RNA which facilitate its removal from DNA after transcription; (b) they stimulate the DNA-replicating enzyme, DNA-primed DNA polymerase.

Putrescine is derived by decarboxylation from ornithine, and spermidine and spermine are metabolic products of putrescine. The activity of the enzyme ornithine decarboxylase and the amount of polyamines are markedly increased in tissues which are undergoing compensatory growth. These changes are among the earliest detectable

after growth stimulation, and probably form a link in the chain of events wherein the growth stimulant elicits the second messengers (cyclic AMP and GMP), which effect an increase in ornithine decarboxylase activity, which stimulates polyamine synthesis and thereby nucleic acid synthesis.

There have been two reports of investigations of ornithine decarboxylase activity and polyamine synthesis during compensatory kidney growth; the results do not support the hypothesis that these events are among the earliest detectable after uninephrectomy, although the experimental design may be at fault.

Brandt et al. (1972), using young adult Holtzman rats, measured ornithine decarboxylase activity at six-hour intervals after UNx. They found a negligible increase at six hours, a maximum, five-fold increase at 24 hours, a drop to normal at 30 hours, and a second three-fold maximum at 36 hours. Sham-operated animals showed "small" increases in activity. Spermidine concentration in kidney tissue showed a sharp maximum at 36 hours, twice as high as normal values.

Melvin and Thomson (1972) found a six-fold increase in enzyme activity in rat kidney four hours after UNx, returning to normal by twelve hours. Sham operation produced a similarly-timed but smaller response, and the authors are of the opinion that some of the enzyme activity is due to the non-specific stress of surgery.

It is possible that the first group of investigators missed a sharp rise in enzyme activity before six hours. One would like to see enzyme activity assays made at frequent intervals over the first few hours after UNx, since it is during this time that an increase should be seen if the polyamines are playing a role in the growth stimulation.

It is an interesting aside that Brandt et al. (1972) found a consistently higher, by 30 per cent, ornithine decarboxylase activity in left kidneys compared to right in sham or unoperated rats. The right kidney is about 2.5 per cent heavier than the left in Sprague-Dawley rats (Kaufman et al., 1974). It is difficult to speculate on the reason for either difference. The possibility must be ruled out that the investigators introduced a bias by always removing the same-sided kidney first, thus changing the contralateral renal blood flow. If the amount of blood contained in the remaining kidney were increased immediately after UNx, then the mass of that kidney would be increased.

There has been no investigation of the effect of blood flow on ornithine decarboxylase activity. Situations of rapid growth which are accompanied by increase in this enzyme's activity also include an increase in blood flow, e.g., in liver remnant after partial hepatectomy. It is possible that the relationship may be causal. Also cyclic AMP mediates many cardiovascular control systems (Sutherland et al., 1968). The combination of shaky hypothesis and

circumstantial evidence induce one to consider the possibility that hemodynamic changes after UNx in the contralateral kidney might play a part in growth stimulation.

The simplest assessment of the effect of UNx on the RNA of the contralateral kidney is measurement of the total RNA content: in rat kidney, it is increased by seven per cent at 12 hours, and by 33 per cent at 48 hours, declining thereafter (Halliburton and Thomson, 1965b;Threlfall et al., 1967; Kurnick and Lindsay, 1968a).

A good review of RNA metabolism after uninephrectomy appears as a chapter in the book Compensatory Renal Hypertrophy (Malt, 1969a). The summary of this review follows:

> "Bulk RNA in the mouse or rat kidney increases rapidly after contralateral nephrectomy. Within 2 days, although the renal mass increases by 10% or less, RNA/DNA increases 20-30%. The elevated RNA/DNA in the mouse persists unchanged for nearly 4 weeks as the mass increases by 50%; total RNA content increases 36% and total DNA content 14%. As turnover time of renal rRNA is about 4 days in both nephrectomized and sham-nephrectomized mice, the accretion of RNA must represent enhanced RNA synthesis. These data show that hypertrophy, defined as an increase in RNA, is the earlier and more prominent process during this period, but hyperplasia is also important later.
>
> An accelerated synthesis of rRNA contributes to the increased number of polysomes in the renoprival kidney. Free ribosomes are labeled with nucleic acid precursors somewhat before membrane-bound ribosomes, but the labeling of both increases perhaps threefold in the first few days after nephrectomy. Deductions about the rate of RNA synthesis wait upon measurements of the specific activity of the nucleotide precursor pools. The normal 3/1 ratio of free/bound ribosomes does not change during compensatory growth. Although equally active in general protein synthesis, bound ribosomes are 13-16 times more active in glycoprotein synthesis than are free ribosomes.

> The synthesis of mRNA may also be increased and may vary in reciprocal relation to the synthesis of rRNA. rRNA synthesis is highest at 2 and 8 days after nephrectomy at the same times that mitotic indices have been reported to be elevated.
>
> The metabolic steps leading to increased RNA synthesis have not yet been defined. Compared with other systems, kidney seems to be poor in the 45 S precursor of rRNA and rich in species between 41 S and 32 S. Labeling of 45 S RNA is enhanced after unilateral nephrectomy as might be expected, but the significance of the diminished labeling of HnRNA 28 S within an hour of nephrectomy is obscure."

Since publication of this book in 1969, some new information has been reported. Toback and Lowenstein (1974a) found that the amount of uracil nucleotide increases within the first hour after UNx. Their data were obtained using renal cortex slices from young adult, male Sprague-Dawley rats. The phenomenon of increase in RNA nucleotide precursors soon after UNx was confirmed by Cortes et al. (1976), using kidney cortex slices from adult male rats. The authors attempted to decrease the differences in precursor quantities in in vitro preparations derived from sham or UNx animals; the result was a significant decrease in the difference between the two groups in uptake of labeled RNA nucleotide precursors. The authors postulate that the increased amount of nucleotides might result from an increased turnover rate of nucleoplasmic heterogeneous RNA (HnRNA). Willems et al. (1969) found that UNx in mice causes accelerated processing of HnRNA at one hour, but not at ten minutes. No function has been definitively associated with HnRNA; this postulated one might be a generalized mechanism for provision of an increased RNA precursor pool size in preparation for increased RNA synthesis. It

has also been proposed that HnRNA plays a role in regulating transcription, but so little is known that no hypothesis may be made to relate this to renal growth.

In the discussion quoted in Van Vroonhoven et al. (1972), R.A. Malt states that the most sensitive assay for a serum factor mediating compensatory renal growth is the disappearance of the heterogeneous nucleoplasmic RNA, but that the test is impractical because of its difficulty.

It is generally accepted that the majority of RNA in mammalian cells is ribosomal (Hoagland, 1960). Over 85 per cent of RNA in rat liver cells is ribosomal RNA (rRNA) (Hirsch, 1967). Although kidney cells may differ, it is reasonable to examine rRNA as an index of RNA metabolism. Both single ribosomes (Halliburton, 1969) and polysomes (Sendecki et al., 1972) are increased at 24 hours after UNx. An increase in single ribosomes within 15 minutes of UNx in the mouse is often cited as the earliest observed effect of UNx (unpublished data referred to in Malt and Lemaitre, 1968).

Hill et al. (1974) found no evidence for an increase in rRNA synthesis from 20 minutes to 48 hours after UNx in mice. They ascribe an observed 20 to 40 per cent increase in RNA content per cell during the first 48 hours to a decrease in degradation of rRNA. As discussed above, polyamines may play a role in increasing rRNA stability.

The finding that rRNA synthesis rates do not increase seems paradoxical in light of the increased precursor pool size. It is clear that the complexities of RNA metabolism after UNx are not yet understood.

The amounts of the various species of RNA in a cell are regulated both by their synthesis rates and their catabolism rates. The various RNAses play a primary role in catabolism, and are therefore relevant to the investigation of RNA changes occurring after UNx, but the interrelationships of the various RNAs and the effects of RNases on them are not completely understood.

Nuclear RNase activity, in both nucleoli and nucleoplasm, is elevated at three hours after UNx in adult male Sprague-Dawley rats; its activity decreases to control levels by 24 hours, and rises again at least until 96 hours (Rosso et al., 1973). Perhaps the initial increase stimulates the accelerated turnover of HnRNA.

Royce (1967) has put forward the interesting hypothesis that elevated plasma levels of RNase result from reduction of renal mass, and the consequent increase in renal handling of RNase by the remaining kidney stimulates compensatory growth. He presents evidence that the proximal tubule cells normally take up RNase from the glomerular filtrate, thus producing a decrease in RNase concentration from the renal arterial to the venous plasma.

In tissues where polyploid and binucleate cells are uncommon, the amount of DNA per cell is constant. This has been shown to be

the case in the kidney cells of unoperated rats (Kurnick, 1951). There is a transient increase in the average amount of DNA per kidney cell nucleus in the early stages of compensatory renal growth in the rat (Ogawa, 1960-61), which is a consequence of some cells being in the 4n state of the G_2 phase of the cell cycle. Therefore, the DNA content of the kidney reflects its number of cells, and an increase in total DNA after UNx would indicate that hyperplasia has occurred.

Threlfall et al. (1967) found no increase until four days (13 per cent), and then further increases at seven and fourteen days up to 25 per cent in young adult, male inbred rats. Kurnick and Lindsay (1968a), using adult, female inbred rats, found no significant difference in kidney DNA content between sham and UNx animals during the first nine days. Miyada and Kurnick (1960) found no increase in DNA for the first six days after UNx, but then an increase of 30 per cent by 16 days. (The report is in abstract form, with no information on the rats used.) A good discussion of this work and others is found in Goss, 1964.

These sparse data, obtained by methods yielding variable results (Kurnick and Lindsay, 1968a), do not allow any conclusion on the effect of UNx on kidney DNA content, but one may reasonably expect an increase concomitant with observed mitoses in the proximal tubular epithelium; i.e., an increase, inversely correlated with age, during the first few days after UNx.

A more sensitive assay for DNA synthesis is measurement of uptake of its precursors. Thymidine is incorporated into DNA via this pathway:

$$dCMP \rightarrow dUMP \rightarrow dTMP \rightleftharpoons dTdP \rightleftharpoons dTTP$$

(TdR ⇅ dTMP; dTTP ↓ DNA)

dCMP = deoxycytidylic acid

dUMP = deoxyuridylic acid

dTMP = deoxythymidylic acid

dTdP = deoxythymidine diphosphate

dTTP = deoxythymidine triphosphate

TdR = thymidine

The usual pathway is via dUMP; exogenous TdR, commonly used to provide the radioactive label for DNA, is incorporated as shown. The rate of incorporation of exogenous TdR into DNA depends on: (a) its rate of passage through the cell membrane; (b) the rate of synthesis of dTMP via dUMP; and (c) the rate of depletion of the nucleotides by incorporation of dTTP into DNA. There is no report in the literature regarding rates of membrane passage of TdR after UNx.

The rate of DNA synthesis is usually determined by incorporation of labeled precursors - a situation with internal contradictions. Only an approximation of the rate may be made from observation of rate of cell division in the kidney, due to limitations of histological methods.

Some of the enzymes of this pathway show increased activities after UNx (Mayfield et al., 1967): dCMP deaminase (catalyzing

dCMP → dUMP), dTMP kinase (catalyzing dTMP → dTDP), and TdR kinase (catalyzing TdR → dTMP) are elevated at 24 and 48 hours after UNx, indicating that synthesis via both endogenous and exogenous pathways is accelerated.

The thymine nucleotide (TdR, dTMP, dTDP and dTTP) pool size must be determined before DNA labeling rates can be interpreted. I find no report in the literature of measurement of DNA precursor pool size in association with compensatory renal growth. Toback and Lowenstein (1974b) have inferred pool size changes from labeled precursor uptake data. They measured in vitro incorporation of ^{14}C-thymidine into DNA of renal cortex slices from young adult, male Sprague-Dawley rats. Incorporation per mg. DNA decreased until 18 hours post-UNx, with a minimum at 12 hours of 21 per cent below control values; the maximum was attained at 27 hours, five times the control. Uptake was still elevated at 36 and 48 hours. The depression of labeled thymidine uptake immediately following UNx is interpreted by the authors as being due to an increase in endogenous thymine nucleotide pool size. However, until these measurements of membrane permeability and pool size are made, one can only assume that the uptake of labeled thymidine reflects DNA synthesis. Reassurance is given by the good temporal correlation among labeled thymidine uptake, autoradiography studies and mitotic indices after UNx.

Some of the earliest biochemical changes observed in the contralateral kidney after UNx are listed in Table 4.

Table 4. Early biochemical changes observed in contralateral kidney tissue following uninephrectomy in the rat

rats used	time after UNx	observation of change in kidney tissue	reference
male, young adult Sprague-Dawley	5 minutes	↑ phospholipid synthesis	Lowenstein and Toback, 1978
male, adult Wistar	10 minutes	↑ cyclic GMP ↓ cyclic AMP	Dicker, 1977 and 1978
male, adult, Sprague-Dawley	15 minutes	↓ cyclic GMP	Schlondorff and Weber, 1976
male, young adult Sprague-Dawley	1 hour	↑ uracil	Toback and Lowenstein, 1974a
male, adult, Sprague-Dawley	1 hour	↑ cyclic GMP ↓ cyclic AMP	Schlondorff and Weber, 1976
male, adult Sprague-Dawley	3 hours	↑ nuclear RNase activity	Rosso et al., 1973
not reported	4 hours	↑ ornithine decarboxylase activity	Melvin and Thomson, 1972
young adult, Holtzman	12 hours	↑ ornithine decarboxylase activity	Brandt et al., 1972
male, adult	12 hours	↑ RNA content	Halliburton and Thomson, 1965b
young adult, Holtzman	24 hours	↑ spermidine concentration	Brandt et al., 1972
not reported	24 hours	↑ single ribosomes	Halliburton, 1969
male, young adult, Wistar	24 hours	↑ polysomes	Sendecki et al., 1972
male, adult, Houston	24 hours	↑ dCMP deaminase ↑ dTMP kinase ↑ TdR kinase	Mayfield et al., 1967

IV Functional changes

No one investigator has attempted to measure simultaneously the complex renal and cardiovascular physiological changes that follow UNx. Such an attempt would be fraught with pitfalls because the techniques available for monitoring these parameters themselves often cause changes in the systems under observation. The best one can do at this time is to examine each parameter separately. I will consider experiments measuring renal blood flow, glomerular filtration rate and sodium reabsorption.

The kidney's vascular system is unique in many respects. The organ's function as a monitor for the size of the erythron and for homeostasis of the cardiovascular system is compatible with its relatively large blood supply. I quote Frank Hinman's description of the renal vessels, from an address he gave to the Kansas City Clinical Society in 1924 (Hinman, 1926):

> "There is no anastomosis between arteries: they are distinctly terminal in distribution, and this type of distribution has no parallel in other gland structures. The short course from the aorta direct to glomeruli, the almost complete absence of nutrient vessels, and the double capillary bed, primary in glomeruli and secondary of two types, cortical and medullary, are unique. The arrangement permits an enormous amount of blood to pass quickly through the kidney, an amount unparalleled in other gland structures, even nineteen times as much as the average supply to other organs, and an average estimated as six times the amount necessary for its own nutrition. The system, furthermore, is an ideal one for the maintenance of blood pressure. The treelike arrangement of the main vessels, the acute angle at subdivisions, the extraordinarily rapid diminution in diameter as branching proceeds, the smaller caliber of efferent vessels compared to afferent, and the absolute terminal distribution of each branch is a perfect construction for this purpose."

There are two reasons that changes in renal blood flow (RBF) are relevant to compensatory renal growth: (a) an immediate increase in RBF may act as a stimulus to the growth after UNx; or (b) RBF may change as a consequence of the remaining kidney increasing in mass.

The kidneys each receive about one-eighth of the cardiac output in humans and dogs. About half the output leaves the aorta before the renal arteries, leaving one quarter in the final segment of the aorta (Krohn et al., 1970). What effect would removal of one kidney have on this blood distribution? If the assumption is made that peripheral resistance does not immediately change, then the proportion of flow in the distal aorta to that in the renal artery will remain the same, i.e., 2:1. The patent renal artery would receive about one-sixth of the cardiac output (an increase of 33 per cent) and the distal aorta, one-third. The venous return is unchanged; presso-receptors of the carotid sinus and the aortic arch are not stimulated. It is difficult to see how hyperemia of the body supplied by vessels distal to the renal arteries can trigger a systemic cardiovascular response. It is possible that the peripheral arteriolar vessels of the lower limbs will constrict in response to increased flow, thus shunting even more blood to the remaining kidney.

Renal blood flow has been tested in dogs using a renal artery probe transducer connected to an electromagnetic flowmeter, and in rats, by measuring para-aminohippurate clearance. The former method can be criticized because it entails clearing the tissues

around the artery in order to attach the transducer, which will probably damage the sympathetic nerve supply to that kidney with consequent alteration of vascular responses.

There are three reports of experiments using mongrel dogs in which renal blood flow was measured during the ligature of the vessels of one kidney, which probably has the same immediate effect on RBF of the contralateral kidney as uninephrectomy. All three report an immediate rise in renal blood flow: Krohn et al. (1970) found an average 30 per cent increase in five dogs; Roding et al. (1971), a 16 per cent increase in nine dogs; Stojkovic et al. (1972), a 32 per cent increase in thirteen dogs. Krohn's and Roding's groups also did actual uninephrectomies and made subsequent measurements; the former found a 33 per cent increase at one to three hours; the latter found a 48 per cent increase at four weeks, and 66 per cent at ten weeks. Two of the three immediate measurements agree with the theoretical prediction of a 33 per cent increase. The maintenance of this increase for at least three hours indicates that the cardiovascular system and the kidney do not react to bring the renal blood flow back to normal. The longer-term measurements at four and ten weeks probably correlate with the kidney's mass increase.

An estimate of renal plasma flow (RPF) may be made by determining the clearance of para-amino hippurate (PAH), which is both filtered by the glomeruli and excreted by the tubules. Nearly all of the PAH in the arterial blood supply to the kidneys is removed as it passes

through, so that the PAH clearance value represents that volume of plasma in which it was dissolved and which circulated through the kidneys in one minute. The renal blood flow (RBF) may then be calculated from the RPF and the haematocrit value.

The PAH clearance does not take into account: (a) blood that passes from the arterial to the venous side without going through a glomerulus or a peritubular plexus; (b) plasma which leaves the kidney as lymph; (c) the diffusion of PAH into erythrocytes (Morrison and Howard, 1966); (d) the necessity to infuse PAH into the rat, which means that there is danger of expanding the extracellular fluid volume, thus increasing RBF by the method itself; or (e) that the use of this technique in rats means that blood sample sizes are small, and errors inherent in the chemical assay for PAH are therefore exaggerated. Clearance of PAH is therefore only an estimate of RBF, particularly in a small animal.

Rous and Wakim (1967) measured PAH clearance in ten mongrel dogs, and found a 31 per cent increase at 24 hours after UNx, 12 per cent at two weeks, eight per cent at four weeks, and 27 per cent at eight weeks. The decline in clearance at two and four weeks is unexplained, and there is no information about the rate of mass increase in the remaining kidney.

Potter et al. (1974) measured PAH clearance in adult rats, weighing 200 to 300 gm., prior to and four to five hours after UNx. Twenty-four rats were infused with a mannitol and PAH solution for

one hour prior to surgery and five hours afterwards. The infusion rate for UNx rats was one-half that for shams. The results are given in Table 5.

Table 5. Cardiovascular changes following uninephrectomy in the rat*

		UNx	Sham
**C_{PAH}	control value	7.2	6.5
(ml./min.)	post-surgery value	9.2	7.2
	per cent change	+28%	+11%
Hematocrit	control value	45.6	43.8
(per cent)	post-surgery value	40.7	41.1
	per cent change	-11%	-5%
**RBF	control value	11.0	11.6
(ml./min.)	post-surgery value	13.2	12.3
	per cent change	+20%	+6%

* Data are from Potter et al. (1974)

** C_{PAH} and RBF values are for one kidney

The decrease in hematocrit during the time of the experiment, greater in UNx than sham, may indicate that plasma volume expension has occurred due to the infusion. Part or all of the increase in C_{PAH} and RBF would be due to the volume expansion, so it is not possible to make any conclusions from these data.

Northrup and Malvin (1976) determined PAH clearance in 200 to 400 gm. rats, 18, 24 and 30 hours after sham operation or UNx. Animals were given isotonic saline infusions for about three hours during clearance studies; the infusion rates were similar to those used by Potter et al., described above. These authors state that the infusion was designed to achieve an expansion of extracellular fluid and saline diuresis. Their mean results for six to nine rats/group are tabulated below:

	UNx	Sham
RPF at 18 hrs. post-surgery:	1.16 ml./min.	1.11 ml./min.
RPF at 24 hrs. post-surgery:	1.46 ml./min.	1.13 ml./min.
RPF at 30 hrs. post-surgery:	1.93 ml./min.	1.45 ml./min.

The difference between sham and UNx values is not significant until 30 hours. There is no raw data given for C_{PAH} or haematocrit.

Autoregulatory mechanisms and/or the sympathetic nervous system may in normal situations (and perhaps not when interfered with by the blood flow measurement apparatus used in dogs) counteract the flow increase by constricting the renal afferent arterioles. This reaction to the increased blood flow could be part of the postulated stimulus, so that even if there were an accurate method available to measure RBF, it might be discovered that no immediate change results from UNx. Careful clearance studies, with no expansion of ECFV, must be done during the first minutes and hours after UNx in order to establish whether or not there are changes in renal blood flow.

Theoretical considerations and experimental results, flawed as they are with technical difficulties, lead one to conclude that there is an increase in renal blood flow immediately after UNx. But it is possible that such an increase in RBF could be dissociated from increase in single nephron blood flow if the increased blood flow was shunted through glomeruli not previously patent. There is evidence in many species of intermittent glomerular blood flow, reviewed by Hartman and Bonfilio, 1959. These same authors used a ten-second exposure to fluorescent stain specific for vascular endothelium to determine the number of patent glomeruli in rats after UNx. They found no change in number of patent glomeruli per unit area of cortex immediately after UNx in adult rats, and an increase four to seven days later. The counts were corrected for kidney mass increase, which dispersed glomeruli over a larger area.

The kinins are a group of vasodilator peptides found in plasma and urine. They are formed from circulating globulins called kininogens by the action of proteolytic enzymes called kallikreins. Plasma kallikrein is formed from an inactive precursor by the action of proteolytic fragments of the active form of clotting factor XII (Hageman factor) (Spragg, 1974). The vasodilator properties of the kinins are well-known: they relax vascular smooth muscle and increase capillary permeability. They produce local vasodilation in a variety of circumstances, including regional warming and the inflammatory response. A role for kinins in renal sodium handling has been proposed (Adetuyibi and Mills, 1972; Marin Grez et al.,

1972). Besides their possible role in sodium homeostasis, these peptides are of interest with respect to compensatory renal growth because of the possibility that they might mediate an increased renal blood flow. Antonello et al. (1975) measured kallikrein activity in rat kidney homogenates 5, 10, 15 and 20 days after UNx. Activity in the remaining kidney was increased 68, 66, 128 and 170 per cent respectively, compared with that in the kidneys removed at UNx. These increases do correlate with observed increases in renal blood flow as the kidney mass enlarges, but the evidence implicating kinins is only circumstantial. It would be interesting to determine the effect of kininases on renal blood flow after UNx: interruption of blood flow increase would indicate that kinins mediate the flow increase and interruption of hypertrophy or hyperplasia would indicate that increase in blood flow plays a role in mediating the growth.

Glomerular filtration rate (GFR) depends on the hydrostatic and oncotic pressures of the blood and of the filtrate in Bowman's capsule. If surgical technique for performing UNx is good, then little blood (except that contained in the excised kidney) will be lost and little body water will be lost from the surgical opening by evaporation; blood and filtrate oncotic pressure should not change significantly. Filtrate hydrostatic pressure will remain the same since the physical aspects of the tubule are unchanged. However, the blood hydrostatic pressure is affected by the arterial blood pressure and flow rate and by the relative diameters of the

afferent and efferent arterioles. The effect of removal of one kidney on many of these parameters is not known, although it seems from the work of Diezi et al. (1976 and 1978) that femoral arterial pressure in the rat increases within five minutes of UNx. Abolition of this hypertensive response by aortic partial clamping diminished but did not totally prevent functional compensation. A prediction of the immediate effect of UNx on GFR can not be made from the available information.

Five groups of authors have reported measurements of GFR in rats during the first 24 hours after UNx. Their results are shown in Table 6. The investigators used either inulin or labeled sodium iothalamate, which have similar clearance properties (Pitts, 1971, Potter et al., 1974). The only cases where there is a possibility that extracellular fluid volume (ECFV) was not expanded are in the experiments of Katz and Epstein (1967), Katz (1969 and 1970), and Diezi et al. (1978), where the infusion rates were relatively slow. It is interesting that these are among the authors who report no change in GFR after UNx, which brings up the possibility that increased renal blood flow and GFR might be a result of the combination of lack of one kidney and an expanded ECFV. This possibility will be further discussed in the section on translation of nephron loss to a growth response by a change in workload.

After 24 hours, when the kidney's mass increase has begun, one would expect to find a concomitant increase in GFR: Katz and Epstein (1967) report a 29 per cent increase at three days, 61 per cent at

Table 6. Change in glomerular filtration rate following uninephrectomy in the rat.

# hours after UNx	Change in GFR*	Other treatment	Reference
½	+8-15%	hypotonic ECFV expansion	Potter et al., 1974
0-3	no change	unknown	Katz, 1969
1-3	no change	1.25 ml/hr/180-430 gm. rat isotonic saline infusion	Katz, 1970
2-4	no change	3 ml/hr/160-240 gm. rat isotonic saline infusion	Diezi et al., 1978
2	+15%	ECFV expansion	Potter et al., 1969
3	no change	0.3 ml/kg/min. isotonic saline infusion	Peters, 1963
5	+13%	isotonic ECFV expansion	Potter et al., 1974
18	+26%	ECFV expansion	Potter et al., 1969
18	+60%	0.3 ml/kg/min. isotonic saline infusion	Peters, 1963
24	+21%	hypotonic ECFV expansion	Potter et al., 1974
24	+24% +44%	H_2O loading, 5% body wt. 0.6% saline, 5% body wt.	Dicker and Shirley, 1971
24	no change	1.2 ml/hr/250-400 gm. rat isotonic saline infusion	Katz and Epstein, 1967

* relative to half the whole-body GFR either in shams or in the same animal before UNx.

seven days and 76 per cent at 14 to 21 days in rats. Dicker and Shirley (1971) report a 77 per cent increase at two weeks in rats.

GFR has increased equally in superficial and juxtamedullary glomeruli in dogs ten days after UNx (Carrière, 1978) and in rats two to four weeks after UNx (Hayslett et al., 1968).

A prime function of the kidney is maintenance of body fluid homeostasis, and one aspect of this is regulation of sodium ion concentration. Renal sodium handling has been of special interest in relation to mediation of compensatory renal growth because selective sodium reabsorption constitutes most of the energy-requiring work of the kidney. The idea that an increase in work acts as a stimulus for kidney growth (the "work hypothesis") will be discussed below in the section on mediation of compensatory renal growth.

There are five reports on renal sodium handling during the 24 hours after UNx in rats, expressing results variously as rate of sodium reabsorption, fractional reabsorption, or rate of sodium excretion. In all except one (Katz, 1969, an abstract in which no relevant information is given), the extra-cellular fluid volume was expanded by water or saline loading. The results are given in Table 7.

From these data, it is not possible to conclude whether or not sodium reabsorption changes immediately after UNx; there is agreement of an increase of about 10 per cent by three hours, and

Table 7. Renal handling of sodium in the rat during the first day after uninephrectomy

# of hours after UNx	Change in Na Handling	Reference
0-3	T_{Na}: no change	Katz, 1969
1-3	T_{Na}: + 11% average	Potter et al., 1969
	*F.R.: -1.1%	
3	Na excretion: -33% (per whole animal)	Peters, 1963
5	**T_{Na}: +10%	Potter et al., 1968
18	T_{Na}: + 29%	Potter et al., 1969
	F.R.: no change	
24	F.R.: no change with water load	Dicker and Shirley, 1971
	+0.2% with saline load	

* F.R. = fractional reabsorption of sodium (proportion of filtered sodium reabsorbed) of UNx compared to control

** T_{Na} = rate of sodium reabsorption of UNx compared to control

further increases with time. There is either no change or a small decrease reported in fractional reabsorption. Sodium excretion per whole animal is decreased at three hours - but increased if only one kidney is considered.

Just as with respect to renal blood flow and glomerular filtration rate, differing values of sodium reabsorption during the first 24 hours after UNx are reported for the rat. No firm conclusion may be made on the immediate effect of UNx on any of these parameters. It is possible that the small size of the rat precludes accurate measurements with the methods presently available. A larger animal, the human kidney donor for example, might yield valuable information. Also, advances in understanding of cardiovascular and renal physiology will allow better interpretation of results.

C. Mediation

I Introduction

Perhaps the most intriguing aspect of compensatory renal growth is its mediation. There are two aspects of the problem, neither of which is yet resolved: (a) what is being monitored - a change in functional demand on the remaining kidney, or a deficit in kidney tissue per se; and (b) how is the monitored change translated into a growth response? I will discuss each of these questions, giving theoretical considerations and experimental results from the literature tending to prove or disprove each hypothesis, and where possible, outlining an untried experimental approach that would be appropriate. The complexity of the phenomenon of compensatory renal growth - with its multitude of changes in physiology, molecular and cell biology, biochemistry and morphology at the cell and tissue level - does not necessarily imply a complex mediation. And yet, the elusive nature of the stimulus tends to make one assume that no single factor mediates the response: Occam's Razor must be applied so that hypotheses are not multiplied unnecessarily.

II Monitoring of nephron loss

a. By functional demand

That the functional mass of an organ should be monitored by the functional demand upon it is a logical idea. This efficient feedback arrangement is known to control both skeletal and cardiac muscle size and the mass of the erythron, for example. In the healthy, unobese animal, the proportion of kidney mass to body mass is remarkably constant, and (in dogs) is about three times more than that required for survival (Peters, 1978). So it appears that the functional capacity of the kidney is far in excess of its normal load. If functional demand is invoked as a controller of kidney mass in normal animals, or as a stimulus for compensatory growth after removal of one kidney, then the assumption is made that capacity is larger than load by a constant proportion, and therefore changes concomitantly with it.

In order to test the functional demand hypothesis, it is necessary first to identify the kidney's functions. Some require the expenditure of energy (work) by kidney cells, while others do not; consideration of functional demand therefore differs from that of work in this respect, since the former is a broader term. As an example of the dissociation of work and function, attention may be called to the role of the renal (Malpighian) corpuscle. This functions to prepare the glomerular filtrate, but the energy required is derived from the work of the left ventricle of the heart, acting

by way of arterial blood pressure.

Renal functions may be classed as follows: (a) elimination of certain soluble, non-volatile substances - the excretory role of the kidney; (b) maintenance of homeostasis of body fluids, including volume, osmotic pressure, electrolyte concentration and pH; (c) secretion into the blood of regulatory substances - the known ones being renin and renal erythropoietic factor. Of all these functions, the reabsorption of sodium accounts for most of the energy used by the kidney (Kiil, et al., 1961; Thurau, 1964; Johnson and Knudsen, 1965). For this reason, sodium reabsorption has received much attention as a possible controlling factor in compensatory renal growth. Still, if functional demand, not just workload, is to be considered, a number of other kidney functions must be investigated. I know of no theoretical reason why ability to monitor an energy-requiring function would have constituted an evolutionary selective advantage over one equally necessary to life but not using energy; to rationalize the biased opinion that sodium reabsorption is being monitored, one must hypothesize that the mechanism for translating the change in reabsorption into a growth response includes energy-production changes as an integral part. The property of energy-requirement is not known to be necessary in control of growth-stimulation systems. And no matter what function is being monitored, it will, by definition, have some unique property, viz., exactly that property which is being monitored.

b. By mass deficit

Although there are a number of general hypotheses extant which implicate tissue-mass-monitoring in a feedback growth-control system, no example has been definitively proven. These hypotheses, which invoke tissue-specific mitotic stimulators (growth factors) or inhibitors (chalones), will be discussed below in the section on circulating substances. Mass deficit may also be monitored by neural or hemodynamic mechanisms, or by changes in kidney-related blood constituents (not themselves growth stimulators), as will be discussed in the next sections.

c. Experimental data

Experiments designed to discriminate between the monitoring of mass deficit and that of functional demand would involve compromising one variable but not the other. For example, the kidney mass could be halved by UNx, but the missing kidney's function retained, by the use of hemodialysis or peritoneal dialysis. An experiment of this type has not been reported; indeed it would be difficult to accomplish because the amount of renal function effected by dialysis would have to be regulated to equal that of the missing kidney. Also, substances elaborated by the missing kidney, such as renal erythropoietic factor and renin, would be present in smaller than normal blood concentrations.

Alternatively, the normal amount of kidney tissue could be left in the body, but the function of half the nephrons disallowed. This

has been attempted (a) by unilaterally reducing glomerular filtration rate to a point where no urine is formed, or (b) by removing one kidney, mincing and returning the tissue to a non-renal site in the animal or (c) by diverting the urine from one ureter back into the blood.

In order to reduce GFR, experimenters have used partial clamping of the renal artery. Diminution of blood flow will result in complex physiological reactions both in the partially clamped kidney by autoregulation and for the whole animal by the renin-angiotensin system and the autonomic nervous system. These adjustments will differ from those resulting from UNx, since the partially clamped kidney will produce larger amounts of renin and changes in afferent activity via the renal nerves. Such animal preparations, originated by Goldblatt et al. (1934), have been used to investigate the etiology of human essential hypertension.

The second method of compromising kidney function while maintaining renal mass, implanting minced or trypsin-dissociated tissue, is far less satisfactory: many cells are damaged and killed in the process, and many others become necrotic before a blood supply can establish itself (unpublished observations by Roberts, Hansen and Hyam). When the minced tissue is injected into the peritoneal cavity, the resulting peritonitis has deleterious effects on the health of the animal. The amount of healthy kidney tissue is less than before the procedure, thus obviating the reason for the experiment.

There are four reported experiments of this nature, all using rats. Breuhaus and McJunkin (1932) measured number of mitoses in a transverse kidney section 14 days after UNx and intraperitoneal injection of crushed kidney. Between 2.4 and 4.5 gm. of kidney from donor animals was injected into rats weighing 100 to 150 gm., i.e., the injected tissue was about five to ten times the weight of the removed kidney. The uninjected rats had an average mitotic index of ten mitoses/section, and the injected had 30/section. No raw data or statistical treatments are presented. The very low values are due to the lack of use of a mitotic inhibitor to accumulate mitoses, and to the timing - two weeks post-UNx, when the mitotic response is essentially over. The infrequency of the event being assayed and lack of statistical analyses do not allow any conclusion, and the discrepancy between removed and injected weight of tissue and the late timing of the assay make it an unsuitable experimental design for the purpose being discussed here.

Williams (1962) spread scissor-minced tissue from donor animals, equal to the weight of the removed kidney of the recipient, in the tissue spaces of the anterior abdominal wall of rats at the time of UNx. Mitotic counts were made at eight-hour intervals for 40 hours. The same procedure was carried out using liver or spleen tissue for implantation, but unfortunately no uninjected controls were done. Mitotic index was lowest for the kidney-injected groups at all times, as was the food intake except at 40 hours. A relationship between poor nutrition and inhibition of mitosis may be inferred.

Goss (1963) gave intraperitoneal injections of viable cells, dissociated by trypsin, in Hank's balanced salt solution (BSS) to rats 30 hours after UNx. Cells from one to two gm. of kidney were used for 100 to 150 gm. rats, about 1.3 to 4 times the amount removed at UNx. Mitotic indices were determined at 48 hours after UNx: kidney-injected rats' mitotic index was about 40 per cent of Hank's BSS-injected animals and 50 per cent of uninjected ones. No data on food intake or body weight change is given, and no statistical analysis was done.

Dicker et al. (1976) reported that intradermal placement of finely chopped kidney cortex cells inhibited contralateral kidney mass increase after UNx in rats. The report is in abstract form, so no data are given.

No conclusion may be made from these reports for the reasons stated above. It should be noted that the device of injecting homogenates (rather than whole-cell preparations) of excised kidney has been used to investigate the existence of both stimulatory and inhibitory factors, and these reports are reviewed in the section below on tissue-specific growth factors.

The last method of compromising kidney function while maintaining renal mass is to divert urine from one kidney back into the blood. This is the only method in which the kidney remains in situ, and the normal blood circulation continues so that the functions of renal erythropoietic factor and renin production are not eliminated.

However, there are methodological short-comings in these experiments.

The earliest attempts at ureteral diversion involved implantation into the gut. In working out this technique, Bollman and Mann (1927) investigated the effect in dogs of bilateral implantation of ureters to four levels of gut - jejunum, ileum, colon and rectum - in order to determine the fate of nitrogenous urine constituents. In all animals, blood urea nitrogen (B.U.N.) rose during the first two days. It fell slightly, but then death ensued by two weeks in the high gut (jejunum and ileum) groups; B.U.N. returned to normal in the colon and rectum groups by two months. Blood creatinine was normal in all groups at all times, and was therefore being excreted via the gut. More reabsorption of urea was occurring when the urine entered the gut at the level of the ileum or above than below; colon and rectum implantation allowed sufficient urea excretion for normal B.U.N. levels. These same authors (Bollman and Mann, 1935) also studied unilateral ureter implantation in the duodenum, an even higher level in gut than those previously studied. In these dogs, B.U.N. rose by day two but was normal again by day four. Since the authors had shown with their bilateral implant preparation that urea was certainly being reabsorbed at this gut level, they assumed that the contralateral kidney was able to excrete twice as much urea as normal. Fortner and Kiefer (1948) confirmed these findings, again using dogs. The presence of moderate to severe hydronephrosis due to occlusion of the implanted ureter was reported by all these investigators.

Block et al. (1953) compared the growth of contralateral kidney in rats after UNx or unilateral ureteroduodenostomy (UUD). They used only five rats per group; their body weights ranged from 170 to 400 gms. at operation, and there was wide variation among body weight change at death three weeks later, including some rats in both groups that lost weight. Expressing contralateral kidney weight as a proportion of body weight in an effort to compensate for these variations, they found an average increase of 19 per cent in the remaining UNx kidneys and an increase of 13 per cent in both UUD kidneys, compared to the average weight of the kidneys removed at UNx (percentage values calculated from the reported raw data). The increase in the UUD group was calculated on the basis of both kidneys, since any compensatory growth would manifest itself equally in both kidneys. With the possibility that hydronephrosis was present (known from previous experiments) and had contributed to the UUD kidney weight gain, it is impossible to conclude from these results whether or not compensatory growth occurred due to an increased excretory demand. Hydronephrosis might cause kidney weight gain both from increased water content and from compensatory growth in healthy tissue interspersed with damaged areas.

No information is given in any of these UUD experiments that would indicate the general state of health of the animals. It is possible that urine could inflame the gut and inhibit absorption of nutrients. Since deficiency in protein intake inhibits compensatory growth, this factor is important.

Another method of urine diversion is to allow the cut ureter to drain into the peritoneal cavity. Using this procedure in rats, no increase in contralateral kidney has been found in mitotic index (Goss and Rankin, 1960), uptake of P^{32} into DNA (Simpson, 1961), kidney weight or activity of Na-K ATPase (Fanestil, 1968). Ewald and Mason (1963) and Mason and Ewald (1965) reported the common occurrence of hydronephrosis and loss of body weight using this technique. Royce (1963) suspected that peritonitis was occurring after ureteroperitoneostomy, and inhibiting any compensatory renal growth which might occur. He had noted striking peritoneal inflammation and concomitant loss of body weight in such circumstances. He tested his theory by giving intraperitoneal injections of talc, known to cause inflammation, and determining its effect on body weight and on renal growth after UNx. He found that inflammation abolished kidney and body weight gain and increase in P^{32} intake into DNA. Starvation gave the same results as talc administration, so Royce concluded that the growth inhibition was due to a reduced protein intake, which was due to poor appetite, which was due to peritonitis. In light of these findings, absence of compensatory growth may not be attributed to lack of stimulation by functional demand, since poor nutrition consequent to an inevitable peritonitis may have abolished the response.

To circumvent this difficulty, Morris (1974, 1976) utilized unilateral uretero-caval anastomosis (UUC) in rats. He measured various parameters six weeks after sham, UNx or UUC operation. The

UUC animals gained only about 75 per cent as much body weight as the other groups. Since the UUC animals' food intake was not monitored, it can only be surmised that it was reduced. If dry kidney weights expressed as a proportion of body weight are compared with sham weights, it is found that the two kidneys together of UUC rats increased 78 per cent as much as the one remaining kidney in UNx rats. Blood creatinine and B.U.N. were significantly greater in UUC than in UNx animals. Water content of the two kidneys in UUC rats was the same, indicating the absence of hydronephrosis. This experiment, in my opinion the best-designed of those reviewed here, indicates that compensatory growth occurs after uretero-caval anastomosis, but to a lesser extent than after UNx. However, it is possible that under-nutrition inhibited the growth; a similarly-designed experiment incorporating pair-feeding, to equalize the nutrition of control and experimental animals, would clear up this point.

Kiil and Bugge-Asperheim, 1968, and Bugge-Asperheim and Kiil, 1968, reported determinations of functional parameters in dogs after ureteroperitoneostomy, but their results must be considered with the reservations stated above relating to the deleterious effects of peritonitis.

Conclusions from these types of experiments relating to the differentiation between functional demand and mass deficit hypotheses must await reliable assays of GFR and sodium handling. Such measurements will indicate whether the growth is mediated in the

same way as after UNx.

d. General considerations

The characteristic of the kidney that is being monitored is not necessarily identical with the type of growth response elicited or, the characteristic that is being monitored to turn off the growth response may differ from that which turns it on. The economy of the body, however, leads one to hypothesize a feedback system in which the stimulus to grow and to stop growing have the same derivation. According to this idea, the functional demand hypothesis is favoured, since, as we have seen above, the original renal mass is not completely restored, while function is.

Johnson and Amendola (1969) have made an interesting point about the nature of the earliest cellular changes after UNx: if functional demand were being monitored, the cells should react by increasing the functional capacity of their cytoplasm by hypertrophy. If loss of tissue mass were being monitored, the cells should react by dividing (as well as by enlargement). The relevant question is whether the early increases in RNA and protein synthesis are directed toward producing structural proteins and synthetic enzymes required for cell division, or toward respiratory enzymes and mitochondrial membrane for increased energy production (assuming that the functional demand is an energy-requiring one). These authors determined numbers of mitochondria in mouse proximal tubule cell sections at two-day intervals after UNx. The percentage increase in mitochondria

was similar to the percentage increase in dry kidney weight, and much higher than increase in cell number. They point out that mitochondrial proliferation is a generalized response of proximal tubular epithelium, while mitosis occurs only in less than one in 100 of these same cells at the peak time of hyperplasia. They postulate that:

"1. Immediately after contralateral nephrectomy, there is an increased functional load placed upon the remaining kidney.

2. The increased work load somehow signals all functional cells to enlarge the capacity of their cytoplasm to do work.

3. The functional cells respond by cytoplasmic growth or hypertrophy, including mitochondrial proliferation.

4. As all functional cells become hypertrophied, eventually the largest reach a size which gives an inefficient ratio of nuclear to cytoplasmic mass. This then triggers the sequence of events leading to cell division in those few cells at the upper end of the size distribution."

The authors cite examples of the "critical mass" stimulus to cell division in lymphocytes and in cultured cells. This idea is also considered by Malt and Miller (1967).

Evolution of the capacity for compensatory renal growth required that it bestowed a selective survival advantage, or at least was not detrimental to survival. Among causes of kidney destruction in mammals would have been infections and trauma. Trauma is likely to be unilateral, leaving damaged tissue on that side. Infection could be either one- or two-sided and most likely segmental, but also leaving non-functional tissue behind. Considering the circumstances under which the capacity for compensatory renal growth must have evolved, unilateral nehprectomy is quite an artifical situation:

no damaged, non-functioning tissue is left behind, and that tissue which does remain is an entire, healthy organ. This consideration tends to support a functional demand hypothesis for growth mediation, rather than one based on monitoring kidney mass.

Since survival during childhood and until reproductive maturity constituted the advantage for evolutionary selection, it is not surprising that the capability for compensatory renal growth is compromised during puberty since its continuation after that would not have afforded significantly more advantage.

As will be apparent in the following discussion of the possible means of translating kidney loss to a growth response, many of the mechanisms could be monitoring either functional demand or mass deficit, or even both simultaneously; therefore the separation of these two aspects of the loss is somewhat artificial.

III Translation of nephron loss to a growth response

a. By a neural mechanism

The possibility exists that the nervous system plays a role in the mediation of compensatory renal growth. It could be the primary stimulus, or it could be acting to mediate some other stimulus.

The nervous system could be postulated to monitor either a deficit in kidney mass, or a change in functional demand on the remaining organ. In the first instance, severence of the renal nerves by UNx would interrupt afferent impulses from the removed kidney; feedback would be possible via efferent autonomic nerves to its remaining mate. Branches of the autonomic nerves have been shown to innervate the proximal tubular epithelium (Norvell et al., 1970).

If the nervous system is postulated to monitor functional demand on the remaining kidney after UNx, then the feedback would have to loop back on that same kidney. The feedback loop might involve alterations in renal blood flow caused by autonomic, principally sympathetic, efferents. In this case, the nervous system would constitute a necessary but not sufficient cause, and its possible enabling role should be kept in mind while considering the sections on monitoring by hemodynamic and renal work changes.

Despite these interesting speculations, there is evidence from

experiments using allografted kidneys that innervation is unnecessary for normal compensatory growth. Norvell et al. (1970) determined that total denervation of kidney does not result from stripping the nerves and adventitia from the renal vein and artery and the ureter; severance of the vessels is necessary since some nerves lie in the vessel walls. They found that kidney auto-transplantation in dogs results in nerve degeneration followed by regeneration beginning at about 12 weeks (Norvell et al., 1969). These same authors (Norvell et al., 1970) examined human kidneys removed at various times after homotransplantation. All kidneys were removed because of immunological rejection, and therefore can not be considered healthy organs. The only kidney in which nerve regeneration was found was removed five years after surgery; the other four ranged from 18 days to 40 months. In this same report, unpublished data was quoted in which nerve regeneration was found as early as 27 days after human kidney transplantation. These patients had died from complications, and the kidney tissue was obtained at autopsy.

Although there are no investigations of kidney nerve regeneration in healthy transplanted kidneys, the available data, and knowledge of nerve regeneration in general, indicate that the process is far too slow for the nervous system to play any role at all in compensatory renal growth.

Further evidence is supplied by experiments showing that compensatory growth does in fact occur in transplanted kidneys.

Klein and Gittes (1973), using adult, male Lewis rats, transplanted one kidney into each bilaterally nephrectomized animal. Five to seven weeks later, the weight increases of the transplanted kidneys were compared to increases in single kidneys remaining after UNx. (The donors' contralateral kidneys and the organs removed at UNx provided the baseline weights.) The groups showed a similar increase: 63 per cent after UNx, and 68 per cent after transplantation.

Mitchell et al. (1974), also using adult male Lewis rats, transplanted a kidney into rats having less than one-fourth the normal amount of kidney tissue - one kidney having been removed and two-thirds of the other infarcted by vessel ligation four months previously. Three months after transplantation, these kidneys were found to have increased in length by nine per cent and in thickness by 16 per cent.

Studies of renal function in human transplanted kidneys show two things: (a) that an isografted kidney (from an identical twin donor) functions the same as its mate left in the donor in all respects tested, except in handling of extracellular fluid volume expansion (Bricker et al., 1956); (b) that isografted kidneys show the same increases in GFR and RBF as their mates at one week and up to six months after surgery. (Flanigan et al., 1968). Allografted kidneys functioned somewhat less well in these respects than their mates, which is thought to be due to immunologic rejection. Since increase in these functional parameters correlates with compensatory renal growth in humans (Donadio et al., 1967), we may conclude that

healthy transplanted kidneys undergo growth comparable to lone kidneys *in situ*. Transplanted kidneys also seem to function normally, with the exception noted, indicating that most vascular reactions of the kidney to various stimuli are not compromised by denervation.

b. By hemodynamic changes

Renal hemodynamic changes may be hypothesized either to monitor kidney mass deficit directly, or to participate in the mediation of compensatory growth whatever the prime stimulus. There is no doubt that renal blood flow increases in parallel with the growth of the kidney, but the question of whether there is an increase which precedes (and possibly mediates) the growth has not been resolved. Interpretation of experiments designed to investigate these possibilities has depended very much on temporal relationships, and since the first evidence of a growth response is seen within a few minutes after UNx, immediate function changes have been looked for. The best evidence for an immediate change relates to increase in renal blood flow - but the timing of even this response is uncertain due to differences in reported experimental results. Experiments relating to renal blood flow changes after UNx were reviewed, and some theoretical considerations discussed, in the preceeding section on description of the phenomenon of compensatory renal growth.

If renal blood flow changes are to monitor kidney mass deficit directly, then the 33 per cent increase in flow which results from contralateral vessel ligature (calculated as discussed in the RBF review section) must result in a chain of events leading to kidney growth. The renal and systemic physiological accommodation to changes in renal blood flow are so complex that an hypothesis can not be formed without good experimental data describing at least some of these physiological changes. Unfortunately, these data are conflicting or incomplete. One needs to know the temporal relationship of changes in renin-aldosterone and vasopressin blood levels; RBF, GFR and systemic blood pressure; sodium and water excretion; extracellular fluid volume. If the interrelationships of these parameters were known, then the role of renal blood flow changes could be postulated, and experiments designed rationally in order to test the hypothesis. The experiments which have been done constitute attempts to manipulate renal blood flow in order to investigate its effects on kidney size.

Reduction of flow by several means has been shown to compromise kidney growth after UNx. Goss and Rankin (1960) removed about one-third of rats' blood volumes at 30 hours after UNx. At 48 hours, the mitotic indices of bled UNx rats were only 2.6 times control values, while rats without blood loss showed a 6.1-fold increase. Interpretation of these results is complicated by the complexity of the renal response to haemorrhage of this extent: not only will renal blood flow be decreased, but conservation of sodium and water will

be effected by aldosterone and ADH.

Another way to reduce renal blood flow is by partial constriction of the renal artery, but this leads, as has been seen, to complex physiological changes affecting many systems, resulting in secretion of renin and prolonged hypertension, as was shown first by Goldblatt et al. (1934).

c. By a change in workload

Selective sodium reabsorption constitutes most of the energy-requiring work of the kidney (Kiil et al., 1961; Thurau, 1964; Johnson and Knudsen, 1965). If removal of one kidney is going to result in an increased workload for the remaining kidney, the increase can be measured in terms of sodium reabsorption. The idea that an increase in work acts as a stimulus to compensatory renal growth (the "work hypothesis") has been reviewed by Johnson (1969). The hypothesis necessarily implies that sodium reabsorption increases immediately after UNx. However, theoretical considerations and experimental evidence given in the section on the phenomenon of compensatory renal growth indicate that sodium reabsorption decreases immediately after UNx; both water and sodium excretion double in the remaining kidney (see, for example, Peters, 1978). Sodium reabsorption does not rise above pre-UNx rates until several days, and then as a result of an elevated GFR. (I do not wish to imply that the questions of the timing of these various changes in function are settled; I am presenting one currently-accepted viewpoint which constitutes a working hypothesis).

A theoretical background for these observed changes follows: In the human with a normal amount of dietary salt - e.g., 3.5 gm. per day - and a normal glomerular filtration rate of 120 ml./minute, 99.75 per cent of sodium in the filtrate will be reabsorbed. Values for rats eating standard laboratory diets are similar: 99.8 per cent reabsorption is reported by Dicker and Shirley (1971).

If diet remains constant, removal of one kidney should mean that the remaining one must excrete twice as much sodium, since a balance must be maintained between the absolute amounts of sodium ingested and excreted. If the GFR remains the same, the tubules of the lone kidney will then be presented with the same amount of filtered sodium as before UNx, and in order to excrete twice as much as before, reabsorption will be reduced by half of the total amount excreted. For example, if each kidney in the intact animal is presented with 500 sodium ions/unit time, 499 are reabsorbed and one is excreted per kidney/unit time - giving a reabsorption rate of 99.8 per cent. After UNx, the remaining kidney is still presented with 500 sodium ions, but now must excrete by itself two ions/unit time, and thus reabsorb only 498, or 99.6 per cent.

The GFR must increase only by 1/500 (0.2 per cent) in order to cause sodium reabsorption to return to its pre-UNx value of 99.8 per cent. Any greater increase in GFR will result in the necessity for the lone kidney to do more work than normal reabsorbing sodium.

The effect of UNx on renal blood flow and GFR have not been

clearly discernable. Determination of sodium reabsorption in rats is made difficult by the large inaccuracies in measuring excreted urine volume relative to the small changes in sodium concentrations expected.

In my experience, rats do not begin to eat until at least 24 hours after surgery, and their drinking habits are disturbed as well. This change in amount of dietary salt and water intake will certainly affect rates of sodium reabsorption and excretion.

The basis for the formulation of the work hypothesis was that there is an increased workload for the remaining kidney after UNx. The finding that workload is decreased may seem paradoxical, but there is no reason why a decrease in renal work for sodium reabsorption should not be considered as a growth stimulus, even though the short-term response to decreased workload might be expected to be hypotrophy rather than the opposite. The situation is by no means so simple, however. Transplantation of a third functioning kidney in the rat does not result in hypertrophy (Silber and Malvin, 1974), so the kidneys were not fooled by this manoeuvre into responding to decreased workload by growing. Also, high intake of dietary sodium by intact rats should decrease renal sodium reabsorption; it has been reported to increase kidney size (Fregly, 1960; Hall and Hall, 1966) and mitotic index (Goss and Rankin, 1960), or to have no effect on kidney weight or DNA content (Halliburton, 1969; deMuchnik, 1974).

Conversely, the effect on kidney weight of increasing sodium

reabsorption in intact animals has been investigated. Adult male Sprague-Dawley rats maintained on sodium-free diets for up to fifty days showed a decreased body weight gain compared with rats on a normal diet (Hsueh and Rostorfer, 1974). Uninephrectomy in the low-sodium group resulted in lower kidney mass increase than controls, but when the kidney weights were expressed as a percentage of body weight, the compensatory growth did not differ from controls. The effects of sodium-free diet would be similar to those of Addison's disease: loss of body water and loss of appetite result in loss of body weight. These general effects obscure interpretation of the experimental results: the investigators did not assay percentage of water in kidney tissue or measure food intake. Because of these side-effects, this experimental design probably can not be successfully used for evaluating the influence of sodium reabsorption on compensatory renal growth.

Another experimental method of increasing sodium reabsorption is to raise it secondarily to increased glomerular filtration rate. However, the methods that have been used to raise GFR have more generalized effects on the cardiovascular system and the kidney. Neither methylprednisone (deBermudez and Hayslett, 1972) nor unilateral ureteroperitoneostomy (Weinman et al., 1973) caused an increase in kidney mass subsequent to increased GFR and sodium reabsorption, but the other effects of these treatments make interpretation of the results impossible. Daily hyposodic peritoneal dialysis of rats for two weeks resulted in an increase in sodium

reabsorption but no change in kidney weight compared with controls dialyzed with normosodic solutions (deMuchnik, 1974).

Even though there is an increasing tendency to view elevated GFR and sodium reabsorption as effects of compensatory growth rather than its cause (Katz et al., 1978; Peters et al., 1978) the view is not proven, and in any case, investigation of these phenomena is interesting and valuable for a better understanding of renal physiology. Also, a great deal of emphasis has been placed on identifying a primary stimulus for compensatory growth, but the stimulus may in fact consist of a series of stimuli operating over the days following UNx when growth is occurring. In fact, there is evidence that the growth is reversible: interruption of cross-circulation after 48 hours in experiments in which one partner has received a bilateral nephrectomy reverses the kidney growth in the intact partner (Dijkhuis et al., 1975). Transplantation of a kidney into an animal in whom a previous UNx has caused kidney growth will reverse the growth (Silber and Malvin, 1974).

It must be noted, however, that Klein and Gittes (1973) found that kidneys transplanted into rats after UNx hypotrophied, while the endogenous kidneys hypertrophied. Their surgical protocol meant that transplanted kidneys experienced cold ischaemia for over two hours, which may have damaged these organs. Silber and Malvin (1974) do not give information relating to their handling of transplanted kidneys.

Renal handling of sodium is controlled by the glomerular filtration rate, by aldosterone and by a hypothetical "third factor"; good discussions of sodium excretion regulation are found in Bricker, 1967; Davis and Knox, 1970; and Mercer et al., 1974.

The increases in sodium and water excretion which result from iso-oncotic extracellular fluid volume (ECFV) expansion may not be explicable without postulating the existence of a natriuretic hormone (third factor) (deWardener, 1977). There are many experimental and theoretical similarities between the physiological adaptations which occur after UNx and ECFV expansion, and it is tempting to postulate a role in mediation of compensatory renal growth for the postulated natriuretic hormone. Lindheimer et al. (1967) demonstrated in dogs that acute increase in GFR has little effect on sodium excretion unless the ECFV is expanded. With a normal fluid volume, there is constant fractional reabsorption of sodium in the proximal tubule, and increased reabsorption in the distal segment. Proximal tubule reabsorption is inhibited in states of ECFV expansion; the mechanism is postulated to be via a humoral natriuretic hormone, called third factor.

Bricker (1967) has proposed that the increase in sodium excretion seen after UNx is another example of the effect of third factor. Experimental support of this hypothesis derives from work by Coe et al. (1968), Guignard and Dirks (1968), Wong and Dirks (1971) and Diezi and Michoud (1974), who showed that natriuresis occurs after UNx in spite of unchanged GFR, decrease in arterial blood

pressure by aortic constriction, or the presence of large amounts of endogenous aldosterone.

d. By a change in a circulating substance

Relevant circulating substances are of two kinds: (a) kidney-related substances, themselves not directly effecting growth, but monitoring kidney loss by virtue of their being added to or removed from the circulation by the kidney (b) kidney tissue-specific growth substances. These circulating substances may act directly or indirectly, as mitotic or cytoplasmic growth stimulators or inhibitors. They may be effective by change in blood concentration, or by change in rate of flow through kidney tissue, secondary to change in renal blood flow. (In the latter case, the factor itself would not monitor loss of kidney, but would mediate the effect of increased renal blood flow.) Kidney-related substances would monitor functional demand, while kidney-specific ones would monitor deficit in tissue mass.

Any substance whose blood concentration is determined, whether by addition or subtraction, by the function of the kidneys fits the qualifications for the category of kidney-related substances. These substances have functions not directly related to kidney size regulation; their role in growth control would be an "opportunistic" one. These substances' primary functions are not kidney-related: their blood levels are regulated primarily in relation to function. The resulting fluctuation in blood levels imposes a large reservation on their acceptability as kidney growth regulators.

Substances known to be elaborated by kidney cells into the blood are renal erythropoietic factor and renin; other substances are those filtered by the renal corpuscle and subsequently reabsorbed back into the blood. Removal of one kidney would decrease the amounts of these in blood, so a negative-feedback situation must be postulated if they were to act (indirectly) as growth stimulators, or positive-feedback if they were inhibitors. On the other hand, substances removed from the blood by the kidney, such as urea, would exhibit the opposite feedback and direction of action properties.

Historically, urea excretion was first considered as a candidate for the regulatory function of kidney size. It used to be thought (see Oliver, 1924, for example) that urea excretion required energy expenditure by the kidney; it is now known that this process is a passive one requiring no work by the kidney, at least in rats and humans (Johnson and Knudsen, 1965; Ganong, 1977b). The heart does the work required for urea excretion by causing blood flow through the kidneys, with a blood hydrostatic pressure high enough to form the glomerular filtrate.

High dietary intake of urea does increase kidney size (MacKay et al., 1931; Allen and Mann, 1935, and reviewed by Katz et al., 1978). However, the extent and character of the growth are dissimilar to that following UNx (Halliburton, 1969); growth is due to cellular hypertrophy alone, and the mass increase is about a third of that produced by UNx. Also, in order to produce any kidney mass increase,

blood urea nitrogen levels must be raised considerably higher than those observed after UNx (Diezi, 1973).

High dietary intake of protein also causes kidney growth (MacKay et al., 1928; Halliburton, 1969) and enhances compensatory growth following UNx (Smith and Moise, 1927; Allen and Mann, 1935). The characteristics of the kidney growth are similar to those following urea feeding (Halliburton, 1969), including the absence of hyperplasia (Halliburton and Thomson, 1965a and b). The major metabolic end product of protein metabolism is urea, so it is probable that the same mechanism is effecting kidney growth in both cases.

Cold acclimitization results in kidney growth in rats (Reiter, 1966; Holeckova and Baudysova, 1975) and hamsters (Reiter, 1968). Since non-hibernating mammals have an increased basal metabolic rate and food intake during cold exposure, the renal growth might well be a result of the increased dietary protein. Experiments could be done to clarify this point in which protein intake is controlled, or in which hibernating animals are compared with those who do not hibernate.

Even though growth resulting from high protein or urea intake or cold acclimitization is dissimilar to that following UNx, its mediation is of interest. A high urea concentration in glomerular filtrate results in an osmotic diuresis, and in some unknown way increases the ability of the kidney to concentrate non-urea solutes. Sodium reabsorption in the proximal tubule and loop of Henle is inhibited by changes in concentration gradients due to increases in

tubular fluid volume and medullary blood flow (Ganong, 1977b). These changes may mimic those caused by UNx and thus cause a growth response. Careful study of the hemodynamic and sodium-handling changes induced by osmotic diuresis may in fact provide clues to the stimulus for compensatory growth.

The kidney removes a large variety of other unwanted substances from the blood, the concentrations of which depend on changes in diet and metabolism. Experimental evidence is lacking and theoretical considerations make it unlikely that they play a role in monitoring kidney loss.

Substances added to the blood by the kidney include those synthesized by specialized kidney cells, and those reabsorbed from the glomerular filtrate. Sodium, an example of the latter type, was considered separately in a preceding section. The arguments concerning the possible role of sodium in kidney growth may be generalized to include any substance reabsorbed by the tubular epithelium, whether or not its handling requires energy.

Substances synthesized by specialized kidney cells and secreted into the blood which have been considered as constituting possible feedback growth control systems are renin and renal erythropoietic factor (REF). There is no good experimental evidence that either of these is implicated: wide fluctuations of either one due to various causes are not known to result in kidney growth. Relatively little work has been done on the possible role of either of these kidney

products in compensatory renal growth, no doubt because neither seems theoretically a very promising candidate.

Renal erythropoietic factor (REF) is thought to be an enzyme (Zanjani et al., 1967) probably produced by the podocytes of the renal corpuscle (Busuttil et al., 1971). It is postulated that the enzyme converts an inactive, liver-produced substrate to erythropoietin, which stimulates bone-marrow production of erythrocytes.

The production of REF bears a direct correlation with the oxygen content of the blood. For example, anemia, high-altitude hypoxia and kidney ischemia all result in increased production of REF. Uninephrectomy should result in decreasing blood levels of REF, and consequently of erythropoietin, after a length of time dependent on the lifespans of these molecules. Erythrocyte concentration in blood would then fall, with a lag period determined by the development time of these cells in the bone marrow. The remaining kidney would recognize the consequent decrease in oxygen content of blood and produce more REF. Mann et al. (1968) examined the relationship between renal mass and blood erythropoietin levels after sham operation or UNx in young adult, female, Sprague-Dawley rats. Seven weeks after surgery, UNx rats' total kidney weight/body weight was 68 per cent of sham. Bioassay of erythropoietin revealed that UNx rats had only 54 per cent of that in shams. And yet, the packed blood cell volumes in the two groups were the same. This discrepancy is not discussed by the authors, and is inexplicable. Determination of the effect of UNx on erythropoietin levels awaits further

experimentation. The dearth of experimental data disallows further speculation on a postulated role for REF in feedback control of kidney size.

Renin is a proteolytic enzyme secreted into the blood and lymph by the juxtaglomerular (JG) cells of the afferent arterioles. It acts on an alpha$_2$ globulin synthesized in the liver, called angiotensinogen, to produce angiotensin I. Other enzymes convert this product to angiotensin II, and then to angiotensin III. Angiotensin I is physiologically inactive; angiotensins II and III are vasoconstrictors, and act directly on the adrenal cortex to produce aldosterone. It is thought that the main function of angiotensin II is its pressor activity, while that of angiotensin III is aldosterone stimulation (Ganong, 1977a).

Secretion of renin is stimulated by sympathetic nerve impulses to the JG cells and by circulating catecholamines. Secretion is inhibited by increased stretch of JG cell walls, increased sodium or decreased potassium reabsorption across macula densa cells (which lie in the convoluted distal tubule), and increased levels of angiotensin II and vasopressin (anti-diuretic hormone).

The current understanding of the function of the renin/angiotensin system, as presented above, is discussed by Davis (1973). Since the system's role in cardiovascular homeostasis is not completely understood, and since renovascular and sodium and potassium handling changes due to UNx are not clear, it is difficult to set an

hypothesis for renin involvement in compensatory growth; cause and effect relationships among renin and renal blood flow and sodium and potassium handling are presently inscrutable. It is certain, however, that renin does play a role in the functional adaptation after UNx. Whether change in renin levels is the primary stimulus to growth, or an intermediary in the chain of events initiated by a different stimulus, can not be stated. Angiotensins, kinins and prostaglandins are all candidates for vasoactive substances at work in this circumstance, but the angiotensins are of special interest in respect to growth mediation since they are controlled by the kidney-produced renin.

Several investigators have attempted to relate renin activity and compensatory renal growth. Schaffenburg et al. (1954) reported that renin, injected at four-hour intervals between UNx and death one or two days later, inhibited kidney mass increase in young adult, female rats. Their experiment used only three rats per group, among which there was large variation. The injected renin was impure. The rats lost body weight (more in renin-treated groups than controls), and even uninjected UNx animals did not attain their preoperative body weights until at least eight days - a far longer time than is usually reported for rats. Royce (1963) has suggested that peritonitis resulting from intra-peritoneal injection of crude renin preparations might be the cause of body weight loss and lack of renal growth since these animals eat little due to loss of appetite. Because of these criticisms, Schaffenburg and his coworkers' results must be accepted

with reservation.

Hsueh and Rostorfer (1974) measured kidney renin content (activity/gm. protein) from two to fifty days after UNx in adult, male, Sprague-Dawley rats; and found no significant change. Blood levels were not measured. It is possible that in this circumstance the JG cells synthesize renin at the rate at which it is secreted, thus keeping the cells' content unvarying. When rats were fed a high-sodium diet, the renin content dropped below normal; low-sodium diet increased renin. A shifting of the equilibrium between renin synthesis and secretion must have occurred, although some allowance must be made for variations in the renin content of the blood and lymph contained in the kidney.

No conclusion regarding a postulated role for renin in compensatory renal growth may be drawn from these reports.

As opposed to the known kidney-related substances just discussed, the existence of kidney-specific growth factors has been hypothesized. The subject of general regulation of tissue mass by tissue-specific growth factors has been well reviewed by Goss (1964 and 1972) and Simnett and Fisher (1973). The various hypotheses involve: (a) intracellular growth stimulators in equilibrium with intra- and extracellular inhibitors, both of which are produced by the cell whose growth is controlled (Weiss, 1955; Weiss and Kavanau, 1957; Kavanau, 1960); (b) circulating mitotic inhibitors (named chalones: from the Greek "to relax") produced by the same cells affected (Bullough,

1964, 1965); (c) mitotic stimulators produced by lymphocytes in response to decreased circulating levels of tissue-specific proteins (Burwell, 1963; Burch and Burwell, 1965). An outline of the major points of these hypotheses follows.

Stimulator/inhibitor equilibrium hypothesis of Weiss and Kavanau

1. Each specific cell type grows by a mechanism in which a compound unique to that cell type acts as a catalyst for growth. Such catalysts are called templates by Weiss and Kavanau. The growth rate is proportional to the intracellular concentration of these templates.

2. Each cell also produces antitemplates which inhibit the catalytic action of the templates by combining with them to form inactive complexes. The antitemplates may be produced as a by-product of cell growth, and may be steric complements to the templates. Contrary to the case of templates, the antitemplates are released from the cell and circulate in the blood.

3. When equilibrium between extracellular antitemplate and intracellular template concentrations is attained, growth will cease. The mechanism when expressed mathematically results in a sigmoid growth curve for the total mass of each cell type; and indeed this is found empirically to be true.

4. Removal of part of the mass of a cell type results in compensatory growth of the remainder because the extracellular concentration of antitemplates is reduced, thus shifting the equilibrium and reducing the intracellular antitemplate concentration and uncomplexing more templates, which will catalyze growth. The postulated antitemplates comprise the inhibitors in a negative feedback system.

The chalone hypothesis of Bullough

Reviews of experiments designed to investigate the existence of chalones affecting a variety of tissues have been written by Bard (1973), Bullough (1973), Iversen (1973) and Rytomaa (1973).

Criticisms of the chalone hypothesis have been put forward by Gelfant (1960).

The main points of the chalone hypothesis follow.

1. This hypothesis involves negative feedback inhibition of mitosis, where the inhibitors of mitosis are termed "chalones". Like Weiss' antitemplates, chalones are tissue-specific, are released into the extracellular circulation, and inhibit growth of the same cell type by which they are produced. In contrast, they are produced by all living cells, not just those which are growing.

2. Their site of action is hypothesized to be at the gene level. All mammalian cells can exhibit only two different modes of being: i.e., mitosis or functional activity. Chalones would inhibit the mitosis, thus allowing the functional activity to occur. Alternatively, the chalone could act as a stimulator for functional activity and inhibit mitotic activity secondarily.

3. Active chalone must be complexed with adrenalin, which, Bullough states, may be correlated with the diurnal mitotic rhythm observed in most tissues.

The immune system hypothesis of Burch and Burwell

Burnet (1961) suggested that "immunological recognition could be derived from an aspect of the process by which all multicellular animals succeed in maintaining a characteristic morphological and functional unity". Burch and Burwell claim that the prime function of the lymphoid tissue is to regulate differentiated tissue growth. In their view (1965): "the size of each distinctive organ and tissue during fetal development, normal growth, and maturity, is regulated - at least in part - by a feedback interaction between specific controlling elements of lymphoid tissue and the related target tissues. We regard the trophic function...of small lymphocytes as

secondary, and in a sense contingent upon their primary function. Hitherto...it has been generally assumed that the immune system carries out a purely police action - apprehending non-self constituents at both the classical antigen and homograft levels; in contrast, we suggest that the defense function can in some ways be regarded as a secondary, although inevitable, concomitant of the primary function of the immune system, which is that of self-recognition and the regulation of tissue size".

The major points of their hypothesis are:

1. Differentiated tissue cells release tissue coding factors (TCF) into the extracellular fluids, and then via the afferent lymphatics to the regional lymph nodes. These TCFs are also on the surfaces of the cells which produce them, which results in contact inhibition of these cells.

2. Certain lymphocytes bearing cell-bound receptor molecules interact with the TCFs, which promotes mitosis of these receptor cells, thus arresting their differentiation into effector small lymphocytes.

3. Effector small lymphocytes bear cell-bound mitotic control proteins (MCP) of the same specifity as the receptors in their parent cells. They pass via the efferent lymphatics to the blood, and to the target tissue.

4. The MCPs on the lymphocytes identify the TCF on the target tissue and neutralize the mitotic restraints imposed by intercellular content (contact inhibition). Therefore, the effector small lymphocyte has two functions: (1) it must recognize and interract with the correct target tissue, and (2) it must alter the cell in some way which stimulates the occurrence of mitosis.

I shall now consider experiments designed to test these hypotheses.

Experiments designed to investigate the existence of a circulating, kidney-specific growth substance fall into five categories, which are: (1) injection of homogenates of kidney tissue into animals; (2) manipulations of the immune system, coupled with UNx; (3) ectopic grafting of small pieces of kidney tissue; (4) exposure of one animal to the blood of another animal, the two having different amounts of kidney mass; and (5) exposure of *in vitro* preparations of kidney tissue to serum from animals having different amounts of kidney mass.

The rationale for injection of kidney homogenate into animals receiving uninephrectomy is that the treatment would inhibit compensatory growth if kidney cells contain chalones. The results of six such experiments using rats are given in Table 8. Inhibition of hyperplasia or hypertrophy, or both, was found in nearly all experimental designs. The one report (Roels, 1965) that the inhibition is tissue-specific is based on a small sample ($n = 7$) of rats injected with liver homogenate. No investigator compared body weights of experimental and control groups; intraperitoneal injections of other substances and of minced kidney have been shown to decrease body weight, probably by appetite depression, which results in nonspecific inhibition of compensatory renal growth. Also, kidney homogenates contain renin; since 1898 (Tigerstedt and Bergman) it has been known that such injections raise the recipient's blood pressure, and although the effect of renin on compensatory renal growth has not been adequately investigated, it may have an

Table 8 Combined effects on the contralateral kidney of rats of uninephrectomy and intraperitoneal injection of kidney homogenate

Recipients	Injection	Assay Method	Effect*	Reference
young adult, male, UNx	1-2 gm. whole kidney, 30 hrs. post-op.	**M.I. 48 hrs. post-op.	— (not tissue specific)	Goss, 1963
adult, male or female, UNx	1 kidney, -24, 0 or 18 hrs. post-op.	Kidney weight 48 hrs. post-op.	0 at 0 & 18 hrs. —at -24 hrs.	Dicker, 1971a and b
adult, male UNx	1/10 kidney, 0 or 24 hrs. post-op.	M.I. 47 hrs. post-op.	— (tissue specific)	Roels, 1965
adult, male UNx	1 kidney, 24 hrs. post-op.	M.I. 40 hrs. post-op.	— (not tissue specific)	Williams, 1962
adult, male UNx	1 whole kidney, cortex or medulla, at operation	Kidney weight, RNA, DNA, protein, GFR 1-14 days post-op.	Whole kidney or cortex: —hypertrophy, 0 GFR; medulla: 0 both hypertrophy and GFR	Dicker, 1972
(not reported) 3/4 Nx	? whole kidney, 18 hrs. post-op.	M.I. 48 hrs. post-op.	—	Steuart, 1958

* — effect = decrease in parameter measured;
0 = no effect; + = increase in parameter measured

** M.I. = Mitotic index

inhibitory effect due to its effects on renal blood flow and sodium handling (see the section on kidney-related factors).

It is known that injection of homogenates or extracts of various organs, including kidney, results in destructive inflammatory lesions specific to that organ (Roitt, 1972). This phenomenon has been described by Dicker and Morris (1974) in the rabbit and guinea pig kidney. This auto-immune process probably exerts generally detrimental effects which are systemic as well as kidney-specific.

However, the cumulative weight of evidence makes it imperative that more rigorous experiments be done along these same lines. Dicker (1972) has refined the method by isolating microsome and soluble fractions of homogenates from either renal cortex or medulla, and further (Dicker, 1976) by concentrating 500-fold by cellulose fractionation the inhibitory activity of cortex homogenate on the "growth" of renal cortex explants (but no data are given in support of this claim).

The second type of experiment designed to investigate the existence of kidney-specific growth factors involves the manipulation of the immune system.

It seems self-evident that a central control system is responsible for coordinated body growth, for the ultimate attainment of body size within the genetically-determined normal range for that species, and for the phenomenon of compensatory growth. This postulated control system must contain a large number of distinctive

information elements, and its coordination must depend on quantitative interrelationships among all the elements. The immune system is uniquely qualified for this function because it has a wide enough range of specifications; the ability to recognize "self", whose pathological expression results in auto-immune diseases; access to nearly all tissues of the body because of the wide distribution of the lymphatics and the ability of long-lived lymphocytes to penetrate freely into all lymph nodes by recirculation (Gowans, 1970) and the ability of all types of lymphocytes to penetrate into many other tissues by unknown control mechanisms.

There are observations which support a role for lymphocytes in mediation of kidney growth, although the evidence is circumstantial. Up to one per cent of cells derived from normal rat kidney, perfused and then trypsin-dissociated, are lymphocytes (Main et al., 1975). Leukocyte infiltration of the remaining kidney after UNx is said to occur (Fox, 1969 - but this observation is attributed to Albarran, 1899, who does not, in fact, describe leukocyte infiltration per se. He does state that he observed inflammatory reaction phenomena - which include polymorphonuclear leukocyte infiltration - but he was using human kidneys contralateral to kidneys destroyed by various disease processes, sometimes infectious).

Carrel (1922) postulated, and provided some preliminary experimental evidence for, a growth-promoting function of leukocytes. Main et al. (1975) confirmed Carrel's findings, using the more sophisticated

techniques of mixed culture and liquid scintillation counting: dissociated rat kidney cells stimulated DNA synthesis of peripheral blood leukocytes (at least 80 per cent lymphocytes) from the same animal.

Experiments designed to test the hypothesis of Burwell (1963) and Burch and Burwell (1965) have been of two kinds: one, in which lymphocytes are exchanged between animals having different amounts of renal tissue and two, in which the immune system is compromised and the animal has one kidney removed to see if compensatory renal growth can still occur. Techniques used to attain the latter goal include X-irradiation, neonatal thymectomy and treatment with anti-lymphocyte serum. The last two techniques are preferred since X-irradiation has many systemic effects unrelated to the immune system.

Transfer of spleen cells from mice having UNx or sham-operation to other mice having the opposite operation was carried out by Babaeva (1973) and Pliskin and Prehn (1975). Babaeva found an increased mitotic index of unoperated recipient kidney proximal tubular epithelium and liver reticulo-endothelium after injection with UNx spleen cells. Pliskin and Prehn X-irradiated their recipient mice, presumably to prevent the occurrence of immune rejection of the injected cells, although the mice used were inbred. They found increased DNA synthesis in both kidney and liver of the recipients after treatment with spleen cells from UNx mice. These results must await confirmation, including control groups of animals, before

conclusions can be drawn.

One must assume that the immune system was compromised by X-irradiation in the experiment by Pliskin and Prehn. This experimental manoeuvre was used by Wachtel and Cole (1965), Fox and Wahman (1968) and Fox (1969) in order to determine whether an intact immune system is necessary for compensatory renal growth. Wachtel and Cole X-irradiated just one kidney of rats and removed its partner; compensatory growth (DNA content and mass increase) was normal. Whole-body X-irradiation with one kidney shielded and the other excised resulted in loss of body weight and inhibition of kidney growth. Dietary restriction producing a similar loss of body weight also resulted in inhibition of kidney growth.

Fox and Wahman used a similar experimental design in mice and included groups of animals receiving isogeneic spleen cells, which apparently restored the kidney mass increase after UNx which was inhibited by X-irradiation. Unfortunately, no information regarding body weight is given, and no statistical analysis of data was done.

Neonatal thymectomy does not inhibit kidney mass increase after UNx in mice (Bump and Malt, 1969), nor does reduction of T-lymphocytes by treatment with anti-lymphocyte serum (unpublished results - Roberts, Hansen and Hyam). Neither of these methods eliminates all T-lymphocytes, so the question remains unsettled.

It is unlikely that the immune system could mediate the earliest

changes seen in the contralateral kidney after UNx since they occur almost immediately, but it is feasible that the immune system could regulate the hyperplasia component of the growth, and/or stop all growth when the remaining kidney has attained an adequate mass. The idea that the immune system plays a role in tissue morphostasis is a provocative one and is a research area needing rigorous investigation.

A technique which has been used to investigate the role of humoral factors in liver regeneration (Leong et al., 1964) is also a suitable one for study of compensatory renal growth: ectopic grafts are made of small pieces of excised tissue; cell division in the graft is then compared with that in the tissue remaining in situ and with that in autografts placed in control, intact animals. This experiment using kidney tissue has not been reported. The technique is feasible; it has been used for other experimental purposes. Simnett et al. (1977) placed ectopic kidney grafts in the frog lung, Muirhead et al. (1960) grafted kidney in the dog lung and peritoneum, and Dicker et al. (1976) used intradermal kidney grafts in rats. Liver regeneration researchers have also done successful grafting at various sites in mammals (Bucher and Malt, 1971). This type of experiment, with suitable controls, would give substantial evidence for or against the existence of a humoral kidney-specific mitotic factor.

Exchange of blood between two animals having different amounts of renal mass will test the hypothesis that compensatory renal growth is mediated by substances carried in the circulation. It must be

pointed out that this general experimental design, depending on the way in which the exchange is effected, may not allow distinction among kidney-specific growth factors, kidney-related mediators, increased functional demand, increased workload, and hemodynamic changes. This type of experiment is discussed in this section only because it has most often been used, rightly or wrongly, as a test for kidney-specific growth factors.

There are three versions of this experiment: serum injection, parabiosis, and cross-circulation of two or more animals. The injection of serum from a donor having uninephrectomy to an intact recipient, or vice-versa, will test for a stimulation in the former and inhibition in the latter. This method, however, is probably not very effective, since the exposure of the recipient to the donor's blood is intermittent and relatively small. A side-effect of intravenous serum injection is hemodilution and expansion of extracellular fluid volume. It is possible that intra-peritoneal injections may result in peritonitis, since many other "physiological" substances cause inflammation when injected at this site.

No effect was found on kidney tissue hyperplasia of unoperated or uninephrectomized rat recipients of serum injections from variously unoperated, sham-operated or uninephrectomized donors by Williams (1962), Goss (1963), Reiter and McCreight (1964), Kurnick and Lindsay (1967) and Connolly et al. (1969). A stimulatory effect on cell division in unoperated rats from intra-peritoneal injections of UNx

serum was reported by Lowenstein and Stern (1963), and Vichi and Earle (1970), and in mice by intravenous injection by Silk (1967).

A constant exchange of blood between animals can be accomplished by parabiosis, although sufficient time must be allowed for capillary anastomoses to form, and the area of joining must be sufficiently large for adequate mixing. The fate of methylene blue injected into one partner is commonly used to confirm adequate mixing, although it has been reported (Thompson and Lytton, 1967) that mixing of the dye is not necessarily an indication of the presence of patent vessel anastomoses. Sibley and Huggins (1946) found that anastomosis of peritoneal cavities was necessary for homeostasis after bilateral nephrectomy in one of a parabiosed pair of rats. Skin conjunction gave insufficient exchange between circulations.

There are three reports of stimulation of kidney growth by removal of kidney tissue in a parabiont. Lytton et al. (1969) reported increased DNA synthesis in the kidneys of the partner of a uninephrectomized rat, but not to the extent of that in the contralateral kidney of the operated animal. No single-rat uninephrectomy controls were done in order to determine whether the contralateral kidney response was less than expected; a stimulatory factor would be diluted in this situation and might give a reduced response, whereas bilateral nephrectomy in one partner would be expected to result in the normal response, since the kidney mass (and function) of the two-animal unit has been halved. However, Lytton and coworkers (1969) report no hyperplastic response of the remaining kidneys after bilateral nephrectomy.

Steuart (1958) removed three kidneys from parabiosed pairs of rats and found an increase in mitotic index compared to unoperated animals at 48 hours, and a greater increase at 72 hours. Bilateral nephrectomy in two rats of parabiosed triplets did not result in an increase until 72 hours. No control values were determined in lone or parabiosed animals.

Kurnick and Lindsay (1968b) measured kidney weight in mice ten days after removing zero, one, two or three kidneys. The increase was proportional to number of kidneys removed, with the following qualifications: (a) when only one kidney was removed, the increase was larger in the remaining kidney of the uninephrectomized mouse than in the two of its partner; (b) when two kidneys were removed, the increase was larger if one kidney was removed from each mouse rather than two kidneys from one mouse. It should be noted, however, that intraperitoneal injection of anaesthetic had to be given to both partners of each pair, indicating less-than-ideal mixing of the circulations.

Negative results were reported by Thompson and Lytton (1967), who measured kidney weight four weeks after uninephrectomy in lone rats or in one of a pair. Weight increase was the same in the contralateral kidney of lone and parabiosed animals, while the partner's kidney showed no increase. There was adequate mixing of methylene blue in these parabionts, but no mixing of erythrocytes.

No data were reported in any of these parabiosis investigations relating to animal body weight or the possibility of uremia; often inadequate sample sizes were used, so that statistical significance of differences was not determined; and appropriate controls were not always included.

Cross-circulation by cannulation of large vessels results in more efficient mixing of blood between animals than does parabiosis by anastomosis of the microcirculation. Johnson and Vera Roman (1968) cross-cannulated carotid arteries and jugular veins in four pairs of adult rats. Labelled albumin injected into one tail vein equilibrated in the pair within 30 minutes. One kidney was removed from each pair at the time of cannulation. Labelling index of renal tubular epithelium was determined by radioautography 48 hours later. The elevated labelling index of the kidney contralateral to the excised one was not statistically different from that in lone, uninephrectomized rats, while the lower index of the two kidneys in the parabiont was the same as that of lone, unoperated rats.

A similar experimental design was used by Van Vroonhoven et al. (1972): cross-circulation was established in young adult rats at the time of sham operation or removal of two or three kidneys, using 12 pairs of animals per experimental group. Uninephrectomy in lone rats was also done. After 48 hours, kidney weight and RNA/DNA ratio were determined. In the remaining kidneys of the pairs in which one partner had received bilateral nephrectomy, both parameters were elevated to

the same extent as in the remaining kidney of the lone, uninephrectomized animals. Removal of three or four kidneys in pairs resulted in even higher increases in both kidney weight and RNA/DNA.

The demonstration of an effect in this report but not in that of Johnson and Vera Roman may be due to the latter's removing only one-fourth of the kidney mass from each pair instead of one-half. Also, the earlier report assayed hyperplasia, while this one employed measures of hypertrophy. The positive findings may be more reliable because they are based on a larger sample size.

Consideration of all reports involving exchange of serum or blood indicates that there is evidence for a role of the circulatory system in compensatory renal growth. Inadequacies of the methods used may account for negative results in many cases, while it is difficult to imagine any reasons for false positive results, except perhaps ECFV expansion in serum injection experiments. Unfortunately, these experiments do not help to differentiate among the various postulated ways of mediating kidney growth.

Finally, I will consider in vitro experiments designed to investigate the existence of a kidney-specific growth factor. An accepted principle of biological experimentation is to control as many physiological variables as possible. It is obvious from the preceding review of reports concerning a postulated renal growth factor that it is difficult to eliminate variation among animals and unwanted side-effects from the procedures used. Most of these

variables which complicate the design and interpretation of *in vivo* experiments are eliminated in *in vitro* preparations. Whole perfused kidneys, incubated slices and fragments of kidney, and primary cultures of kidney epithelium have been used for investigation of a postulated humoral factor mediating compensatory renal growth. In preparations in which there is no functional organization of the tissue, effects of growth factors can be dissociated from the influences of normal renal function. Primary cultures of tubular epithelium would seem ideal for this purpose: a hyperplastic effect on this cell type - the same one which is stimulated by UNx to divide *in vivo* - will be unobscured and undiluted by other cell types ("background noise" is eliminated).

Tissue-slice and fragment preparations are necessarily short-term since many of the cut-surface cells are damaged, and there is danger that cells in the interior will die due to lack of adequate diffusion between these cells and the incubation medium. Perfused whole-kidney preparations deteriorate after a few hours (Shames et al., 1976 a and b).

On the other hand, the health and viability of monolayer cultured cells can be monitored, by microscopic examination for example. Replication of primary cultures can be assured by various assays, thus allowing adequate sample sizes with low variance.

The reader should keep these qualifications in mind during the following review of *in vitro* experiments relating to the

mediation of compensatory renal growth.

Shames et al. (1976 a and b) used isolated dog kidney preparations to examine the effects of 90 to 240 minute perfusion with blood from dogs unoperated or uninephrectomized 24 hours previously. They measured nucleic acid synthesis by labelled adenine uptake, and the functional parameters of RBF, GFR, and sodium handling. The kidneys used for the control and experimental groups were from animals not handled similarly: the control kidneys were from dogs bled of one-half to one-third of their blood volumes just before the kidneys were removed, while the experimental (UNx group) kidneys were from untouched animals. This large blood loss would have had profound effects on the kidney. Functional parameters were reported in one paper to be unaffected by UNx blood perfusion (1976b), while in the other paper GFR and sodium reabsorption are reported to be increased (1976a). Tritiated adenine uptake by the UNx kidneys were significantly greater than by controls (1976b). This may be an indication of increased RNA or DNA synthesis, or both. Interpretation is hampered by the difference in kidney-donor handling between the two groups.

Kidney slices have been the most extensively-used preparation for these investigations. The results of four groups of investigators are given in Table 9. All groups used kidney slices and serum from rats weighing 125 to 200 grams (except possibly Lowenstein and Lozner, who did not give weight data). Kidney tissue was incubated with sham

Table 9 The use of *in vitro* rat kidney slice preparations in investigations of a postulated circulating kidney growth factor.

source of rat serum	source of kidney slices and length of incubation	assay of isotope uptake	ratio of UNx/ sham uptake	reference
UNx and sham	cortex 120 mins.	*thymidine	1.55	Lowenstein and Lozner, 1966
24 hrs. post-UNx and sham	cortex 120 mins.	*thymidine *uridine	1.29 1.30	Preuss et al. 1970
24 and 48 hrs. post-UNx and sham	whole kidney 120 mins.	*thymidine	1	Soulillou et al., 1975 and 1976
12, 24 or 48 hrs. post UNx and sham	cortex 60 mins.	*uridine	1	Cortes et al., 1976

* denotes labelling with an isotope

or UNx serum and labeled uridine or thymidine for one or two hours, and uptake into RNA or DNA was assayed and the ratio of UNx/sham calculated. As can be seen in the table, two groups of investigators reported increased nucleotide uptake with UNx serum, and two reported finding no differential effect. Since the authors give little information regarding the handling of serum and kidney tissue, the sample sizes and the variance of results, it is not possible to speculate on the reason for the difference in findings.

Fragments of kidney tissue have been explanted in clotted plasma by Ogawa and Nowinski (1958), or incubated in medium by Preuss and Goldin (1975, 1976). The explants were of pieces of rat medulla, about one mm. square, in clotted rooster plasma and chicken embryonic extract. Three days after explantation, the cultures "growing most abundantly" were chosen and divided into groups of four to seven each and treated with serum obtained from adolescent rats which had been unoperated, sham-operated or uninephrectomized two days previously. After three days, the proportion of dividing cells in the explants was determined. About 2000 epithelial-like cells were observed in each explant. In two out of three comparisons, the UNx serum groups had higher mitotic indices than control (unoperated or sham) groups. The differences were not large (the range of values lay from 0.7 to 5.5 per cent), and there is no indication of variance or the statistical significance of the differences, although the authors state that there is a wide scattering in the mitotic indices of controls. Criticisms of this

experiment include the use of medulla rather than cortex fragments, of small numbers of non-replicate cultures, of an unnecessarily complex and variable medium, and of such a long interval between addition of serum and assay of effect.

Some of these objections were overcome by Preuss and Goldin (1975, 1976), who prepared adult rat renal cortex fragments by forcing tissue through a sieve with holes 1.5 x 2 mm. Thirty to sixty mg. of tissue fragments were incubated for 90 minutes in oxygenated Krebs-Ringer solution with tritiated thymidine (^{3}HTdR) and plasma ultrafiltrates from rats either sham-operated or uni-nephrectomized 10 hours to 14 days previously. Liquid scintillation counting was used to determine ^{3}HTdR uptake in groups of three to six samples. Both sham and UNx groups were elevated between 10 and 36 hours, with UNx consistently above sham but not significantly different from it (1976); or, UNx was reported to be elevated from days one to 14 with a maximum at day five, and sham slightly elevated on days three to eight (1975). There was wide variation among groups. Autoradiography of samples showed that half the thymidine was being taken up by proximal tubule cells, and the remainder by other tubule epithelial cells and glomerular cells.

The authors ultrafiltered the plasma used in these experiments to prevent clotting during incubation with tissue fragments. Substances of molecular weight greater than 25,000 were removed by the procedure. (The authors mistakenly considered that renin was stimulating DNA synthesis in their cultures: since the molecular

weight of renin is about 40,000, it could not have been present in the plasma ultrafiltrates being tested.)

The only published report (Lyons et al., 1974) of the use of primary cultures of kidney epithelium in the investigation of compensatory renal growth used cells derived from the hamster. Incorporation of tritiated uridine into RNA was increased in five-day-old cultures when plasma was added from hamsters uninephrectomized 24 hours previously. Plasma from sham-operated animals was used as a control. Two to four cultures per group were incubated with hamster plasma for periods varying between two and 24 hours. The largest increases of UNx vs. sham serum treatment were seen at two and eight hours, with smaller increases between and after these times.

Although there are criticisms to be made, the results of these in vitro experiments lend support to the hypothesis that a circulating growth factor plays a role in mediation of compensatory renal growth. For the reasons outlined above, in vitro techniques, and especially primary culture, offer advantages over in vivo situations for the investigation of such growth factors; in fact, such theoretical considerations and review of the literature of the last 100 years on this subject both indicate that in vitro methods offer one of the most promising lines of research on this topic. Consequently, the subject of this thesis is the development of a primary culture system suitable for use in confirmation of the existence of a humoral kidney mitotic growth factor.

MATERIALS AND METHODS

A. Rat surgery

I. Animals

Male, albino, Fischer strain rats were used for all experiments. They were obtained either directly from Charles River Laboratories via Canadian Breeding Laboratories (St. Constant, Quebec), or were bred at Memorial University of Newfoundland from stock derived from Charles River Laboratories. These inbred rats are reported to be able to accept reciprocal skin grafts. They were used when young adults of five to eight weeks, of body weight in the range 75 to 150 gm. (Figure 4.). They had free access to Purina rat chow and tap water at all times.

II. Anaesthesia

All surgical procedures were carried out under open diethyl ether anaesthesia. The minimum dose and length of anaesthesia required for each procedure were used.

III. Nephrectomy and sham operation

A rat was weighed and anaesthetized, and its abdomen shaved. A midline incision was made, through which the kidney was gently separated from surrounding fat and the adrenal gland, the renal pedicle ligated with 00 silk and the kidney

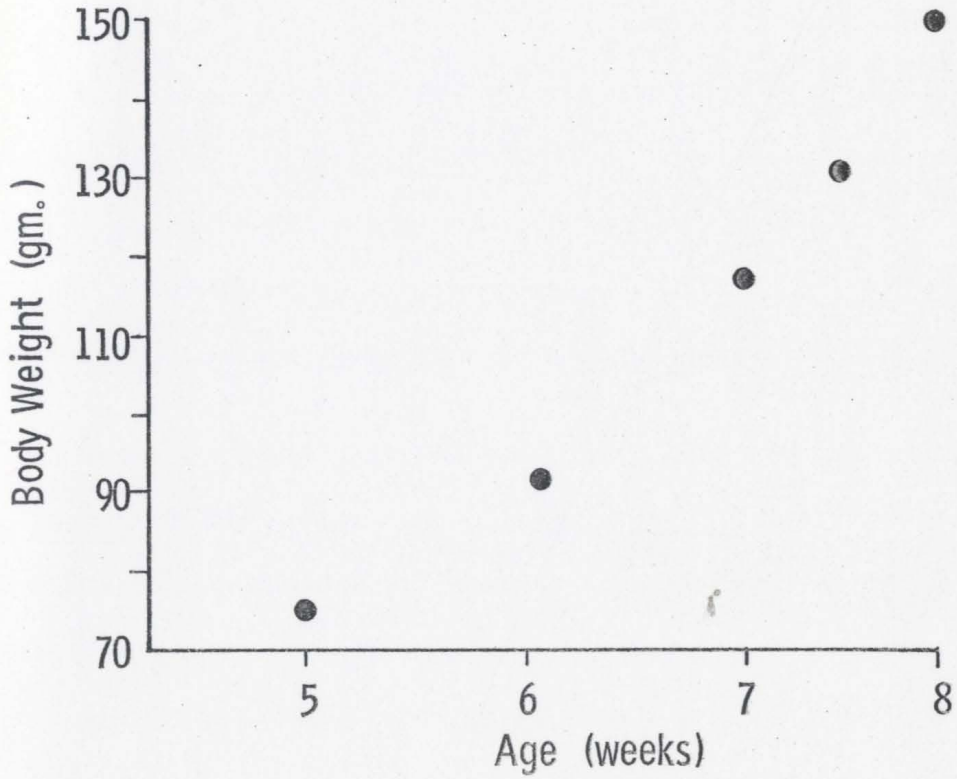

Figure 4. Growth rate of Fischer rats. The data were supplied by Charles River Breeding Laboratories, Inc., Wilmington, MA.

excised. Care was taken to ensure that the adrenal gland and its blood supply remained intact. The anterior abdominal wall was then sutured with silk and the skin closed with 9 mm. Autoclips (Clay Adams, Parsippany, N.Y.). A maximum of five minutes' anaesthesia was required for the procedure. The right kidney is on the average heavier by about four per cent than the left in the Fischer rat (unpublished observations; Roberts, Hansen and Hyam), and by about 2.5 per cent in the Sprague-Dawley rat (Kaufman et al., 1974), but apparently not in Lewis rats, whose kidneys are reported to be of equal weight (Klein and Gittes, 1973). The right kidney was removed at unilateral nephrectomy; thus it was always the left kidney which underwent compensatory growth, and the bias caused by disparity of kidney weights was made consistent.

Sham operation was performed using the same procedure, except that the kidney was only palpated with the finger: it was neither cleared of surrounding tissues nor ligated. The sham procedure was made to last approximately the same length of time as unilateral nephrectomy (UNx).

All operations were carried out between 10 a.m. and 12 noon in order to minimize variability due to the effects of diurnal variation on mitotic rates (Saetren, 1972).

IV. Bleeding

A rat was weighed and anaesthetized. Blood was obtained from the aorta through a midline incision: the region of the iliac bifurcation was cleared, and the rat was exsanguinated with a sterile 23-gauge needle and syringe. Approximately five ml. of blood were obtained from each rat. The blood was placed in a sterile screw-cap tube, allowed to clot on ice, and centrifuged at 1700 g to obtain clear serum. Sera from rats which had received the same experimental treatment were pooled and used without delay in the preparation of cell culture medium as described below.

Whenever kidney removal was required at the time of death, the nephrectomy was performed before bleeding.

V. Kidney weights

Immediately after excision, kidneys were cleared of any adhering tissue, including the capsule, gently blotted on a gauze swab, and weighed to the nearest 0.1 mg. on a tared analytic balance.

B. Cell culture

A kidney removed from a rat under sterile conditions was cleaned of fat and decapsulated in a laminar flow hood. It was then minced into pieces approximately 0.2 mm. in size with an automatic tissue sectioner (Sorvall, Allied Scientific, Scarborough, Ontario). The minced tissue was transferred to a 100 ml. bottle and rinsed twice with 10 ml. calcium- and magnesium-free Hank's basal salt solution (BSS) containing 0.05 per cent trypsin and 0.02 per cent EDTA (Gibco Canada, Burlington, Ontario). The tissue was suspended in 40 ml. of this solution and incubated at 37°C., using a magnetic stirrer at slow speed. After 15 minutes, any undissociated tissue was allowed to settle and the supernatant cell suspension was decanted. The remaining tissue was once again incubated with an additional 40 ml. of solution for 15 minutes, and the resulting second cell suspension was added to the first harvest. The pooled suspensions were centrifuged for ten minutes at 1000g. The supernatant was thrown away and the sedimented cells suspended in 12 ml. of RPMI 1640 culture medium containing 5 per cent fetal calf serum, 100 units/ml. of penicillin and 100 µg./ml. of streptomycin (all obtained from Gibco Canada). Microscopical examination revealed that this suspension consisted primarily of rounded-up, single cells. The trypan blue dye exclusion test indicated that a minimum of 95 per cent of these cells were viable.

Cultures were set up in either 24 cm^2. plastic flasks (Falcon,

Fisher Scientific Co., Dartmouth, N.S.) or in 2 cm^2. plastic Leighton tubes (Nunc, Roskilde, Denmark). The cell suspension was added to the culture medium described above at a concentration of 5 per cent v/v; i.e., all the cells harvested after trypsinization from one kidney would be finally diluted in 240 ml. medium. Using a dispenser (Repipet, Fisher Scientific Co.), the final suspension was measured into the culture vessels, either 10 ml. into each flask or 2 ml. into each tube. The dispenser was agitated from time to time during this procedure in order to minimize settling of cells. The cultures were gassed with 5 per cent CO_2 in air and then tightly closed and incubated at 37°C. The medium was changed three and five days later, and the cultures were harvested at six days. Before each of these manipulations, any culture whose medium had an abnormal pH, as indicated by phenol red colour change, was discarded. Figure 5. is a scheme of the culture procedure.

The use of pipettors and dispensers resulted in efficient handling and good reproducibility in replicate cultures. No infections ever occurred in cultures, due to the use of antibiotics, of closed vessels during incubation, and of a laminar flow hood for all manipulations.

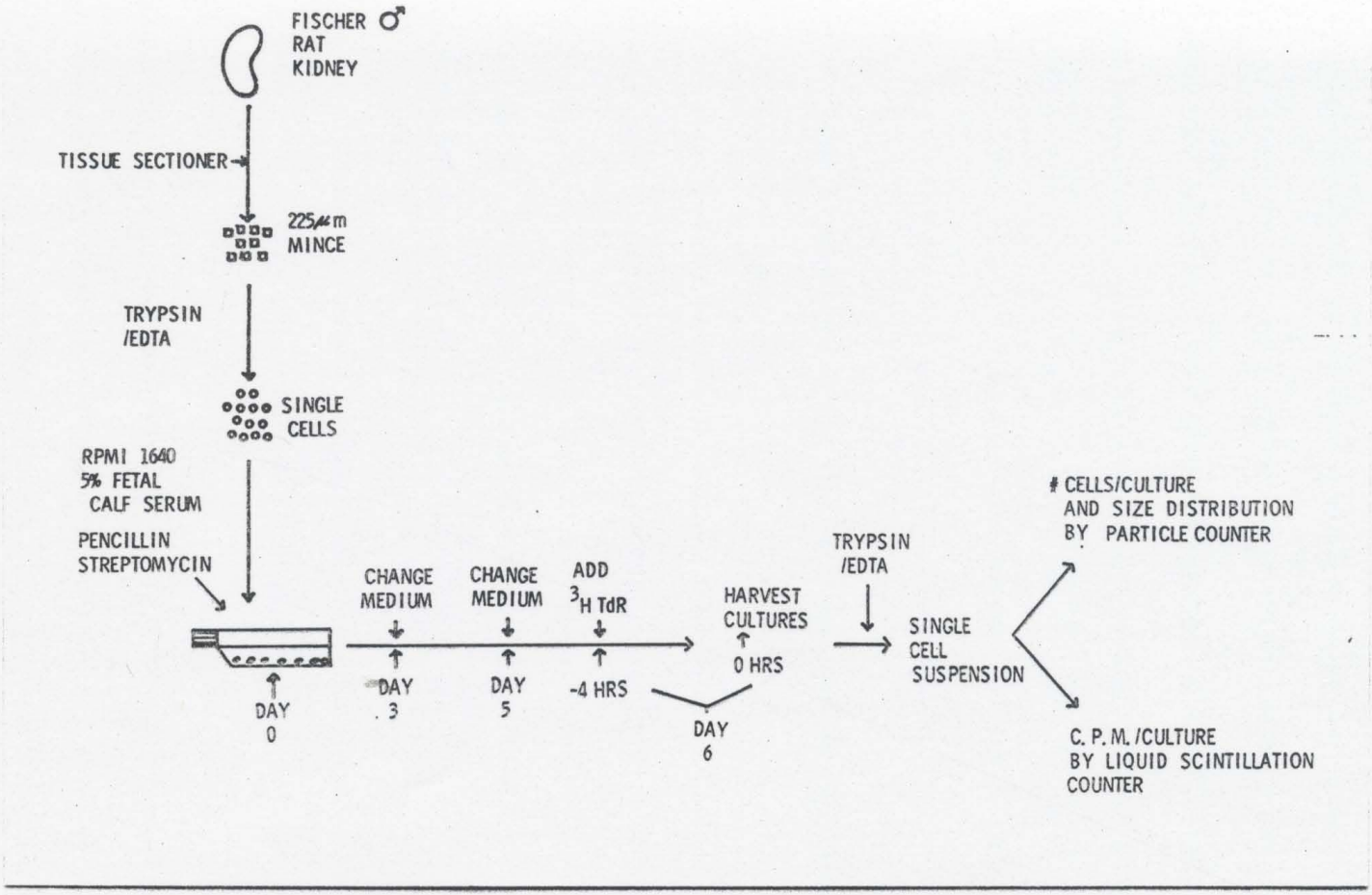

Figure 5. Scheme of procedure used for primary culture of rat kidney epithelium.

C. Radioisotope techniques

I. In vitro

When the cultures were six days old, tritiated thymidine (^{3}HTdR, 6.7 Ci/mmole, New England Nuclear, Dorval, Quebec) was added four hours before harvest. The isotope was diluted for use to 10 µCi/ml. with sterile distilled water. An automatic hand pipettor (Oxford Laboratories Sampler, Fisher Scientific Co.) was used to add 2 µCi to each flask or 1 µCi to each tube.

At the time of harvest, the culture medium was poured off and, for both flasks and tubes, replaced with 5 ml. of calcium- and magnesium-free Hank's solution containing 0.05 per cent trypsin and 0.02 per cent EDTA (Gibco). The cultures were incubated 20 minutes at 37°C., at which time all the cells had become detached from the plastic surface of the culture vessels and were in suspension.

If a cell count was to be made, a 1 ml. aliquot was removed. The remaining volume (4 ml.) of cell suspension was processed for liquid scintillation counting. Using a filtering manifold (Millipore, Mississauga, Ontario) with fibre-glass filters (Reeve Angel, Fisher Scientific Co.), the cells were washed once with 0.9 per cent saline, three times with cold 5 per cent trichloroacetic acid and twice with 95 per cent ethanol. Each wash consisted of 10 ml. of solution delivered

by an automatic dispenser (Oxford Pipettor, Fisher Scientific Co.). Each filter, containing the precipitated and washed DNA from a single culture, was placed in a glass scintillation vial and 1 ml. of solubilizer (NCS, Amersham Searle, Oakville, Ontario) was added with an automatic hand pipettor (Clay Adams, Fisher Scientific Co.). After standing at room temperature overnight, 10 ml. of scintillation fluid (Spectrofluor, Amersham Searle) was added to each vial with an all-glass automatic dispenser (Repipet, Fisher Scientific Co.). The samples were refrigerated in the dark for 24 hours to minimize chemiluminescence, and then counted in a refrigerated Beckman LS-355 liquid scintillation system. Either counts per minute (C.P.M.) as obtained directly from the counter were used as data, or disintegrations per minute (D.P.M.) were calculated using an external standard.

II. In vivo

To estimate the rate of new DNA synthesis in the intact animals, rats were injected intraperitoneally with 25 μCi ^{3}HTdR/100 gm. body weight (6.7 Ci/mmole, New England Nuclear) four hours before killing. The excised kidney was immediately weighed and cut in two; one half was fixed for histological examination (mitotic index determination), and the other was weighed and frozen at -15°C. until further processing, which was done within a week. A motor-drived Potter homogenizer was

used to prepare a homogenate of each kidney in 5 ml. cold 5 per cent trichloroacetic acid (TCA), and then extracted with 2.5 ml. hot 5 per cent TCA (90°C. for 15 mins.) according to the method of Schneider (Leslie, 1955). After centrifugation at 6000 g, 0.5 ml. of supernatant was mixed with 10 ml. scintillation fluid (Aquasol, New England Nuclear). After refrigeration in the dark for 24 hours to minimize chemiluminescence, the samples were counted in a Mark I Nuclear Chicago counter. The data were expressed as C.P.M., or as D.P.M. after correction for quenching. Since the quench correction was found not to vary significantly, the data were usually expressed as C.P.M.

D. Cell counting

When cell counts were to be made, as noted above one ml. of trypsinized cell suspension was removed from each culture at harvest. It was diluted with 9 ml. of suspending solution (Isoton, Coulter Diagnostics, Inc.), and the cell count was made with an electronic cell counter (model ZBI Coulter Counter). The amplification, aperture current and threshold range were chosen in order to eliminate cell debris and undissociated clumps of cells. An analyzer and plotter (Coulter Channelyzer) were used to plot the cell size distribution. Suspensions of pollen grains of known size were used as standards - paper mulberry, 12 to 13μm, and ragweed, 19 to 20μm (Coulter Diagnostics, Inc).

E. Histology

I. Intact kidney

Histological sections of kidney were prepared for determination of mitotic index. An excised kidney was weighed, halved, fixed in Bouin's fluid for 24 hours, and imbedded in wax. Sections of 7 µm. were cut and stained with haematoxylin and eosin. The standard procedures as outlined in Carleton's Histological Technique were followed (1967).

II. Cultured cells

Stained preparations of cultured cells were made as follows: cultures were set up (in plastic flasks) by the method described above. For fixation, the medium was poured off and a microscope slide-sized piece was cut out of the flask's growing surface with small bone snips. The cells were air-dried, using an unheated blower, and fixed in absolute methanol for 10 minutes. The preparation was dipped in pH 6.8 phosphate buffer for 2 minutes, then stained in Giemsa diluted 1:10 with the same buffer, and finally rinsed with the buffer.

Preparations for phase and electron microscopy were trypsinized cultured cells fixed in suspension in Karnofsky's fixative, post-fixed with osmium tetroxide and imbedded in resin according to the method described by Rowden and Lewis (1974).

III. Microphotography

All photography was done with a Zeiss Phomi II. Kodak Plus X Pan film was used with a green filter for phase microscopy or a red for stained preparations. Colour photographs were made with Kodak Ektacolor Professional Type S film.

F. Preparation of tissue homogenate fractions

Soluble and microsome fractions of rat kidney and liver homogenates were prepared according to the method of Dicker (1972). Kidney or liver tissue was removed from a rat using my standard surgical procedure. The kidney cortex was dissected away from the medulla, which was discarded. The tissue was weighed and homogenized with a motor-driven Potter homogenizer in cold 0.25 M. sucrose. Sucrose was added to give a volume of 10 ml. for every gram of tissue. The homogenate was centrifuged in the cold at 8000 g for 10 minutes to remove cell debris, nuclei, lysosomes and mitochondria. The supernatant was then centrifuged at 40,000 g for 30 minutes. The sediment, containing most of the microsomes (free ribosomes and fragments of endoplasmic reticulum with attached ribosomes) was resuspended in 0.25 M sucrose to give the original volume - i.e., 10 ml. of the final suspension contained microsomes derived from 1 gm. of kidney cortex or liver. The supernatant from centrifugation (the soluble fraction) was also made up to the original volume (10 ml./gm. tissue) with 0.25 M sucrose. The homogenate fractions were kept cold throughout the procedure, and were used in experiments immediately after preparation.

G. Preparation of xanthopterin solutions

The method described by Haddow et al. (1972) was used to prepare solutions of xanthopterin, a pigment isolated from insect wings. Ten mg. of the compound (Sigma, St. Louis, Mo.) was dissolved in 1 ml. of N/3 NaOH. An equal volume of N/3 HCl was added, and the resulting fine suspension of 5 mg. xanthopterin per ml. N/6 saline was autoclaved. For addition to cultures, this suspension was diluted with Hank's balanced salt solution (Gibco) to give the required concentration in a 0.02 - 2 ml. volume.

H. Data presentation and statistical analysis

The mean and standard error of the mean were calculated for each group of cultures treated similarly and simultaneously. Data from cultures grown at different times were never combined. Since samples of 30 or less were used in this study, Student's t distribution was employed to test significance of differences between any two groups of cultures being compared. The two-tailed distribution was always used, since in many experiments it was not known whether experimental treatment would increase or decrease the parameter assayed when compared with a control group. All differences with values ≤ 0.10 were considered significant.

Calculations were performed with a Hewlett-Packard instrument, model 9810A.

EXPERIMENTAL DESIGN AND RESULTS

A. Compensatory renal growth in Fischer rats.

Some aspects of compensatory renal growth vary with age (see discussion in review chapter); and since rate of body growth varies with strain of rat, it is reasonable to assume that compensatory renal growth may also vary. Therefore, I have established certain growth parameters for the Fischer rats used in the research for this thesis. Most of the published work in this area has been on the more commonly-used rat strains, such as Sprague-Dawley and Wistar, which have a higher rate of body growth than Fischer rats.

I. Kidney weights

Animals were randomly divided into two groups of thirty each: sham(S) and uninephrectomy (UNx). On day 0, the rats were weighed and numbered by ear-clipping, and the operations were performed alternately. The removed kidneys were weighed. Five rats from each group were killed on days 1, 2, 4, 7, 14 and 21; the rats and their remaining kidneys were weighed. The surgery was always done in the morning, and for the first four days particular attention was paid to killing the rats at intervals of precisely 24 hours after initial surgery.

Figure 6.illustrates the increase in contralateral (left) kidney weight expressed as a percentage of body weight at various periods after sham or UNx operation. Means and standard errors of the mean are shown. The two groups differed significantly when compared by t test ($p < 0.1$) on days 2 to 21, but not on day 1, 24 hours after surgery. The two groups of rats gained body weight at the same rate, as shown in Figure 7.

II. Hyperplasia

Two measures of kidney cell hyperplasia were studied: mitotic index and uptake of tritiated thymidine.

For determination of mitotic index, forty rats were randomly placed in two groups of twenty: S and UNx. The appropriate surgery was performed during the morning, and 44 hours later, the animals received an intraperitoneal injection of the mitotic inhibitor colchicine; the dose was 0.1 mg. colchicine/100 gm. body weight. At 48 hours after surgery (4 hours after colchicine injection), the left kidneys of both groups were excised and weighed. The interval of 48 hours was chosen because we had found that cells were dividing at a maximal rate between 40 and 50 hours (Roberts, Hansen and Hyam, unpublished results). Half of each excised left kidney was used to prepare stained sections, which were coded and from which counts were made of mitotic figures in 25 fields at a

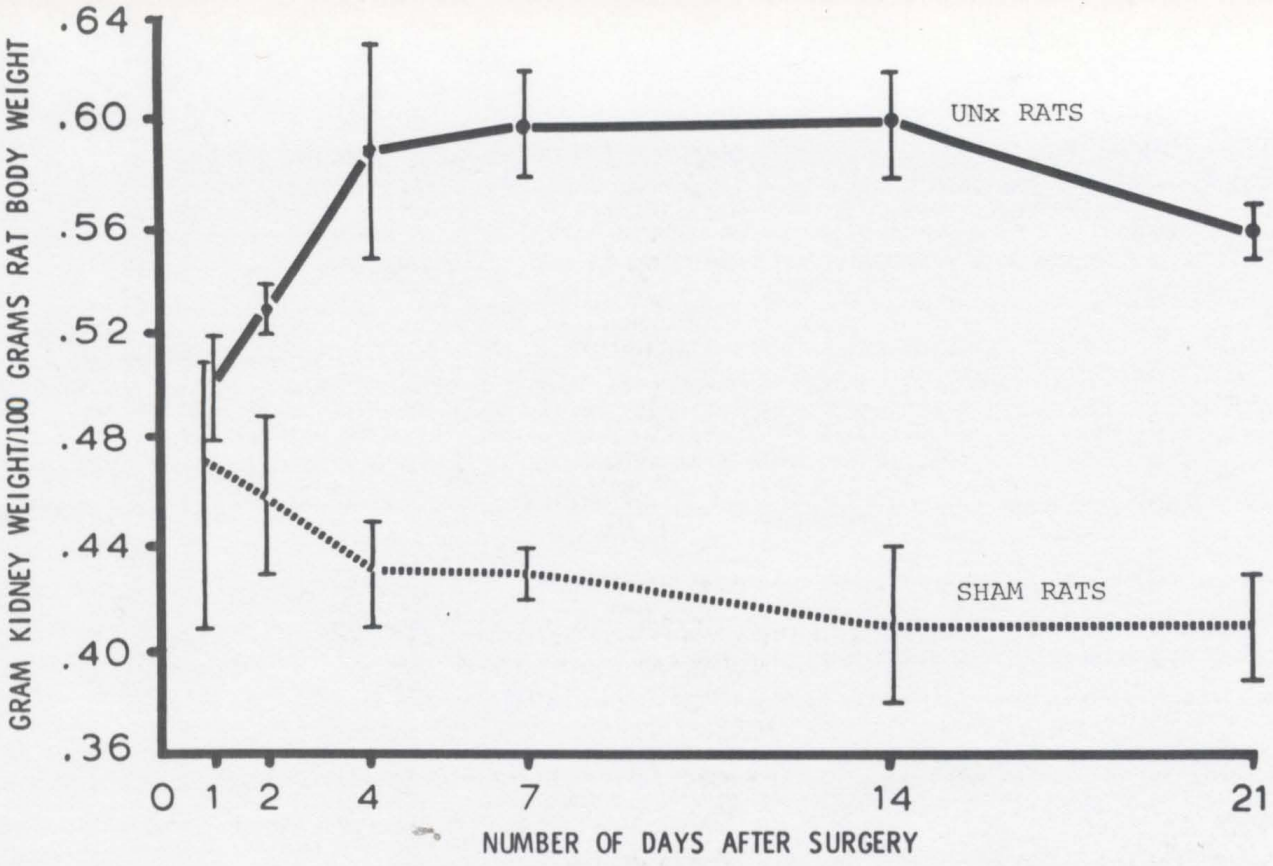

Figure 6. Effect of uninephrectomy on contralateral kidney weight in Fischer rats. A significant increase in contralateral kidney weight is seen in young adult, male Fischer rats 2 to 21 days after uninephrectomy, when the kidney weights are compared to those of sham-operated animals. Each data point represents the mean $\pm$ S.E.M. for four rats, and ● designates a significant difference between uninephrectomy and sham values.

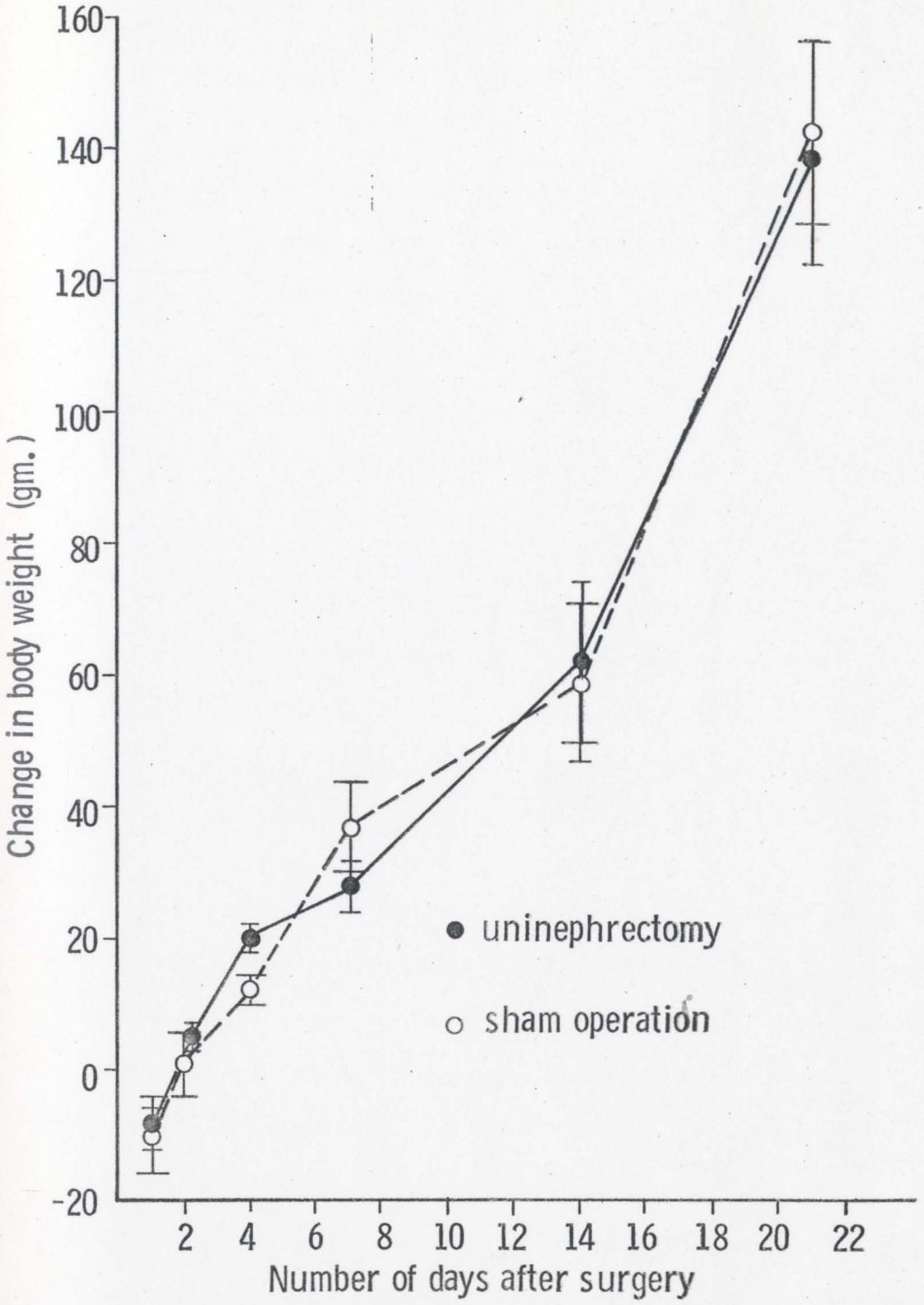

Figure 7. Effect of uninephrectomy on Fischer rat body weight. The rate of body weight gain in young adult, male Fischer rats does not differ significantly when sham operation is compared with unilateral nephrectomy. Each data point represents the mean ± S.E.M. for four rats.

magnification of X500. Most of these were recognizable as colchicine-inhibited metaphases, with stubby chromosomes in a pale, swollen cytoplasm. The scanning was done in a castellation pattern over the cortex alone, since, with rare exceptions, all mitoses were found in this area of the kidney. The code was broken and the results averaged; there was close agreement between the results of two observers. The means and standard errors of the means were calculated for each group, and Student's t test applied.

Figure 8. shows the results of 20 rats in each group by scatter diagram, where each point represents the value for one rat averaged from two observers making blind counts. Although there is some overlap between the groups, the means differ at a significant level ($p < 0.05$).

In an experiment designed to measure tritiated thymidine (^{3}HTdR) uptake by kidney cells, sham and UNx operations were performed on twenty rats each; 44 hours later they were injected with ^{3}HTdR (see Materials and Methods) and at 48 hours the left kidneys were removed and weighed. Half of each kidney was used for histological examination, and the other half was weighed and processed for liquid scintillation counting. The ^{3}HTdR uptakes of the two groups were compared, and were considered to be an index of their rates of DNA synthesis during the 4 hours before death.

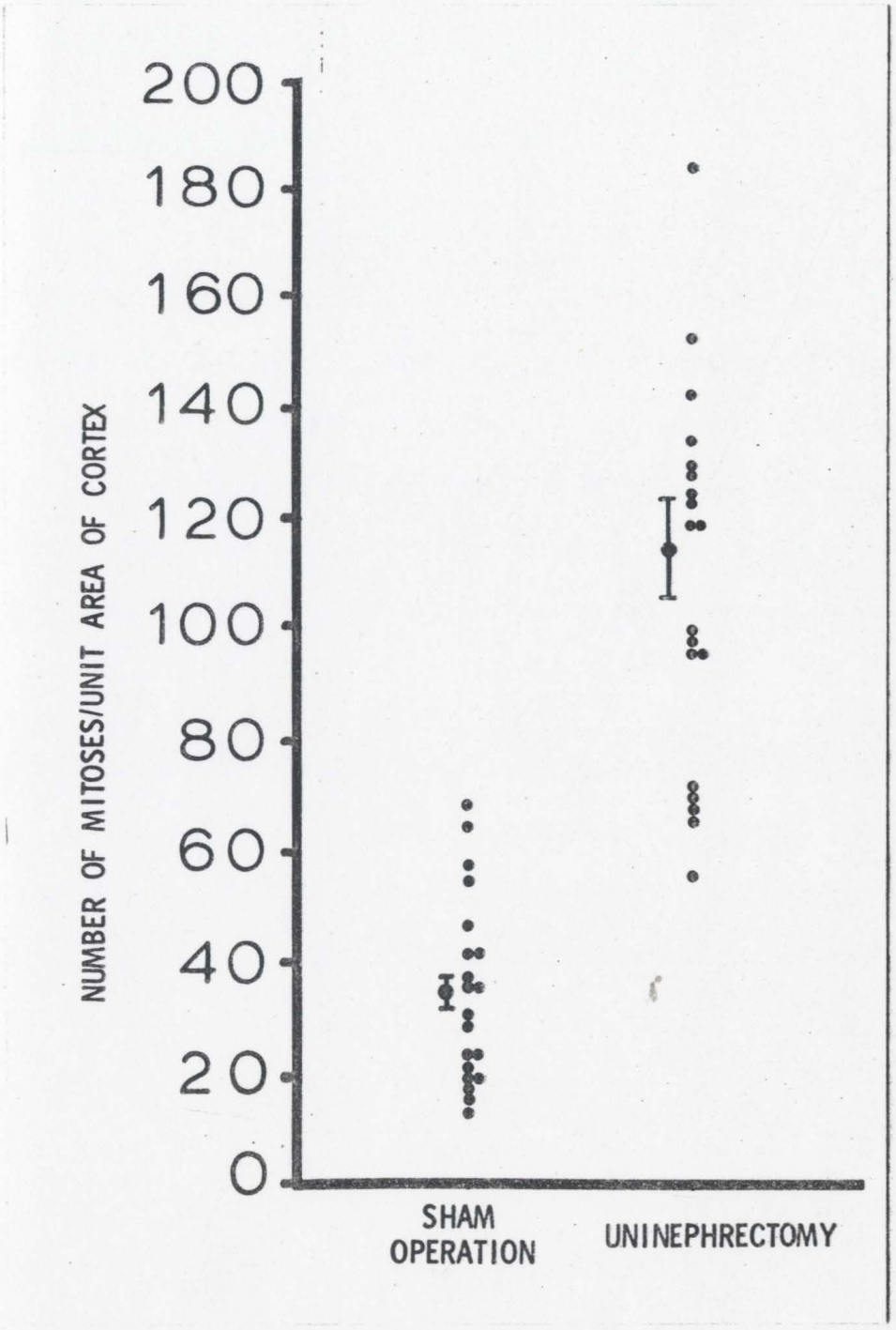

Figure 8. Mitotic index of contralateral kidney after uninephrectomy. Mitotic index of renal tubular epithelium is significantly increased 48 hours after uninephrectomy. Each data point represents one male, young adult, Fischer rat. Mean and S.E.M. are designated for each group.

Figure 9. shows that there is variation in each group, but no overlap, and the mean uptake of UNx rats is greater than shams at a significant level ($p < 0.05$).

III. Adrenal gland weights

There was a danger that the manipulations required for unilateral nephrectomy would interfere with the adrenal gland's blood supply, resulting in atrophy of the gland and upset in hormone production. In order to investigate this possibility, twelve rats were randomly divided into two groups: one received sham operation and the other, uninephrectomy. Forty-eight hours later both adrenal glands were excised from each rat, cleaned of adhering fat and weighed.

When tested by Student's t test, no difference was found between the adrenal gland weights from sham and UNx rats, or between the right and left glands from either group. These weights, their mean values and standard errors of the mean are given in Table 10.

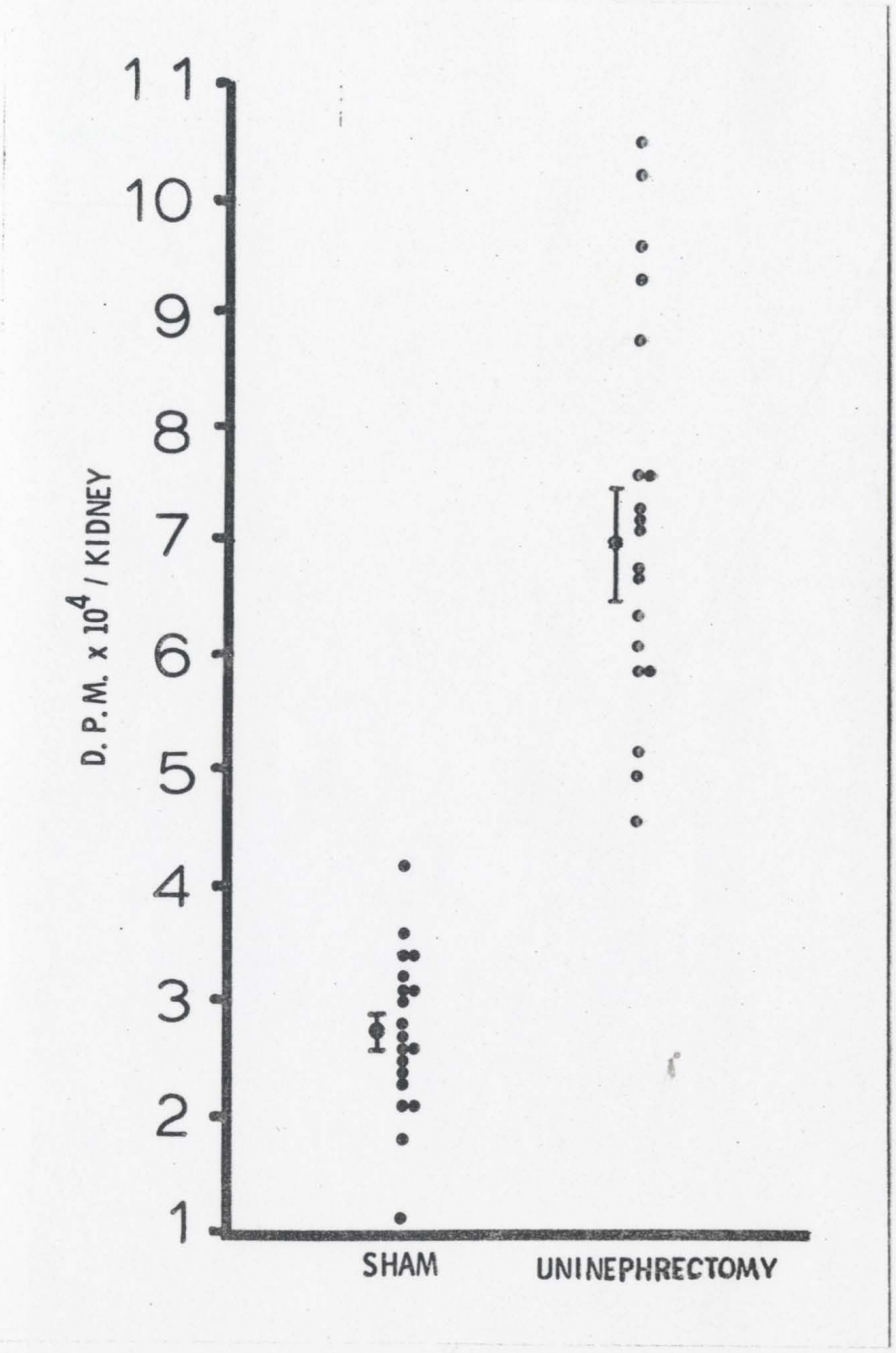

Figure 9. Uptake of tritiated thymidine by contralateral kidney after uninephrectomy. Uptake of tritiated thymidine by contralateral kidney 48 hours after uninephrectomy. Each data point represents one male, young adult, Fischer rat. Mean and S.E.M. are designated for each group.

Table 10. Absence of effect of uninephrectomy on adrenal gland weight in Fischer rats

Type of surgery*	Sham		UNx	
side of adrenal gland	**Right	Left	**Right	Left
adrenal gland weights (mg.):	23.5	17.5	24.8	21.8
	17.9	20.3	23.8	25.5
	22.7	21.9	21.7	19.8
	24.1	20.4	24.1	24.4
	19.0	24.4	12.7	16.2
	18.4	22.6	23.6	19.6
	21.1	25.1	21.8	21.1
mean weights (mg.)	21.0	21.7	21.8	21.2
S.E.M.	1.0	1.0	1.6	1.2

* Surgery was performed 48 hours previously.

** The kidney from the right side was removed at uninephrectomy.

B. Characteristics of primary rat kidney cultures

I. Morphology

The morphology of the cultures was of interest for two reasons: (a) to characterize the growth of the cultures, and (b) to confirm that the cells were epithelial, and not fibroblast-like or endothelial, cells.

In the course of all experimental work, randomly-chosen cultures were routinely examined by phase microscopy to confirm the health and growth status of the cells.

In one experiment, cultures were fixed *in situ* with methanol on days 4 through 7, stained with Giemsa and photographed to show the change in appearance with age (Figure 10.). (Details of this and other methods described in this section are given in the Materials and Methods section.) The number of cells increases until about day 5 and the size until day 6, finally forming a nearly complete monolayer. The cells have an epithelial appearance, which is further demonstrated in Figure 11., which is a phase photomicrograph of a 0.5 μm. section of fixed cultured cells on day 5. Microvilli can be seen in these photographs, and also in Figure 12., an electron micrograph of cultured cells on day 5.

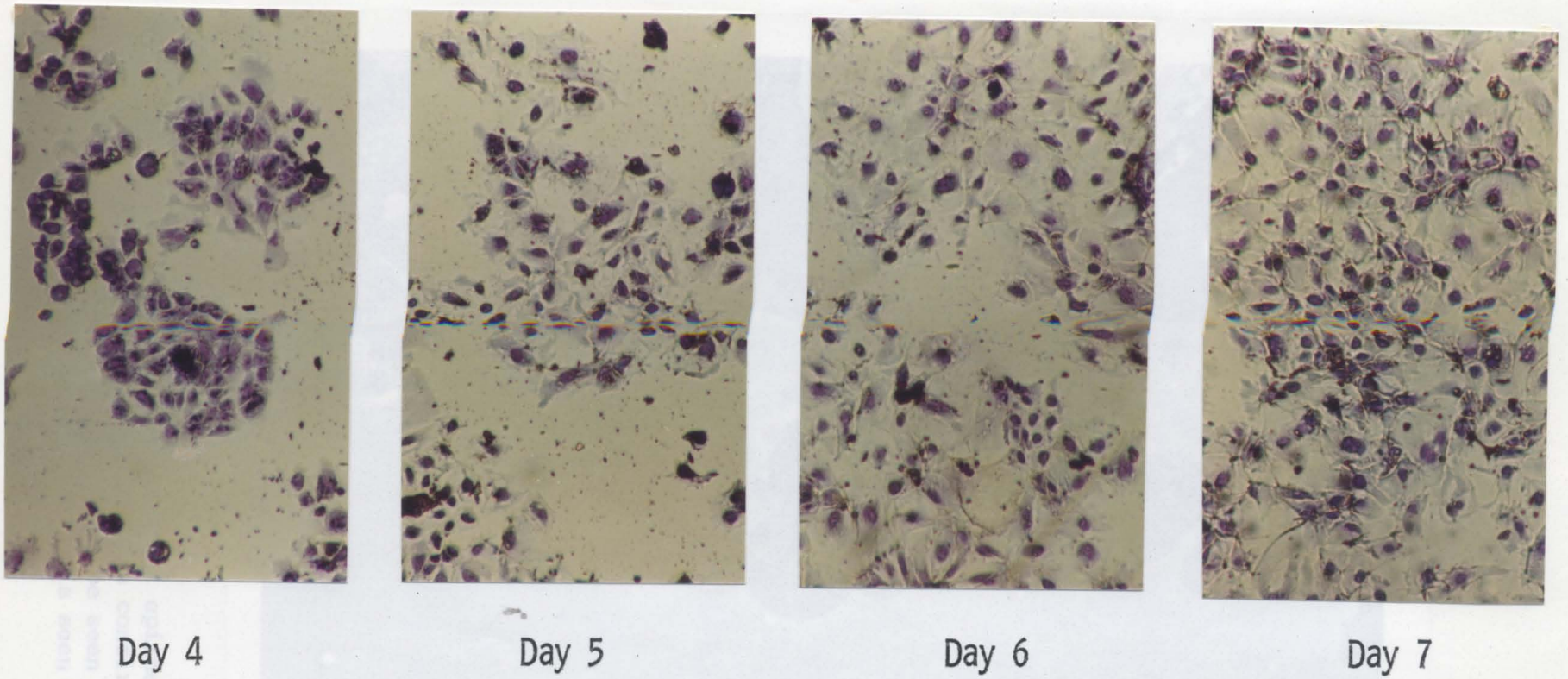

Figure 10. Change of appearance of primary kidney cultures with time. Both size and number of these cells increase with age of culture. The cells' epithelial appearance can be seen.

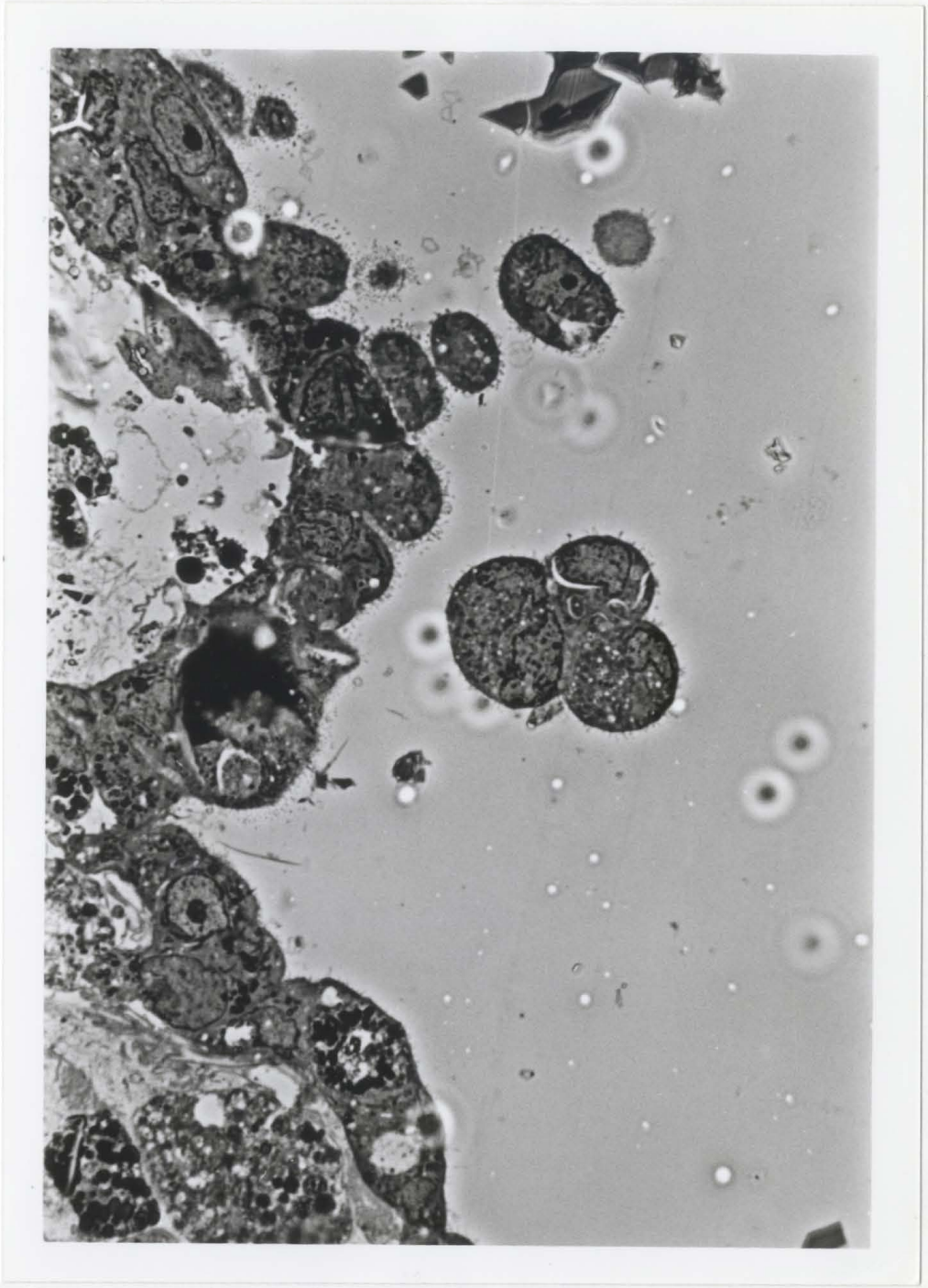

Figure 11. Phase photomicrograph of cultured renal epithelial cells. The epithelial nature of these cells is confirmed by their palisade arrangement. Microvilli can be seen on their unattached surfaces. A dividing cell is seen at the bottom. Magnification X550

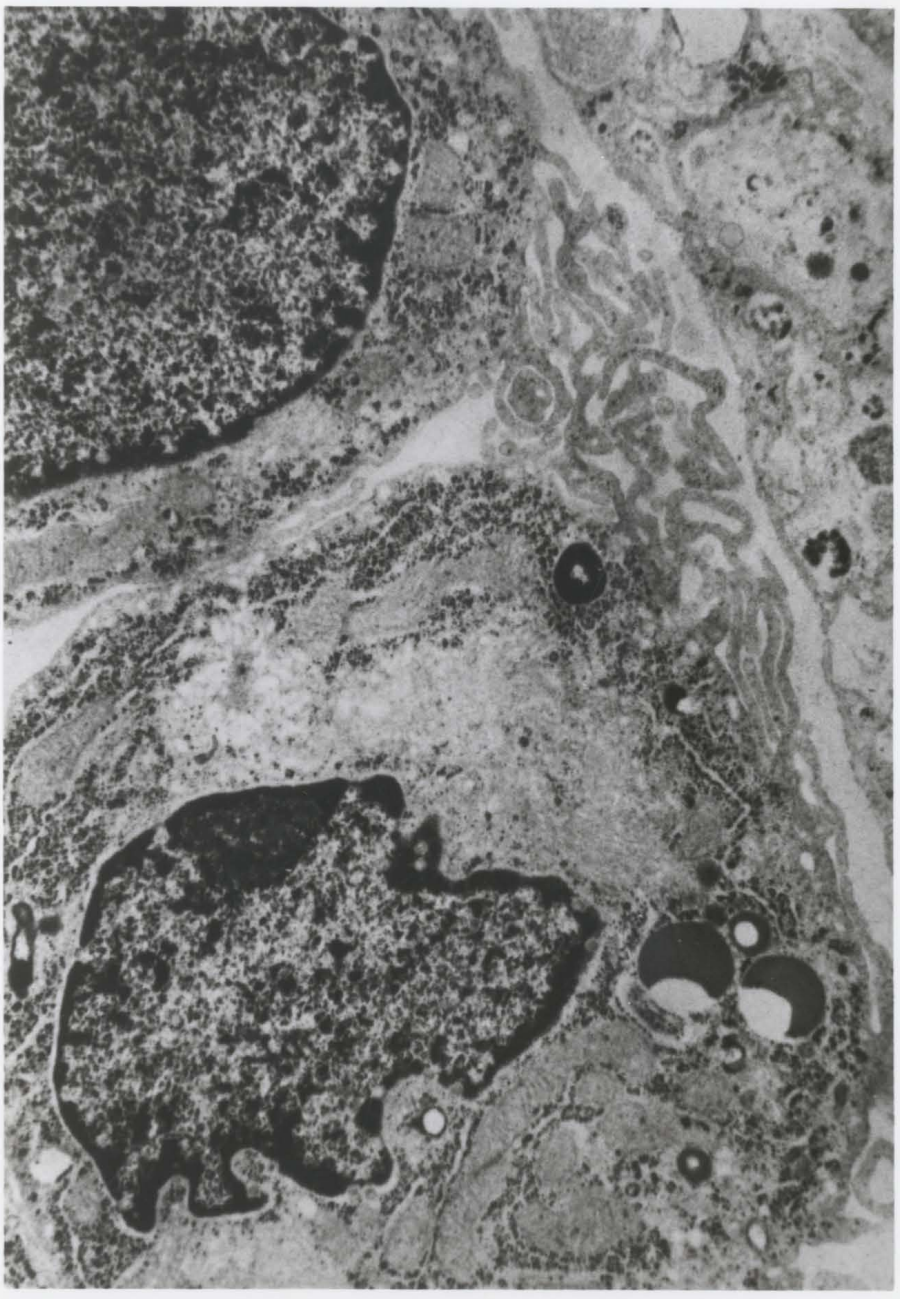

Figure 12. Electron micrograph of cultured renal epithelial cell. The nuclei of two adjacent cells are seen. Microvilli fill the intracellular space. Magnification X18,000

II. Quantitative growth characteristics

Cell size and number and tritiated thymidine uptake of cultures were determined daily in order to characterize the growth of kidney epithelium *in vitro*. A Coulter Counter Channelyzer was used to plot the size distribution in a trypsinized kidney epithelial cell suspension. Then, 36 cultures were set up in flasks using this suspension. Six cultures were harvested on each of days 2 through 7, and the cell size distributions plotted. Since the six plots for each harvest were similar, an "average" (by eye) distribution was drawn for each day.

Both the range of and mean of cell volume increased during the period 2 - 7 days. The mean cell diameter of trypsinized cell suspension measured with an electronic cell counter and sizer increased from about 13μm to about 17μm as shown in Figure 13. (The assumption is made that the cells are spherical, and therefore the cell diameter is an accurate index of its volume.) This measured cell hypertrophy correlated with that observed by microscopy.

Unit number of cells and C.P.M. per culture were determined using cultures set up in Leighton tubes. Ten were harvested on each of days 2 through 7. Since only the relative assay values were needed to show the growth pattern, absolute number of cells and D.P.M. were not calculated.

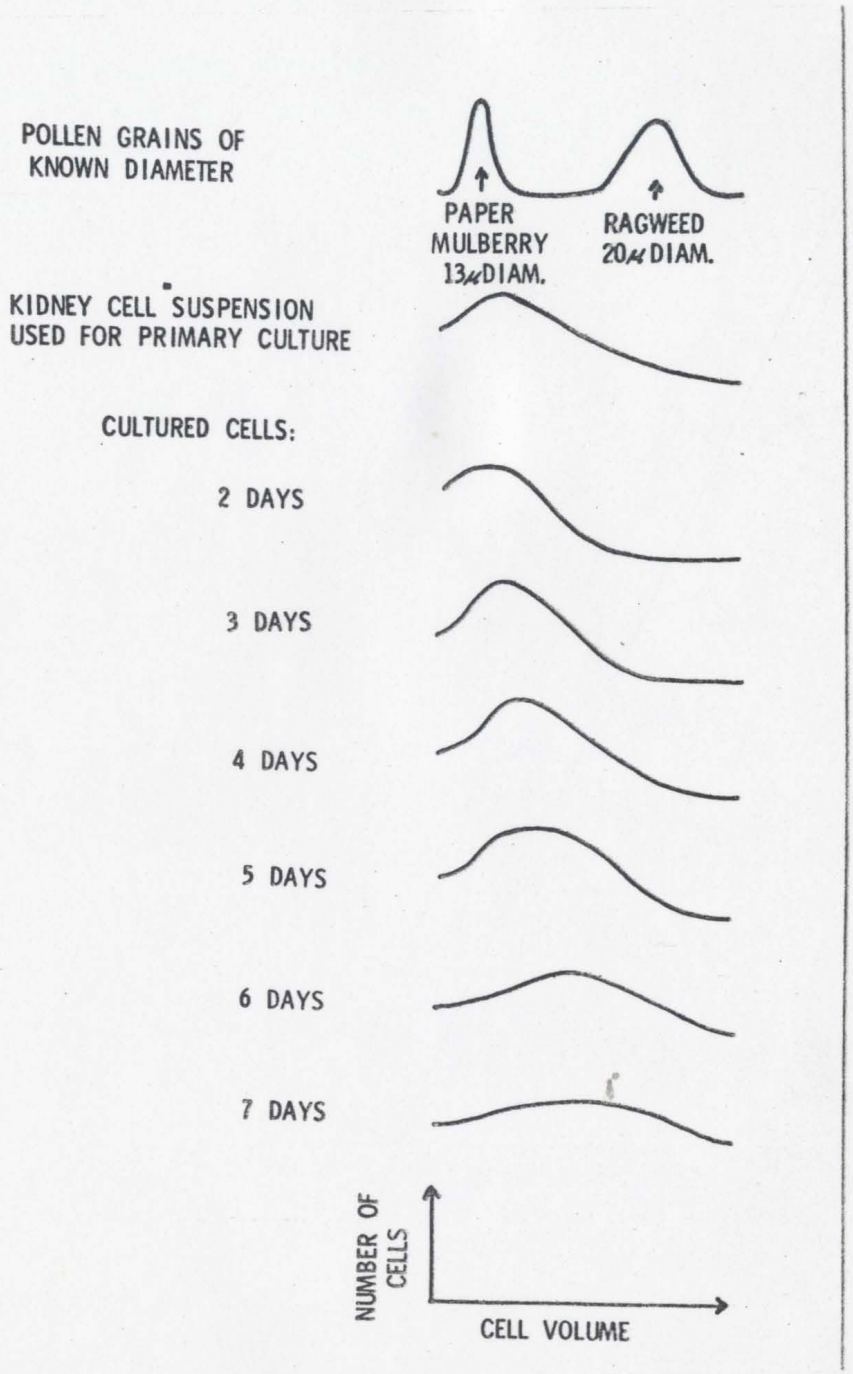

Figure 13. Change with time of cell size distribution in primary kidney cultures. Increase in cell size with age of culture is demonstrated using an electronic cell counter and sizer. Pollen grains are used for comparison.

The mean values are plotted in Figure 14. The highest rate of DNA synthesis occurs on day 4, one day before that of the greatest number of cells.

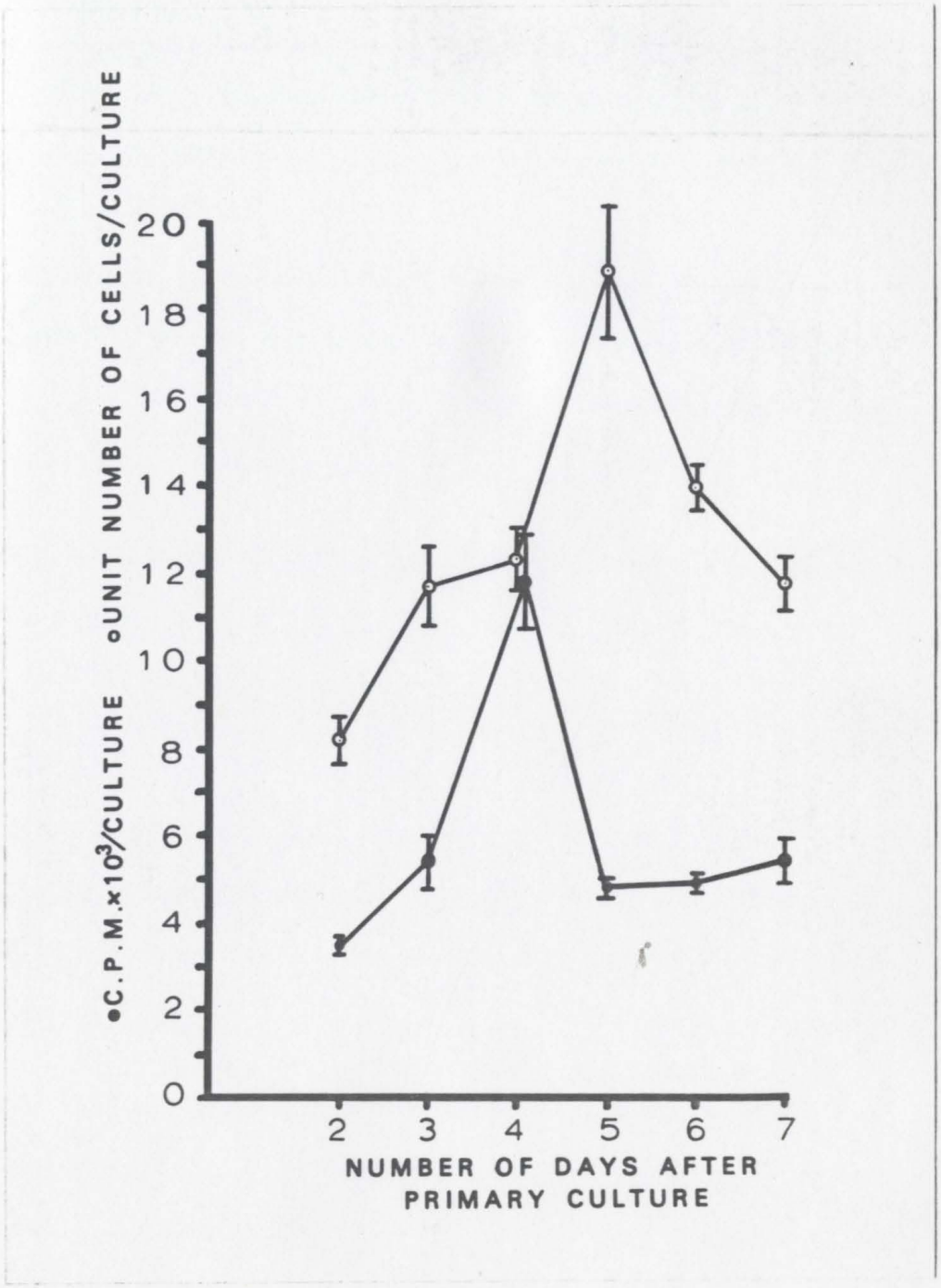

Figure 14. Change with time of cell numbers and uptake of tritiated thymidine in primary kidney cultures. The maximum rate of DNA synthesis precedes the maximum of cell numbers by one day. Mean and S.E.M. for each group of cultures is shown.

C. In vitro radioisotope assay

I. Effect of dose

Sixty cultures were set up in Leighton tubes. On day 6, four hours before harvest, they were randomly divided into six groups of ten cultures each, receiving the following doses of ^{3}HTdR in 0.1 ml. volumes: 0.125, 0.25, 0.50, 1.00, 2.00 or 4.00μCi. Counts per minute per culture were determined, and the means calculated and plotted. A linear increase in uptake is shown in Figure 15.

II. Effect of cold thymidine chase and proportion uptake of total dose

Sixty cultures were set up in Leighton tubes and divided into three groups of 20 each, receiving 0.50, 1.00 or 2.00 Ci ^{3}HTdR on day 6. Four hours later, 0.2 mg. of cold thymidine was added to 10 cultures from each group. This thymidine was combined in solution with the trypsin and EDTA used for harvesting.

The same doses of ^{3}HTdR added to the cultures (0.50, 1.00 and 2.00 μCi) were dispensed into ten liquid scintillation vials per group, a total of 30 vials. These samples, containing no cells or medium, were then treated exactly as the remainder of those in the experiment: solubilizer and scintillation cocktail were added, and the C.P.M. determined. The means

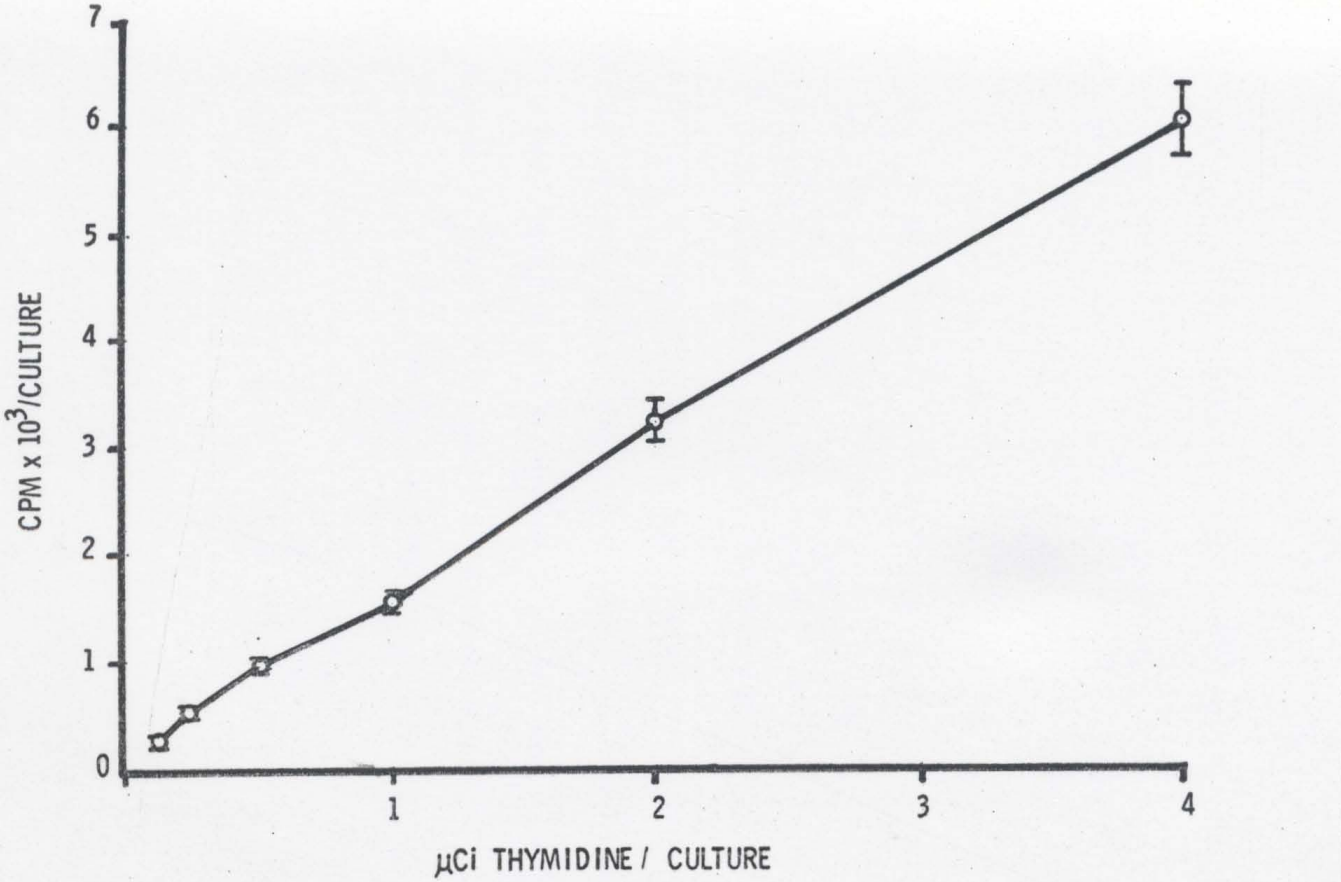

Figure 15. Dose-effect of tritiated thymidine on its uptake by primary rat kidney cultures. The mean and S.E.M. are shown for each group of ten cultures.

were calculated for each dose: (a) using the normal procedure, (b) followed by a cold chase, and (c) when added directly to the scintillation vials without any cell suspension.

Only at the highest dose did the cold chase effect a decrease in ^{3}HTdR uptake: cold chase cultures were 138 per cent of controls at 0.5µCi, 104 per cent at 1µCi and 88 per cent at 2µCi.

The percentage uptake of the total dose was consistently small: 0.49 per cent at 0.5µCi, 0.43 per cent at 1µCi, 0.53 per cent at 2µCi.

The blank control values ranged from 6 to 27 (mean 14) C.P.M., with no correlation with dose. It is usually the aim for the experimental sample to have a radioactivity at least ten times that of the background (Wolf, 1964), so the dose of 1µCi was chosen as optimal: the mean experimental C.P.M. at this dose is about 1500 C.P.M., which is about 100 times the blank control mean of 14 C.P.M. The cold chase effect was also taken into consideration in making this choice.

III. Effect of length of exposure

Fifty cultures were set up in Leighton tubes. Beginning on day five, ^{3}HTdR was added to groups of ten cultures at 20, 16, 12, 8 and 4 hours before harvest on day six. The mean C.P.M. per culture for each group was determined and plotted.

There was a non-linear increase in uptake with time, with the smallest variance at four hours (see Figure 16). This four-hour length of exposure was considered optimal since the mean counts per minute value is sufficiently high (about 1800 C.P.M./culture), and because the biological "natural" variation among the cultures makes it imperative to reduce variation wherever possible.

D. Search for a positive control: xanthopterin

I. Optimal length of exposure

Sixty cultures were set up in Leighton tubes. Thirty were chosen at random on day four, and 0.01 mg. xanthopterin (Xn) in 0.02 ml. HBSS added to each. The dose was determined by calculating the relationship of the culture medium volume to the blood volume of rats used by Haddow et al. (1972) and scalling down the dose given in vivo proportionally. The remaining thirty were control cultures, and 0.02 ml. HBSS was added to them. Ten each of control and experimental cultures were harvested 4, 28 and 52 hours later. The cultures were exposed to ^{3}HTdR for the four hours prior to harvest, and C.P.M. and number of cells per culture were determined.

Figure 17. is a plot of ^{3}HTdR uptake vs. unit number of cells per culture. At four hours, the Xn-treated cultures show higher mean C.P.M. and cell number values than the control group; it is obvious from the plot, however, that the high C.P.M. values are associated with high cell numbers. Since cell division could not be stimulated and completed within such a brief time, it must be by chance that four cultures with high numbers of cells and a high rate of DNA synthesis associated with this, were placed in the Xn group.

At 24 hours, we see the same correlation between number of cells and ^{3}HTdR uptake. Although the distribution of Xn-

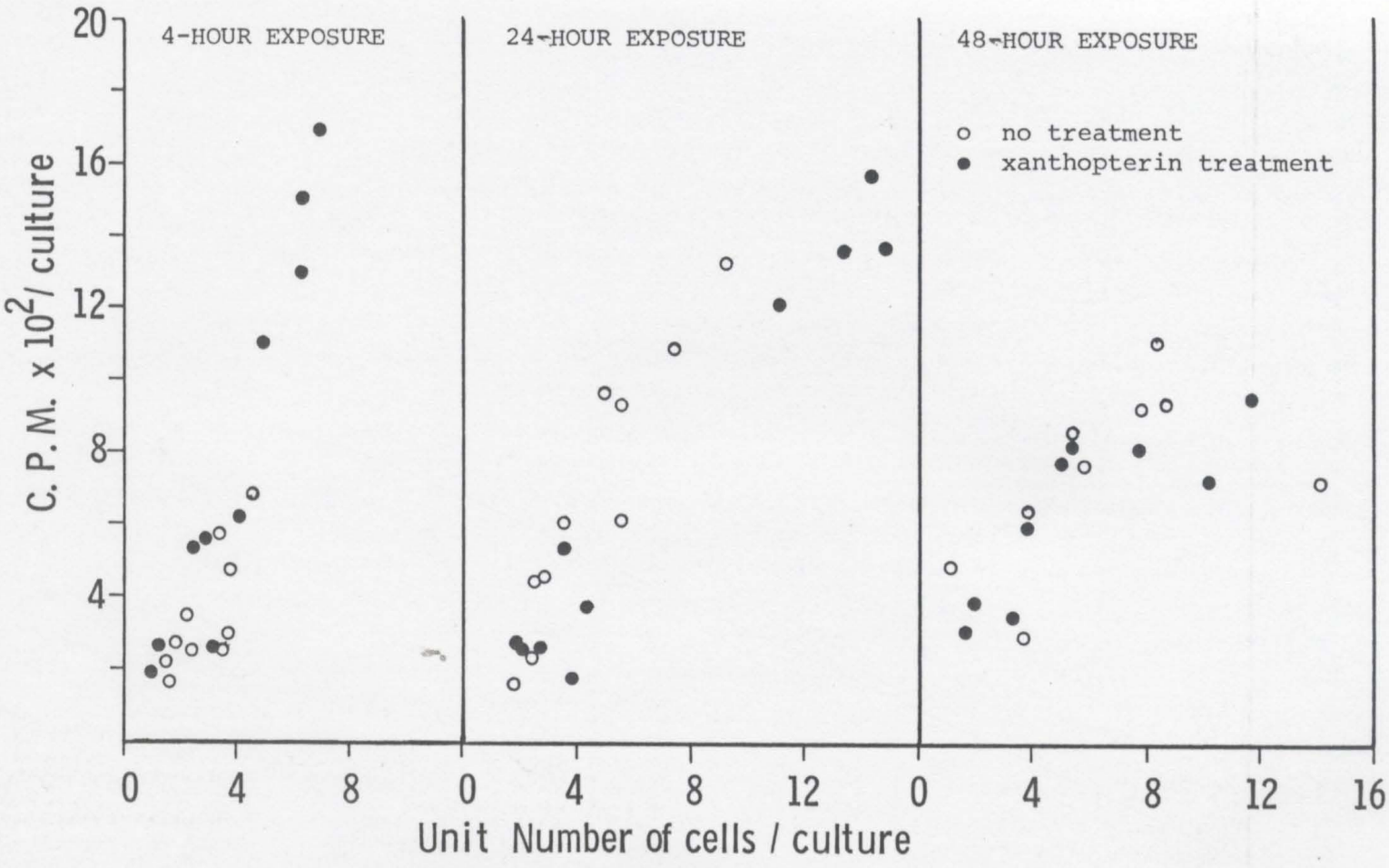

Figure 17. Effect of various lengths of exposure of primary kidney cultures to xanthopterin. Uptake of tritiated thymidine correlates with number of cells per culture. Treatment with xanthopterin has no effect on either parameter with exposure times of 4 - 48 hours.

treated cultures is bimodal, the mean values for ^{3}HTdR uptake and number of cells do not differ significantly from the control means. At 48 hours, the two groups are the same and show the same distributions.

II. Dose response curve

Fifty-six cultures were set up in Leighton tubes, and eight assigned to each of seven groups: on day 4 each culture received 0.001, 0.010, 0.025, 0.050, 0.075 or 0.100 mg. Xn in 0.02 ml. (See Materials and Methods section for technical details.) The cultures were harvested 28 hours later, and C.P.M. and number of cells per culture were determined. The results are shown in Figure 18.,where each point represents one culture. There is large variation among cultures in each group. No stimulation occurs at any dose, and the lowest and highest doses may be inhibitory.

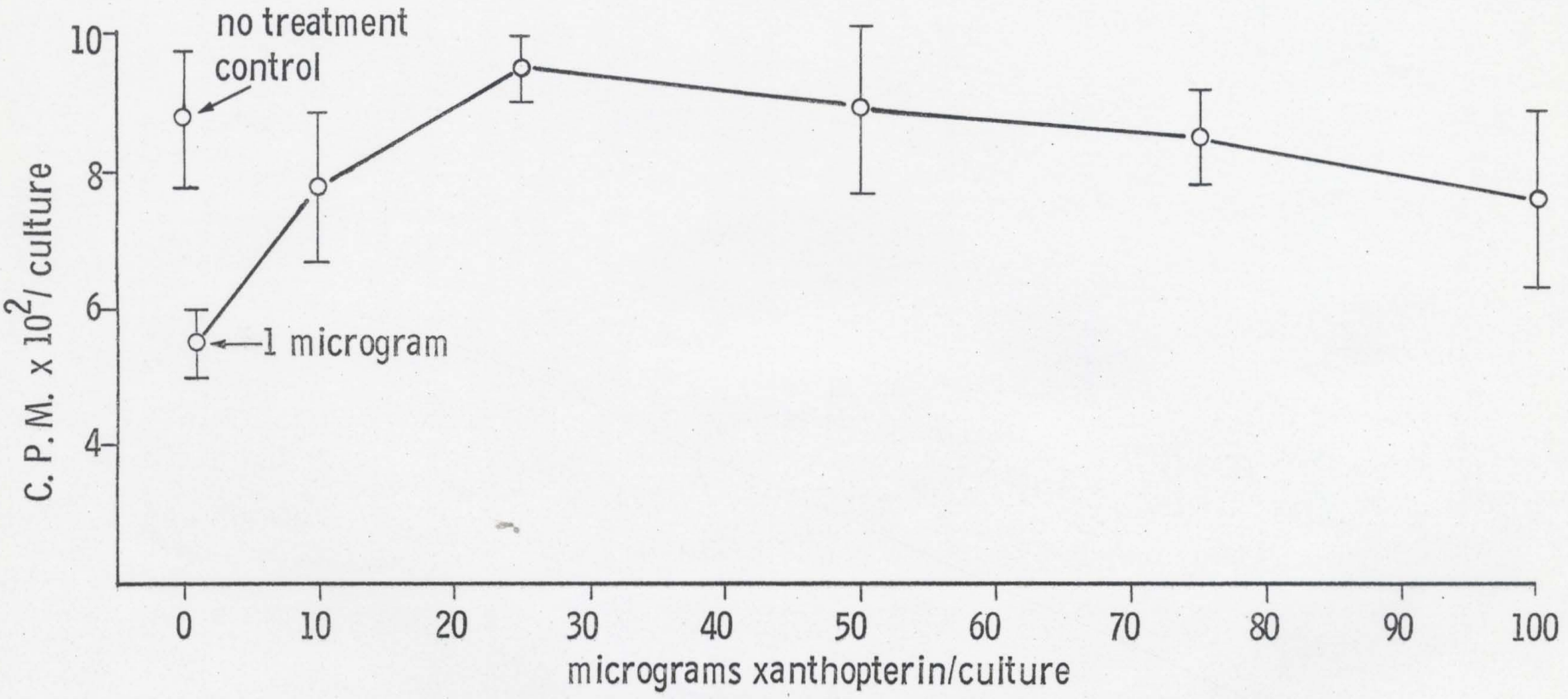

Figure 18. Effect of various doses of xanthopterin on uptake of tritiated thymidine in primary kidney cultures. Only cultures receiving the smallest dose tested differ significantly from those with no addition ($p = 0.02$). A stimulatory effect of xanthopterin is not seen at any dose.

E. Effect of tissue homogenate fractions on cultures

Reports in the literature indicate that intraperitoneal injection of the soluble fraction of kidney, but not liver, inhibits the compensatory renal growth that should follow UNx in rats (Dicker, 1972). The following experiments were designed to determine whether the soluble fraction of kidney homogenate would inhibit cultured kidney cell division. Liver soluble fraction was also tested.

The soluble fraction was prepared, as described in the Materials and Methods chapter, from homogenate of rat kidney cortex or liver tissue in 0.25M sucrose. Various doses of these preparations, derived from tissue ranging in weight from 0.1 to 100 mg., were added to five-day-old cultures with an automatic pipet (Selectapette, Clay Adams, Parsippany, N.J.). Control cultures had 0.25 M sucrose added. All doses were diluted so that the same volume was added to every culture, which was ten per cent of the medium volume. After twenty hours, ^{3}HTdR was added to the cultures according to the usual procedure, and harvesting was done at 24 hours. The uptake of ^{3}HTdR for each group is shown in Figure 19. The addition of sucrose decreased ^{3}HTdR to 83 per cent of that of untreated controls. The largest dose of both kidney and liver fraction, that from 100 mg. of tissue, gave lower than sucrose control uptakes; all other doses of kidney, and 10 mg. liver, resulted in uptakes not significantly different from controls; and the two smallest doses of liver

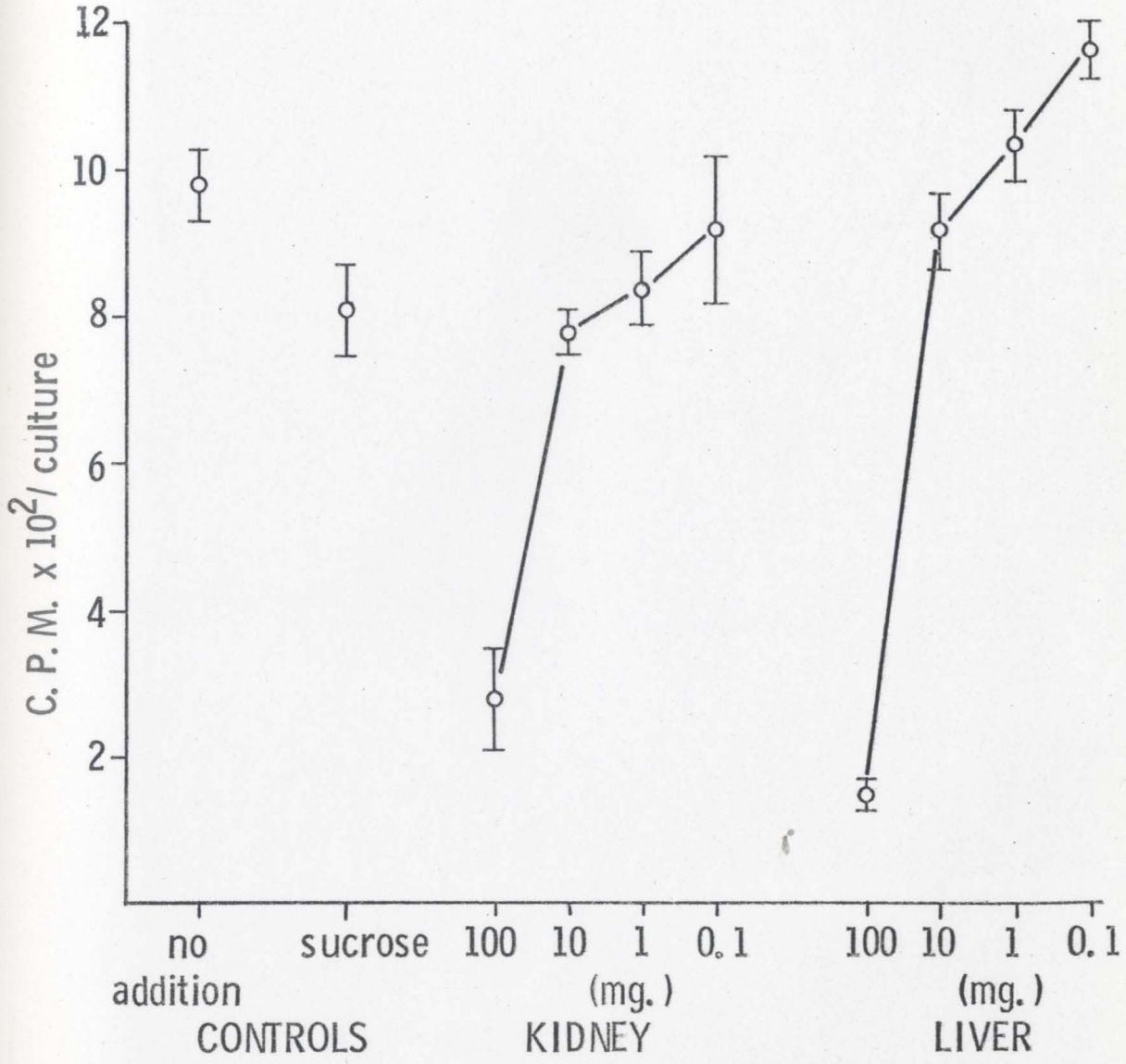

Figure 19. Dose-effects of soluble fractions of kidney and liver homogenates on uptake of tritiated thymidine by primary kidney cultures. Cultures were treated with soluble fraction of homogenates of the designated type and weight of tissue. The mean and S.E.M. is shown for each group of ten cultures.

fraction, 1 and 0.1 mg., were stimulatory. Both tissues produced dose response curves.

F. Rat serum experiments

I. Comparison of rat and calf sera

Sixty cultures were set up in flasks. On day three, twenty rats were sham-operated. They were bled 48 hours later, and their pooled serum added to thirty of the cultures, replacing fetal calf serum. Twenty-four hours later, on day six, the cultures were harvested, C.P.M. determined and the groups compared. Figure 20.shows a scheme of the procedure.

Cultures treated for 24 hours with serum from sham-operated rats had a significantly higher ^{3}HTdR uptake than parallel cultures maintained continuously in fetal calf serum. Each point represents one culture in Figure 21.,and the means differ at a level of $P < 0.01$.

II. Absence of non-specific effect of surgery.

In order to investigate the possibility that physiological disturbances due to the stress of surgery or the anaesthesia might have a stimulatory or inhibitory effect, fifty cultures were set up in flasks. On day three, ten rats were sham-operated. On day five, forty-eight hours later, these rats and ten unoperated rats were bled, and the sera added to twenty cultures for each group. Twenty-four hours later, the cultures were harvested, C.P.M. determined and the groups compared.

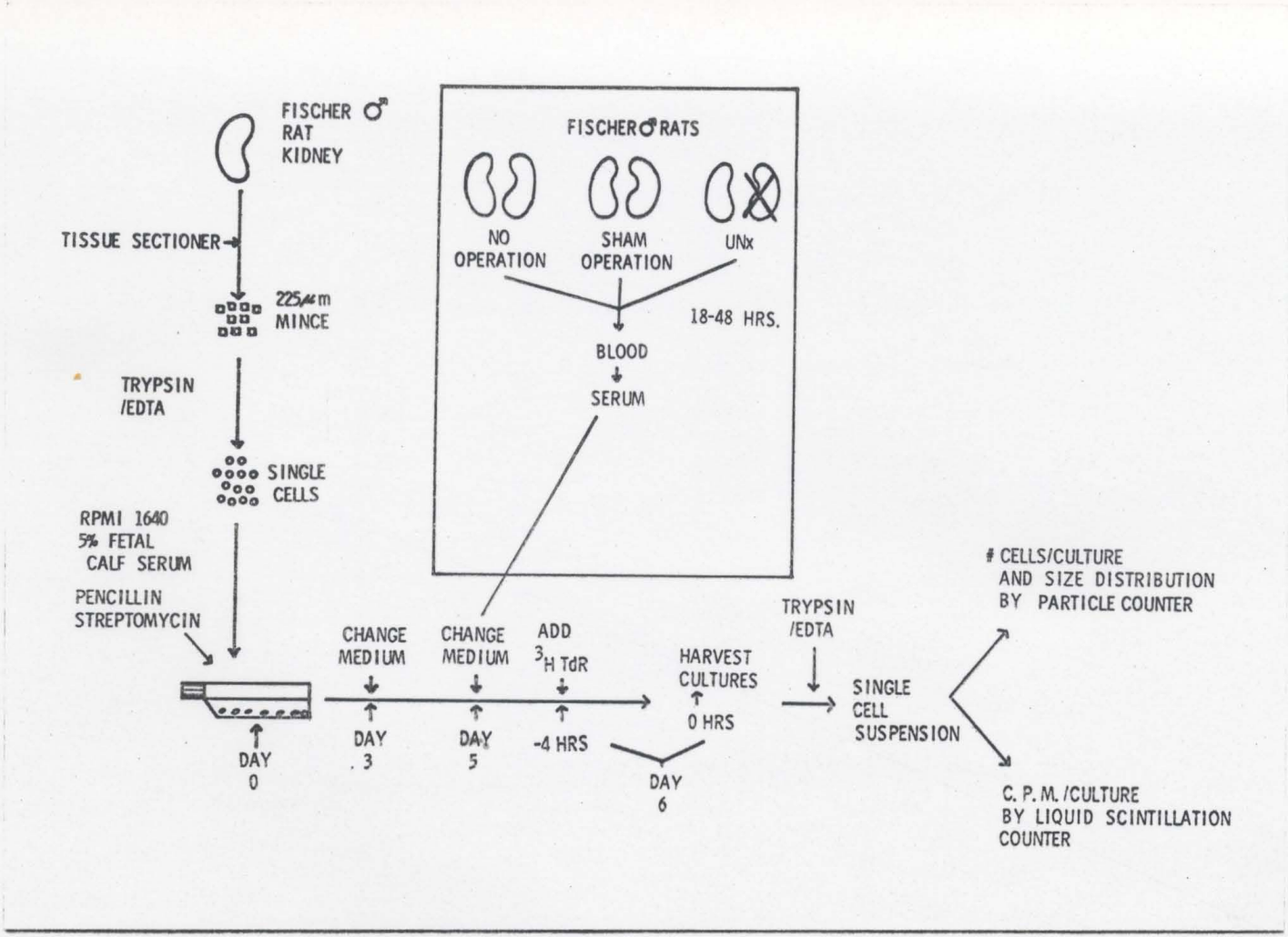

Figure 20. Scheme of treatment of primary kidney cultures with rat serum.

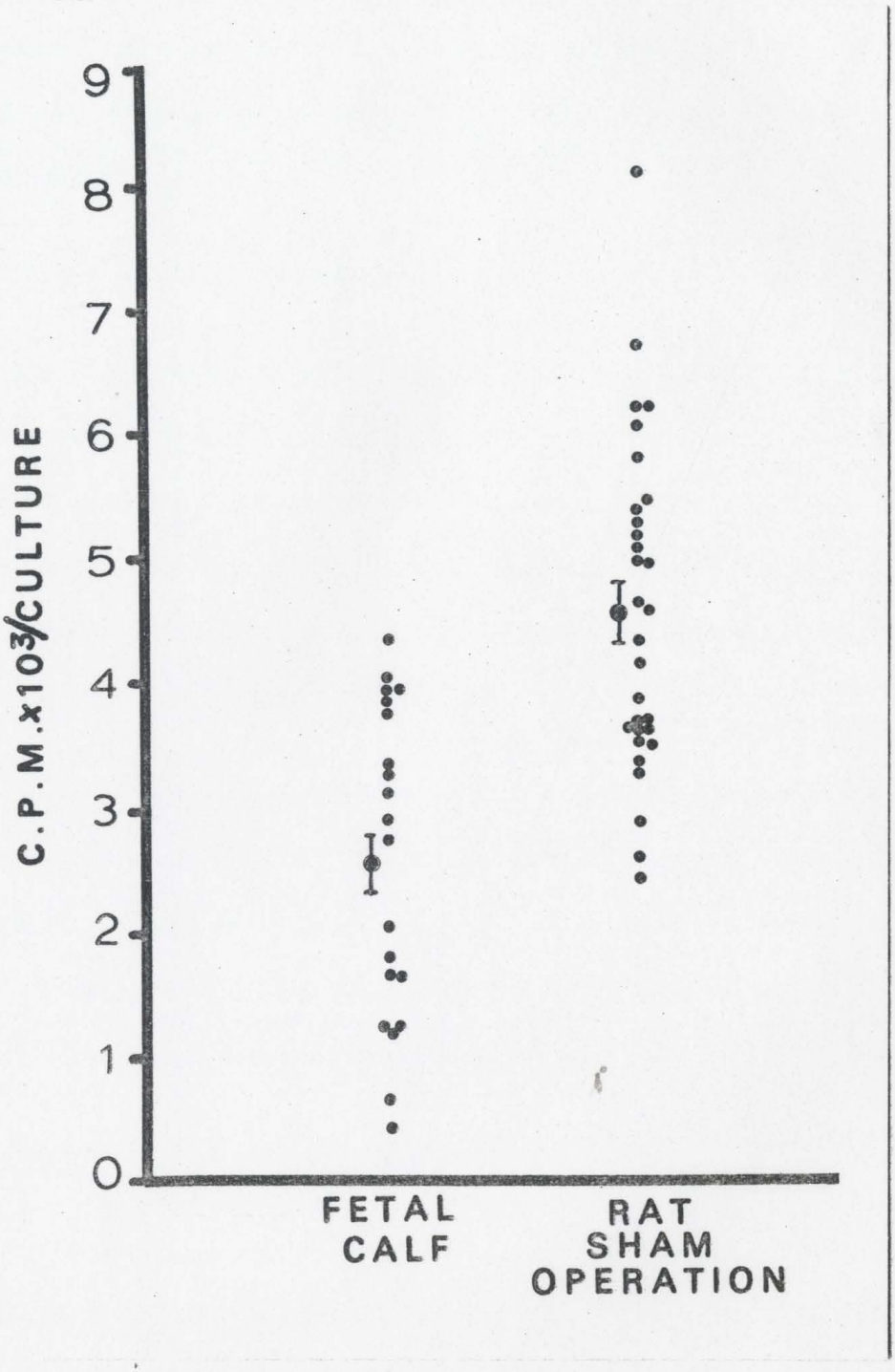

Figure 21. Comparison of effects of rat and fetal calf sera on primary kidney cultures. Serum from rats sham-operated 48 hours previously causes greater uptake of tritiated thymidine in cultures than does fetal calf serum. Each data point represents one culture, and the mean and S.E.M. of each group is shown.

The results are given in Figure 22,, where each point represents one culture. The groups do not differ ($p > 0.4$), indicating that there is no growth stimulation or inhibition due to surgery.

III. Effect of uninephrectomy

Eight experiments were done in which pooled sham and UNx sera obtained 48 hours after surgery from at least three rats were added to groups of 12 to 30 cultures on day five. Twenty-four hours later the cultures were harvested and C.P.M. determined. The ratio of mean ^{3}HTdR uptake - UNx/sham - was calculated for each experiment.

Table 11.summarizes the data from these experiments, and the 48-hour portion of Figure 23.shows the results, where each point represents the mean ratio for one experiment. The range of means ratios is from 1.03 to 1.40, all of which except one differ significantly from 1.00.

IV. Time course of UNx serum effect

Experiments were carried out as in the preceding section using serum pools obtained 18, 24 or 36 hours post-operatively. Two, six and one experiments were done, respectively. The ratios, C.P.M. of UNx/sham, for these three time intervals plus those for 48 hours are shown graphically in Figure 23.; the data are given in Table 11. Each point represents the ratio

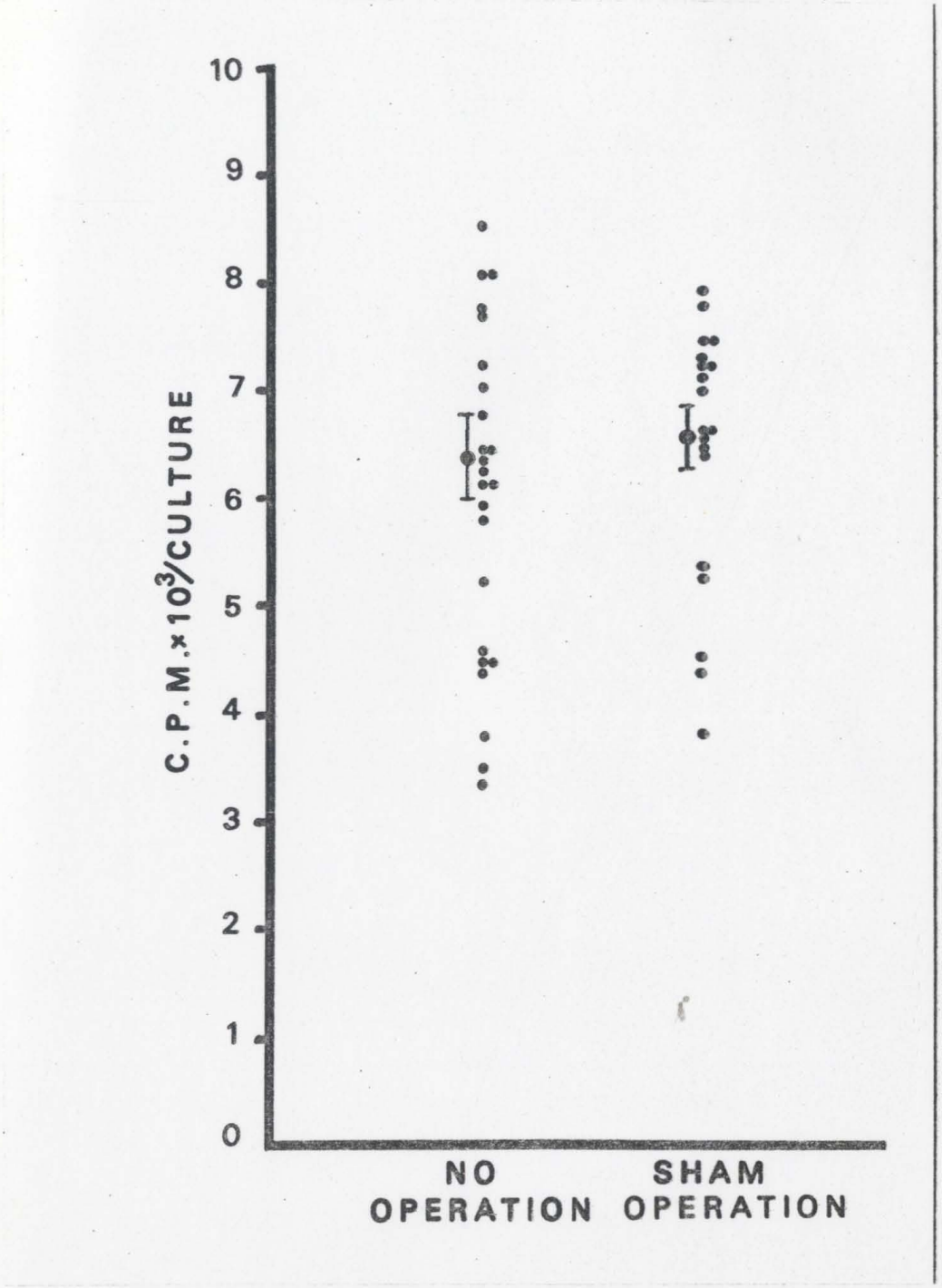

Figure 22. Absence of effect of surgery on primary kidney cultures treated with rat serum. There is no significant difference between uptake of tritiated thymidine in cultures treated with serum from unoperated rats or from rats sham-operated 48 hours previously. Each data point represents one culture, and the mean and S.E.M. of each group is shown.

uninephrectomized rat serum.

Experiment #	# hrs. post-op. serum collected	Sham serum # of cultures	Sham serum mean C.P.M./culture	UNx serum # of cultures	UNx serum mean C.P.M./culture	C.P.M. ratio UNx/S	C.P.M. ratio Significance P Value
1	18	12	760	12	750	0.99	0.45
2	18	15	840	15	810	0.96	0.90
3	24	15	630	15	680	1.08	0.10*
4	24	15	618	15	626	1.01	0.80
5	24	10	750	10	750	1.00	0.90
6	24	40	700	40	660	0.94	0.20
7	24	15	390	15	330	0.85	0.30
8	24	15	760	15	600	0.79	0.20
9	36	30	7140	30	7140	1.00	0.45
10	48	12	109	12	153	1.40	0.10*
11	48	30	303	30	389	1.28	0.01*
12	48	15	440	15	550	1.25	0.01*
13	48	12	154	12	191	1.24	0.10*
14	48	30	4600	30	5520	1.20	0.005*
15	48	30	259	30	299	1.15	0.05*
16	48	20	6610	25	7260	1.10	0.10*
17	48	30	1750	30	1800	1.03	0.45

* denotes a significant difference between sham and UNx groups

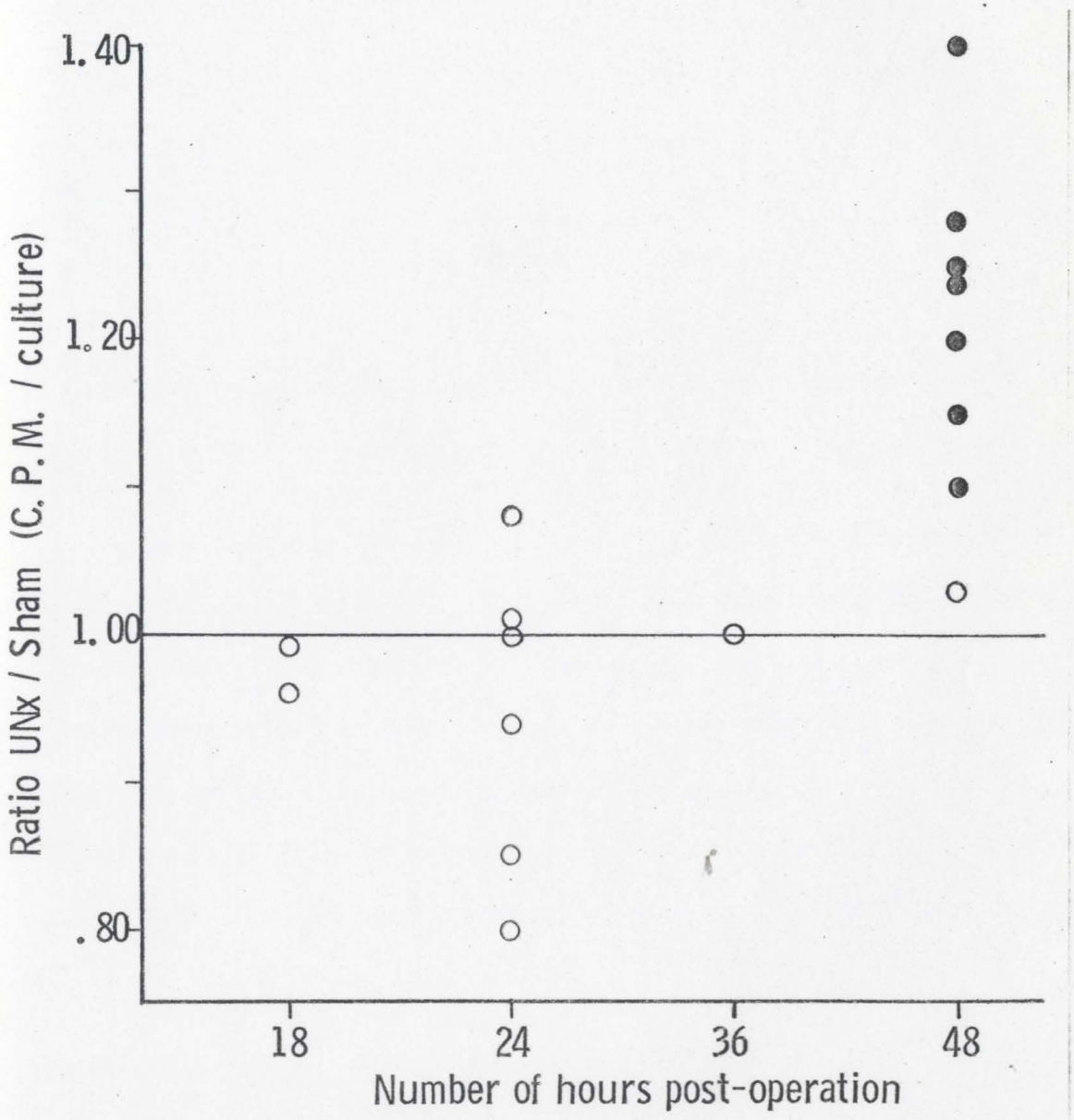

Figure 23. Differential effect on primary kidney cultures of treatment with sham-operated or uninephrectomized rat serum at various times after surgery. ● denotes a ratio derived from means of two groups which differ significantly. o denotes a ratio of means not significantly different.

from one experiment. There is no consistent growth stimulation until 48 hours.

V. Efforts to optimize UNx effect

Most of the kidney cells which divide in vivo following UNx are proximal tubule epithelial cells which lie entirely in the cortex. It is possible that the stimulatory effect of UNx serum on cultures could be increased by raising the proportion of these cells in the cell suspension used to set up cultures. Preparation of cultures from the cortex alone, instead of from the whole kidney, would achieve this end.

Sixty cultures were set up in Leighton tubes, thirty with cell suspensions derived from whole kidney and thirty from cortex alone. The dissected tissue was weighed and the appropriate dilution made so that the cells in each preparation were derived from the same weight of tissue. Each of these groups was divided into two, and 48-hour sham or UNx serum was added. The same two serum pools were used for both groups.

Tritiated thymidine uptake was determined and the group means compared. Figure 24.shows the results, with each point representing one culture. Comparison by t test of the two sham groups, and of the two UNx groups, reveals no significant difference; and in both cortex and whole kidney cultures, the stimulatory effect of UNx serum compared with

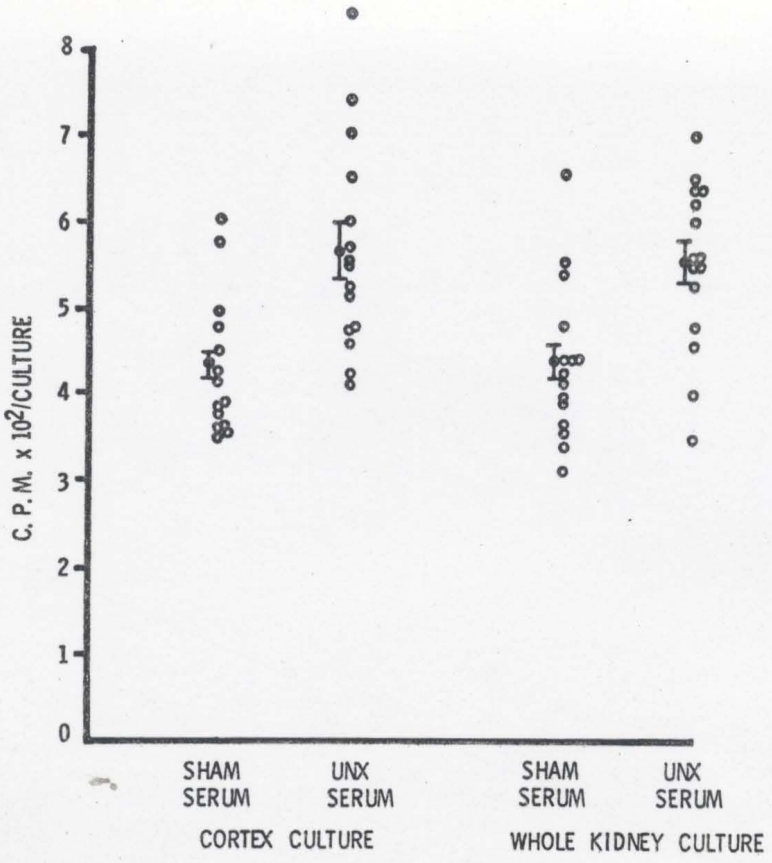

Figure 24. Comparison of effects of sham and uninephrectomy sera on primary cultures derived from whole kidney or from cortex alone. There is no significant difference between the two types of cultures when treated with the same serum pool. In both cases, uninephrectomy serum causes a significantly higher uptake of tritiated thymidine than does sham serum. Sera were obtained 48 hours after surgery. Each data point represents one culture; group means and S.E.M. are shown.

sham is significant ($p < 0.01$ in both cases).

The differential effects of control and UNx sera might also be increased by lengthening the time of exposure of cultures to serum from the usual 24 hours. An experiment was done in which cultures were maintained in the various serum types for six days. This type of experiment might also give information regarding the nature of the serum factor: is it stimulatory and in UNx serum, or is it inhibitory and in control sera? In the latter case, the differential effect would be seen in young cultures before the growth maximum is reached at four days; and in the former case, after the growth peak.

One hundred sixty cultures were set up in Leighton tubes; forty cultures each were set up in fetal calf serum, or rat unoperated, sham or UNx serum. The rat serum was obtained forty-eight hours after surgery. The medium was changed as usual on days three and five, keeping each group of cultures in its particular type of serum. Ten cultures from each group were harvested at 24-hour intervals on days three, four, five and six. C.P.M. were determined and the results plotted, as shown in Figure 25.

No significant differences exist between any two culture groups on days three and four. On days four, five and six, UNx serum cultures had higher ^{3}HTdR uptake than controls: the

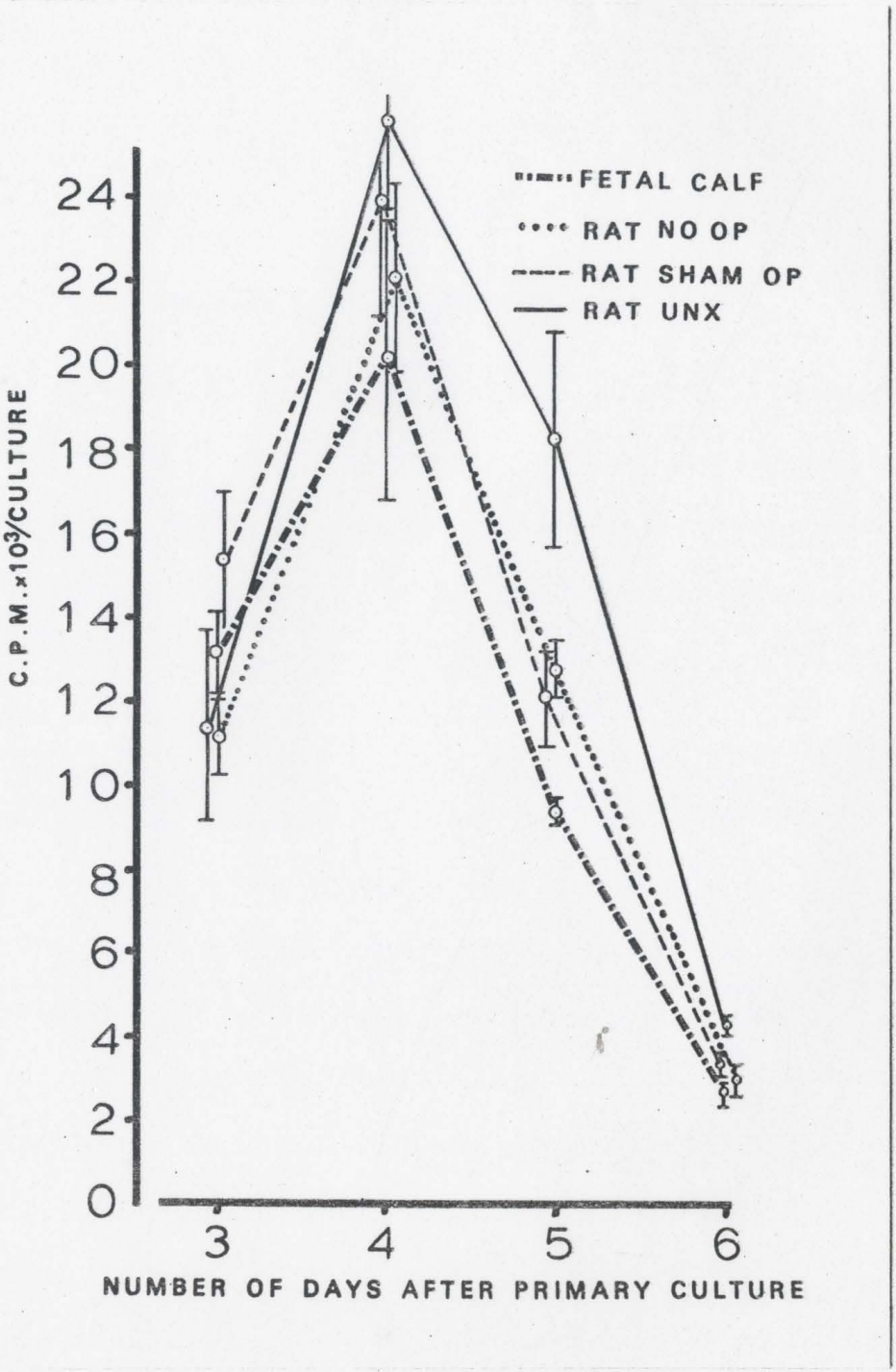

Figure 25. Differential effects of incubating cultures with various sera for three to six days. Serum obtained from rats uninephrectomized 48 hours previously causes significantly higher uptake of tritiated thymidine than controls on days five and six. Mean and S.E.M. are shown.

difference was not significant on day four, but the levels of significance were $p < 0.01$ on day five and $p = 0.02$ on day six. Sham and unoperated rat serum cultures did not differ significantly on any day. Cultures with fetal calf serum had the lowest uptake on days four through six.

VI. Effect of bilateral nephrectomy

It is possible that the postulated serum factor mediating compensatory renal growth is produced by the remaining kidney tissue. If so, bilateral nephrectomy would abolish the serum stimulatory effect on cultures. However, if the factor is derived from another source, removal of both kidneys should increase the stimulatory effect.

Two batches of ninety cultures were set up in tubes. On day three or four, groups of rats received either sham operation, UNx or bilateral nephrectomy (BiNx). The animals were allowed water but no food in the interval between operation and death since preliminary experiments had shown that fed BiNx rats died before 48 hours, probably due to uremia increased by ingested protein catabolism. Rats were bled on day five (some 24 and others 48 hours after surgery), and the various serum pools were added to groups of thirty cultures each. C.P.M. were determined, and the results compared and plotted (Figure 26.).

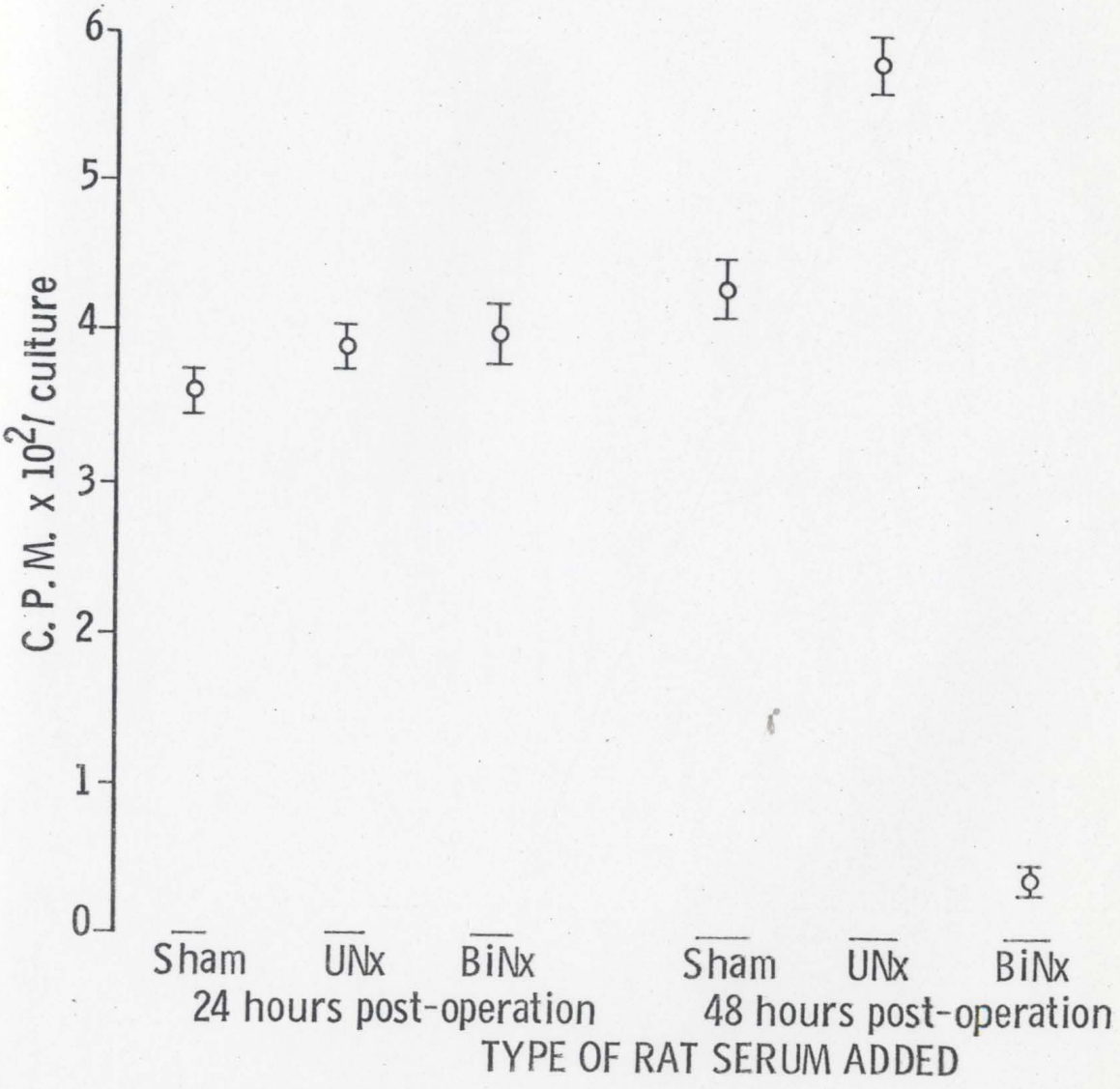

Figure 26. Effect of bilaterally-nephrectomized rat serum on primary kidney cultures. The effect of 48-hour BiNx serum on uptake of tritiated thymidine in cultures is an inhibitory one. Serum from rats 48 hours after UNx significantly stimulated uptake. The groups do not differ significantly at 24 hours. The mean and S.E.M. is shown for each group of 30 cultures.

At 24 hours, there is no significant difference between either sham or UNx, or UNx and BiNx cultures. At 48 hours, UNx serum cultures have a significantly ($p < 0.01$) higher ^{3}HTdR uptake than shams. BiNx serum has halted ^{3}HTdR uptake in cultures; C.P.M. in these cultures is no higher than background. Serum urea nitrogen content was determined for each 48 hour pool by a hospital clinical laboratory Autoanalyzer method. Sham serum contained 20 mg./100 ml. serum, UNx-21 mg. per cent, and BiNx - 272 mg. per cent.

G. Human serum experiments

Human data is understandably scarce in the area of investigation of compensatory renal growth. Limited information has recently become available from kidney transplant donors, e.g., radiological renal measurements and function estimations. Any information is of interest since the long-term goal of this research is to understand compensatory mechanisms in renal disease and in loss of renal tissue.

Human serum was made available for this investigation through Dr. H. Gault, Director of Dialysis Unit, General Hospital, St. John's.

Blood was obtained from two healthy, adult, human kidney transplant donors. Patient A was bled prior to surgery (time 0), and 24, 48 and 72 hours later. Patient B was bled 6, 30 and 72 hours post-operatively. A healthy adult human control was bled four times at 24 hour intervals. The sera were frozen at -70°C., and added to thirty cultures per serum sample within one week. Three separate experiments were done - one for each of the series of serum samples - and thirty cultures with fetal calf serum were used as controls each time. The mean C.P.M. was determined for each group, and the results were expressed as a percentage of the initial sample in each series.

The results are shown in Figure 27. There is variation among the normal human serum-treated cultures, but no significant difference between the first sample (time 0) and any of those following it. Both patients' sera produced increasing ^{3}HTdR uptake in cultures with time. Levels of significance for patient A were $p < 0.01$ when

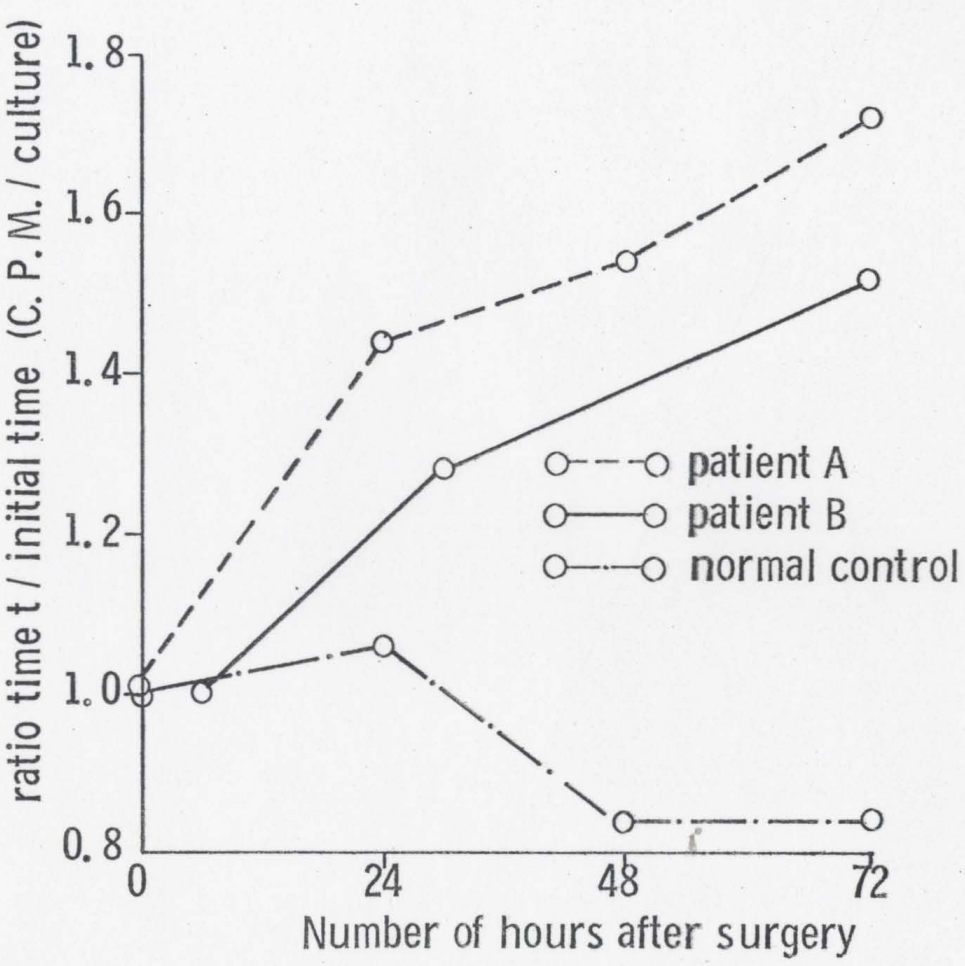

Figure 27. Effects of control and unilaterally-nephrectomized human sera on primary kidney cultures. Uptake of tritiated thymidine is stimulated significantly in cultures treated with serum from human kidney transplant donors. Comparison was made between the first serum sample of each individual and those which succeed it. There is no significant difference in the control series. The mean ratio is shown for each group of 20 - 30 cultures treated with a single serum sample.

24, 48 and 72 hour sera were compared with time 0 serum; for patient B, the levels were $p < 0.01$ at 30 and 72 hours, when compared to six hours.

DISCUSSION OF RESULTS

A. Compensatory renal growth in Fischer rats

The inbred albino rats used in the research for this thesis were Charles River CDF strain animals, derived in 1960 from sixty-eighth generation Fischer 344 rats. Immunogenetic homogeneity of the strain is assured by the supplier, who uses reciprocal skin grafting as a check.

A characteristic of the Fischer strain is its low rate of body weight gain compared with other strains, e.g., with Sprague-Dawley rats. Figures 1. and 4. show body weight data obtained from the literature for male Sprague-Dawley rats (Potter et al., 1969), and for Fischer rats (data supplied by Charles River Breeding Laboratories, Inc., Wilmington, MA).

In spite of the difference in rate of body weight gain between these two strains, there is no difference between the regressions of dry kidney weight (for two kidneys) upon body weight (Potter et al., 1969). Therefore, comparisons may be made of kidney weights expressed as a proportion of body weight.

There are age-related changes in compensatory renal growth, as discussed in the Review chapter. Since the rate of both body and kidney growth vary with strain, it is important to relate the various parameters of compensatory renal growth not only to body weight, but also to age.

The knowledge of growth rates is also vital for interpretation of the exaggeration of kidney growth which follows uninephrectomy; allowance must be made for the "background" upon which compensatory growth is overlaid. Compensatory growth in young animals can not be compared directly with that in older ones, for example.

Kidney weights expressed as a percentage of body weight for Fischer rats undergoing sham operation or UNx are shown in Figure 6. (Results chapter). All rats lost body weight from the effects of surgery; sham-operated rats showed an increased kidney weight as percentage of body weight on days 1 and 2. I interpret this to mean that although the rats lost body weight, their kidneys did not decrease in weight, or decreased to a lesser extent. There is a slight decrease in kidney weight as percentage of body weight over the next 21 days. This agrees with reports that the rate of kidney weight gain does not keep pace with that of body weight (Review chapter).

The left kidney, remaining after uninephrectomy, increased from about 0.44 per cent of body weight to about 0.59 per cent by four days; this is an increase of about one-third. Others have reported increases of between 30 and 40 per cent in rats (see Review chapter). A slight decrease is seen between two and three weeks, which may be interpreted to mean that compensatory growth has ceased by two weeks.

The mitotic index in the kidney cortex of rats 48 hours after sham or UNx surgery is shown in Figure 8. The UNx group mean is about 3.3 times the sham mean. There is overlap between the two groups, and a large scatter, especially among UNx rats. One is made aware of the wide biological variation in normal animals, including in this compensatory growth response.

Sources of variation have been recognized and controlled whenever possible as follows: (a) operations and sampling were done consistently at the same time of day to allow for diurnal variation; (b) particular attention was paid to the time interval between operation and sampling - it was 48 hours $\pm$ 15 mins. These rats do not show a steep peak of mitotic activity after UNx; the index begins to increase between 36 and 40 hours, is four times sham at 44 hours, and five times sham at its maximum at 52 hours (unpublished observations: Roberts, Hansen and Hyam).

Could some of the variation be due to clustering of mitoses? The counts were done over the area of 25 fields at 40x. D. Hyam (unpublished results) prepared a composite photograph of an entire longitudinal section of a kidney treated in the same way as the experimental ones. When the dividing cells in the cortex were marked, observers detected no indication of clustering; so this may be discounted as a source of error.

The correlation of proximal tubule cell hyperplasia with age was discussed in the Review chapter: in the growing unoperated rat,

the relative contribution of hyperplasia to kidney growth decreases at about six or seven weeks of age, with the onset of puberty. The rats used in the experiments reported here ranged in age from five to eight weeks, and in weight from 80 to 150 gm. Sexual maturity is attained sometime between eight and eleven weeks in these rats.

Figure 9.illustrates the uptake of tritiated thymidine (^{3}HTdR) by kidney cells in rats having had sham operation or UNx 48 hours before. As in the measurement of mitotic index, there is a large range of values among the UNx group, but the UNx mean value is about 2.6 times the sham - slightly less than the 3.3 difference seen with mitotic indices, but with less overlap between groups. The difference in increase over sham values between the two methods of assaying cell division is due to differences between the cell cycle times required for M and S phases, and the use of colchicine to accumulate cells in M over a four hour period. Both results are consistent with those reported for other rat strains of similar age (Bucher and Malt, 1971).

Uninephrectomy must be done with some care in order not to interfere with the adrenal gland lying in the fat surrounding the removed kidney. In the Fischer rat, the adrenal gland's blood supply usually derives from the aorta above the renal artery, but sometimes there is a small branch leading from the renal artery to the gland. In these cases, the renal vessels were ligatured distal to the gland branch in order not to interfere with its blood supply. The care taken was evidenced by the similarity in weight of the adrenal gland

from the operated side and its partner opposite (Table 10). Also, there was no difference between UNx and sham-operated rat adrenal gland weights. The danger of compromising adrenal function during uninephrectomy must be guarded against since mineralocorticoids are necessary for the normal mitotic response to UNx (Goss, 1965), at least in rats drinking tap water, as in the experiments reported in this thesis, rather than saline.

B. Characteristics of primary rat kidney cultures

Most of the published work on compensatory renal growth has been done on adult rats. If experimental data using *in vitro* methods is to be of value, it must be able to be related to the *in vivo* situation. I have taken this into consideration in designing my culture system. I have used kidney tissue from the same age and strain of rat that provided experimental sera - even though primary culture of adult tissues is usually less successful than culture of fetal or neonatal tissues. I have assured myself that the cells which are growing in culture are tubular epithelium, including probably a high proportion of proximal tubular cells. I have selected bleeding and incubation times with consideration of the cell division phenomena observed *in vivo*.

The kidney is composed of a large number of different kinds of cells, including epithelial types (podocyte; parietal Bowman's capsule; proximal, descending and ascending limb and distal tubule; macula densa; collecting duct dark and light cells; basal and surface transitional epithelia of papillae, calyces and pelvis; endothelium of the lymphatics and the glomerular and other blood vessels), connective tissue cells (fibroblasts in the kidney and composing its capsule; macrophages; mast cells; lymphocytes), smooth muscle cells around blood vessels -including juxta-glomerular renin-producing cells, nerve cells, polkissen and mesangial cells and pericytes. The use of whole kidney to initiate primary culture might be predicted to result in mixed culture of many of these cell types.

However, when this technique is used, as in this thesis, the result is a nearly homogeneous culture of cells of epithelial appearance. Relatively few (much less than one per cent) fibroblast-like and lymphocyte-like cells are seen, and only an occasional macrophage is observed.

The two cell types most frequent in kidney are blood vessel endothelium and tubule epithelium, and it is these latter cells which are growing under the culture conditions used; vascular endothelium has a distinctly different appearance *in vitro* (Willmer, 1965).

Electron micrographs (Figure 12) reveal long microvilli on most of the cells in these cultures; this is consistent with their derivation from proximal tubule, for the luminal surface of proximal convoluted epithelium is covered with abundant, long microvilli *in vivo*, while distal tubule cells have no brush border and only sparse, stubby microvilli.

Carley and colleagues (1976) report that an untransformed rat kidney epitheloid cell line displays a direct correlation between rate of cell division and presence of microvilli, and an inverse correlation between intracellular cyclic AMP level and microvilli. These authors postulate that cyclic nucleotides are controlling the cell surface morphology, as well as the rate of cell division. Since the observation of the ultrastructure of my cultured cells was done on day five, near attainment of confluency, the cells were not rapidly dividing and cyclic AMP levels were probably high; one would not expect to see *abundant* microvilli.

Other morphological characteristics observed in the cultured cells which are consistent with tubular cells *in situ* are apparent in Figure 11.: the cells are seen to align themselves as an epithelial sheet, with an orientation so that the microvilli project from the free surfaces, while the bases of the cells are adherent to culture debris. A dividing cell seen in this photograph still remains within the epithelial sheet; this appearance may also be seen in tubules from kidneys undergoing compensatory renal growth. Cultured fibroblasts, on the other hand, detach themselves from their neighbors and round up during cell division.

Visual observation of cultures led to the conclusion that after five or six days there were fewer, but larger, cells present than on day four. Final achievement of confluency appeared to be a result of cell size increase on days six and seven. This was confirmed by use of an electronic cell counter and sizer. The mean cell size on each day shown in Figure 13 (Results chapter), increased from about 13 µmdiameter (assuming a spherical shape) to about 17 µmat seven days.

The mean number of cells per culture was maximal on day five (see Figure 14, Results chapter), the decrease thereafter due to attrition of cells combined with a decreasing rate of cell division. The maximal rate of tritiated thymidine uptake was seen on day four, preceding the cell number maximum by one day and thus correlating well with it. Changing the medium, including serum, on day five precluded the possibility that the cultures' rate of cell division was decreasing due to lack of essential medium components. The growth

decrease was due to contact inhibition (Stoker, 1973; Carley et al., 1976), since the cultures were confluent over much of their areas by that time.

Establishment of these growth characteristics was necessary in order to select the appropriate time to treat cultures with experimental sera: (a) if the UNx serum contained a mitotic stimulator, its effect might be masked if it were tested in cultures in which the rate of cell division was increasing, i.e., during days one to four; (b) if sham or unoperated rat serum contained a mitotic inhibitor, its effect could be best expressed on day four or five, when cell division was maximal. Since treatment on day five would be appropriate for testing serum for the presence of either a stimulator or inhibitor, this day was chosen.

C. *In vitro* radioisotope assay

Uptake of tritiated thymidine was chosen as the assay for cell division in cultures because of (a) its good correlation with other criteria for cell division, e.g., mitotic index and cell numbers, both *in vivo* and *in vitro* (see data in Results section); (b) its frequent use as an assay for rate of DNA synthesis (and cell division) in published work relating to compensatory renal growth; (c) the efficiency and accuracy of this method, compared with direct measurement of cell division or cell numbers in cultures, as determined in this laboratory.

A criticism of the use of tritiated thymidine uptake as an index of DNA synthesis is that pool size changes in unlabelled (cold) thymidine will be inversely reflected by rates of uptake of the labelled compound. That is, if the thymidine concentration in serum from UNx rats is decreased compared with sham serum, the ^{3}HTdR uptake of cultures treated with UNx serum will be higher than those with sham serum, an effect unrelated to the rates of cell division in the cultures. Thymidine concentration in blood of rats having had sham operation or UNx has not been directly measured (see discussion of this in Review chapter), but the good correlation *in vivo* of mitotic index and labelled thymidine uptake after the first 24 hours post-UNx gives some reassurance that thymidine pool size changes are not producing a false indication of DNA synthesis. Further reassurance was derived from experiments in our laboratory in which I counted the total number of cells at harvest of cultures treated with sham

and UNx sera: the UNx cultures contained more cells than sham cultures after 24-hour serum treatment (Figure 28). The range of values was wider than that for ^{3}HTdR uptake, and the ratio of UNx/sham mean values was less high - but the correlation did exist.

Selection of the optimal dose of, and exposure time of cultures to, tritiated thymidine was done on the basis of straightforward dose-effect experiments. First, an exposure time of four hours was used to study the effect of dose: ^{3}HTdR uptake increased linearly with dose between 0.1 and 4 µCi/culture. This probably reflects increased rate of transport into cells as concentration in the medium increases since the amount added to the cultures is very small: 1.5 x 10^{-10} moles of thymidine provide 1 µCi of tritium, and only about 0.5 per cent of the dose is taken up in the range tested. A "chase" with 0.2 mg. (9.3 x 10^{-7} moles or about 5000 times the dose of labelled thymidine) of cold thymidine causes a decrease in uptake of tritiated thymidine at a dose of 2 µCi but not at 1 µCi, probably due to non-specific binding of thymidine to DNA at the higher dose. (An increase in uptake with chase treatment occurred at 0.5 µCi, and is inexplicable.) All things considered, the 1 µCi dose was chosen for use in these culture conditions.

Variation of time of exposure to ^{3}HTdR from four to twenty hours resulted in increased uptake with increased time but with the slope of the curve decreasing and the variance increasing as time increased. Consideration of these findings resulted in the selection of a four hour exposure time to ^{3}HTdR.

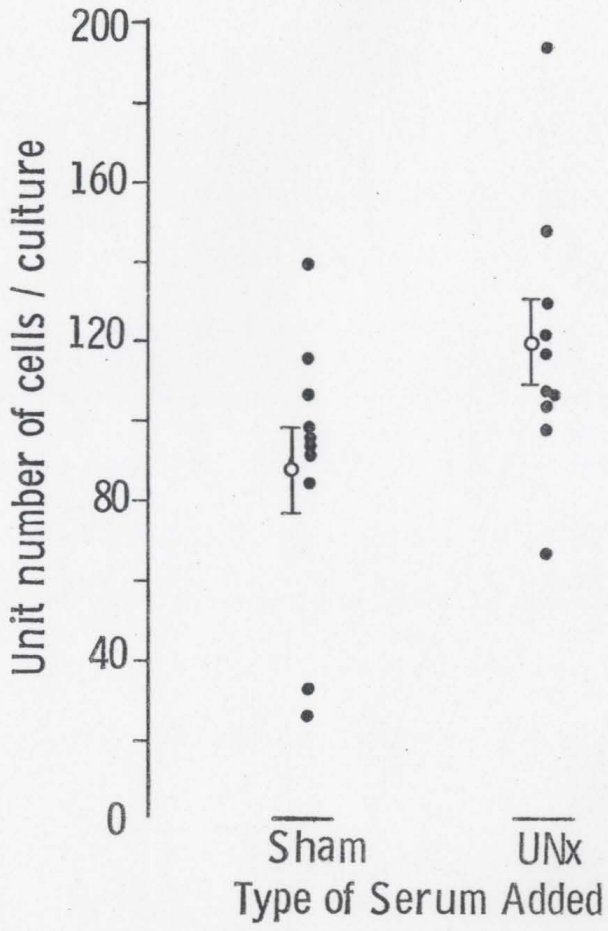

Figure 28. Differential effect of treatment with sham-operated and uninephrectomized rat serum on number of cells per culture at harvest. Serum obtained 48 hours after UNx has a significant stimulatory effect compared with sham serum. Each data point represents one culture, and the group means and S.E.M. are shown.

D. Search for a positive control: xanthopterin

Xanthopterin was first identified as a butterfly wing pigment, and subsequently as a component of mammalian urine. Its structure is shown in Figure 29. It and some other structurally-related compounds were found to stimulate mitoses in the rat kidney tubular epithelium (Haddow et al., 1972). Preliminary experiments showed that the effect of peritoneal injection into rats of xanthopterin was similar to that of uninephrectomy in time course and cell specificity, with an even greater rate of cell division. Xanthopterin is structurally related to folic acid, another compound which has been found to stimulate mitosis of the renal tubular epithelium (Baserga et al., 1968; Threlfall, 1968). Both folic acid (Taylor et al., 1966) and xanthopterin are known to crystallize in the tubules and cause blockage and damage, but carefully-designed experiments seem to have ruled this out as the primary cause for cell proliferation after folic acid administration (Hirsch and Hook, 1969; Paul et al., 1969); compounds related to xanthopterin which do not crystallize in the kidney retain the mitosis-stimulating property (Haddow et al., 1972).

The mechanism of action of these compounds, although not necessarily the same, may be a direct mitotic stimulus to the tubular epithelium. Haddow et al. (1972) have proposed that xanthopterin may act by virtue of its ability to inhibit xanthine oxidase, an enzyme which converts adenine derivatives to hypoxanthine and xanthine in the catabolic pathway leading to uric acid. The enzyme inhibition

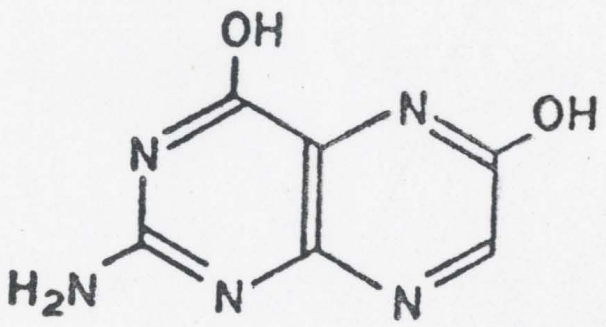

Figure 29. The structure of xanthopterin. This compound, a butterfly wing pigment and a normal component of human urine, stimulates cell division in renal tubules *in vivo*.

would presumably result in decreased concentrations of these products. Xanthines inhibit the action of phosphodiesterase, an enzyme which degrades cyclic AMP, resulting in increased intracellular cyclic AMP. A decrease in xanthine concentration should result in a decrease in cyclic AMP, which has been correlated with an increase in cell division (see Review chapter). The diuretic property of xanthines (such as caffeine and theophylline) may be relevant; they are thought to act by affecting sodium reabsorption or GFR or both. These ideas concerning the possible relationship of xanthine concentration and compensatory renal growth are not founded on any experimental data; their elaboration and testing constitute an interesting direction for future research.

My interest in xanthopterin not only concerned the compound itself, but also lay in the possibility that it is a direct-acting mitotic stimulator, in which case it could be used as a positive control in my culture system. The possibility of non-specific inhibition of growth of cultures is great, so that a positive control is desirable to separate false-negative results from "real" negative ones. To this end, I attempted to find conditions under which xanthopterin would stimulate kidney epithelial cell division in vitro. The attempt, albeit a preliminary one, showed no indication of stimulation of ^{3}HTdR uptake of the cultures by various doses of (Figure 18.) and lengths of exposure (Figure 17.) to xanthopterin.

E. Effect of tissue homogenate fractions on cultures

Experiments in which intraperitoneal injection of kidney homogenate resulted in inhibition of compensatory renal growth were discussed in the Review chapter. The possibility was raised that the growth inhibition was non-specific, and arose from poor nutrition consequent to peritonitis, or from side-effects of an auto-immune response in the kidney elicited by immunization with isologous kidney homogenate.

In an effort to circumvent these possible side-effects of in vivo treatment with kidney homogenates, Dicker and Morris (1974) tested soluble fraction of kidney homogenate on the growth of explants of renal cortex from neonatal rats and mice. They found inhibition of growth with cortex homogenate fraction from all species tested, but not with preparations of renal medulla, liver, spleen, duodenum or heart muscle. The amount of inhibition correlated with the protein content of the homogenate. The explants used in the assay were characteristic of those derived from adult tissues: their growth consisted primarily of an outgrowth of fibroblasts. The authors state that the cultures also contained some epitheloid cells, but relatively few. It is therefore difficult to relate these findings to the situation in vivo, where it is tubular epithelium and not fibroblasts which exhibit hyperplasia after UNx. This system is detecting a fibroblast inhibitory factor. My culture system contains only epithelial cells, and so constitutes a better system with which to test for a tubular cell-specific mitotic inhibitor

postulated to effect kidney morphostasis *in vivo*.

When various doses of soluble fractions of kidney cortex and liver homogenates were added to cultures, an inhibition, which was not tissue specific, of ^{3}HTdR uptake resulted from the largest dose tested, and a stimulation of uptake resulted from the two smallest doses of liver fraction only (Figure 19.). No other dose of kidney or liver resulted in uptake differing significantly from control values when each value was compared with the control by t test. However, if the results of treatment with either kidney or liver fraction is inspected in the figure, a dose-response effect is apparent.

There is no indication of tissue-specific growth inhibition in this experiment. The stimulatory effect of small doses of liver homogenate fraction is intriguing, and apparently not reported in the literature. Non-specific inhibition of growth of cultured cells is common but not well-understood. Further work is indicated in order to elucidate this non-specific growth inhibition as well as the low-dose stimulation by liver extract.

F. Rat serum experiments

Cultures incubated with control rat serum ordinarily showed greater uptake of tritiated thymidine than those growing in fetal calf serum. Figure 21. shows this effect in five-day-old cultures treated for 24 hours with serum from rats sham-operated 48 hours previously. Figure 25. shows the same effect when cultures were set up and incubated continuously for three to six days in either fetal calf or rat sham serum. It is not surprising that these cultured cells grow better in homologous serum: it is a common finding that such serum offers advantages in the culture of cells derived from adult tissues (Paul, 1965).

Significant differences between the effects of serum from unoperated and sham-operated rats on cultures were never seen. Figures 22. and 25. demonstrate this consistency in the two serum types both when cultures were exposed to them for only 24 hours, and when cultures were set up and maintained in them. The conclusion from these data is that the surgical techniques themselves, as well as the resulting disruption of the operated animals' feeding habits, have neither an inhibitory nor stimulatory effect on uptake of tritiated thymidine in cultures treated with serum from operated rats.

The chief finding of the work reported in this thesis is a consistent, significant stimulation of uptake of tritiated thymidine effected by treatment of cultures with serum from rats uninephrectomized 48 hours previously, but not from those operated on 18, 24 or 36 hours

previously. Figure 23. summarizes these results. The stimulation by 48-hour UNx serum is consistent at a significant level when the 363 cultures are considered in groups of 12 to 30 cultures, as they were arranged in the eight separate experiments reported. Some of the necessity for doing these numbers of cultures derives from biological and technical variation: the range of values within groups is wide, and there is therefore always overlap between groups. Every effort was made to decrease technical variation among replicate cultures, for example by using the most accurate volume dispensing equipment available, an improvement over the pipettes and hypodermic syringes commonly used for volume measurement in tissue culture work.

Awareness of the biological variation, evident in the effect of UNx on uptake of tritiated thymidine in vivo, led to the use of a minimum of three donors for each serum pool. In spite of this precaution, the ratios of 48-hour UNx/sham ^{3}HTdR uptake in cultures range from 1.03 to 1.40.

Experiments done in our laboratory (Roberts, Hyam and Hansen, unpublished results) show that the kidney cortex mitotic index in Fischer rats is still increasing at 48 hours; a maximum is reached between 52 and 56 hours. The S phase of the cell cycle in rat kidney epithelium is about seven hours (Pilgrim and Maurer, 1965) which indicates that maximum stimulation for cell division in Fischer rats occurs sometime around 48 hours at the latest. The scatter seen in these results might obscure the stimulatory effect of UNx serum when less than maximal, i.e., at 18 to 36 hours after UNx. There

are other possible explanations for the absence of effect at these times: (a) relative to 48 hours, few cultures were assayed at each preceding time, thus increasing the likelihood that small differences would be undetected; (b) the effect of UNx on cell division may have been decreased at 18 and 36 hours due to diurnal variation (see Review chapter) since these rats were operated on between 10 p.m. and 12 midnight, rather than in the morning hours as were 24- and 48-hour rats. This would probably have affected an increase only in the 36 hour experiment, since an increase in mitotic index *in vivo* is not seen until much later than 18 hours.

Absence of stimulation before 48 hours may be interpreted on other bases than postulating a mitosis-stimulating factor in UNx serum. As discussed above, a decrease in the thymidine concentration of serum at 48 hours after UNx would result in increased uptake of added labelled nucleotide cultures treated with this serum. Such an increased uptake would be falsely interpreted as an increase in DNA synthesis of the cultured cells. Endogenous thymidine depletion in serum might be due to its increased transport into kidney cells of the donor rat where it will be incorporated into newly-synthesized DNA. Although endogenous thymidine is not the primary precursor for deoxythymidine triphosphate (see also Review chapter), it does become incorporated in DNA as evidenced by the incorporation of exogenous labelled thymidine. However, circumstantial evidence from *in vivo* comparisons of isotope uptake and mitotic index is against the possibility that thymidine pool size changes account for changes

in its uptake *in vitro*. This can not be entirely ruled out until serum thymidine levels are determined after UNx or until mitotic indices of cultured cells are determined.

A phenomenon may be postulated in which there would be stimulation of DNA synthesis in cultured cells as a side-effect of the high rate of synthesis of DNA in the kidney of the serum donor. This idea, which has not been well-defined in the literature, involves secretion of a substance by dividing cells which will stimulate mitosis in other cells of the same type. Tumanishvili (1960) and Cain et al., (1976) have discussed the idea and reported experiments purportedly designed to test the hypothesis by determining the effect of tissue homogenate injection on homologous tissue, the same design as used by investigators searching for evidence of chalones. These authors report stimulation of homologous tissue by homogenates, but their hypothesis would have been better-tested by comparing the effects of homogenates of tissue with low and with high rates of mitosis, e.g., of normal compared with contralateral kidney after UNx or of normal with regenerating liver after partial hepatectomy. A stimulatory effect under these circumstances has not been reported, but an inhibitory effect has (Saitren, 1956).

A mechanism such as this would serve to amplify a mitotic response, and therefore must be tissue-specific if the stimulatory factor is humoral. Since it is a positive-feedback situation it is necessary to propose an additional mechanism for turning off cell division at the appropriate time.

If this assay were to be used for further characterization of the humoral growth factor which has been postulated, and which has been indicated by these experimental results, then optimization of the culture conditions would be desirable. Besides the efforts to minimize biological and technical variation already described, two other means were investigated: (a) raising the proportion of cells susceptible to the factor (i.e., proximal tubule epithelium) in the cultures; and (b) increasing the exposure of the cultures to the serum factor.

The first of these methods was unsuccessful in increasing the differential effect of sham and UNx sera: cultures derived from whole kidney or from cortex alone had similar uptakes of tritiated thymidine when treated with sham or UNx serum. Previous experience had shown that medullary cells did not grow well in these culture conditions. Many of the cells in the medulla have a hyperosmotic environment *in situ*, and may require similar conditions *in vitro*. So, the two types of cultures probably contained primarily cortical cells, whether or not medullary cells were initially present.

The second attempt at optimizing culture conditions did result in an increased differential effect of UNx and control sera. Cultures set up and maintained in the various serum types - fetal calf and unoperated, sham-operated and UNx rat serum - were harvested on days three to six; UNx serum produced higher tritiated thymidine uptakes on days four to six than did controls, the differences being significant on days five ($p < 0.01$) and six ($p = 0.02$) (see

Figure 25.).

This experiment also sheds some light on the question of the mode of action of the serum factor. The effect of a mitotic inhibitor, present in control sera, would be apparent on day three, and possibly on day four, when cultures are growing at an increasing rate; i.e., cultures treated with control sera would show a lower uptake of isotope than would UNx cultures. This is not the case; instead the differential effect of the sera is certainly seen on days five and six, and possibly on day four. Mitotic stimulation by UNx serum, rather than inhibition by control sera, is being detected in cultures which are in the phase of declining rate of cell division.

Another attempt to increase the differential effects of control and UNx sera consisted of treating cultures with increased concentrations of sham or UNx sera for 24 hours on day five. Concentrations of serum in the medium which were tested are two, five, ten and fifteen per cent; all other experiments in this thesis used five per cent serum. Pools of serum from sham and UNx rats operated on 48 hours previously were combined with medium in the desired concentrations, and the same total volume was added to cultures of each group. Uptake of tritiated thymidine was assayed and a t test was performed to compare sham with UNx C.P.M. for each serum concentration. The results are plotted in Figure 30.

Increasing the serum concentration from five to fifteen per cent appears to inhibit thymidine uptake in the sham cultures but not in

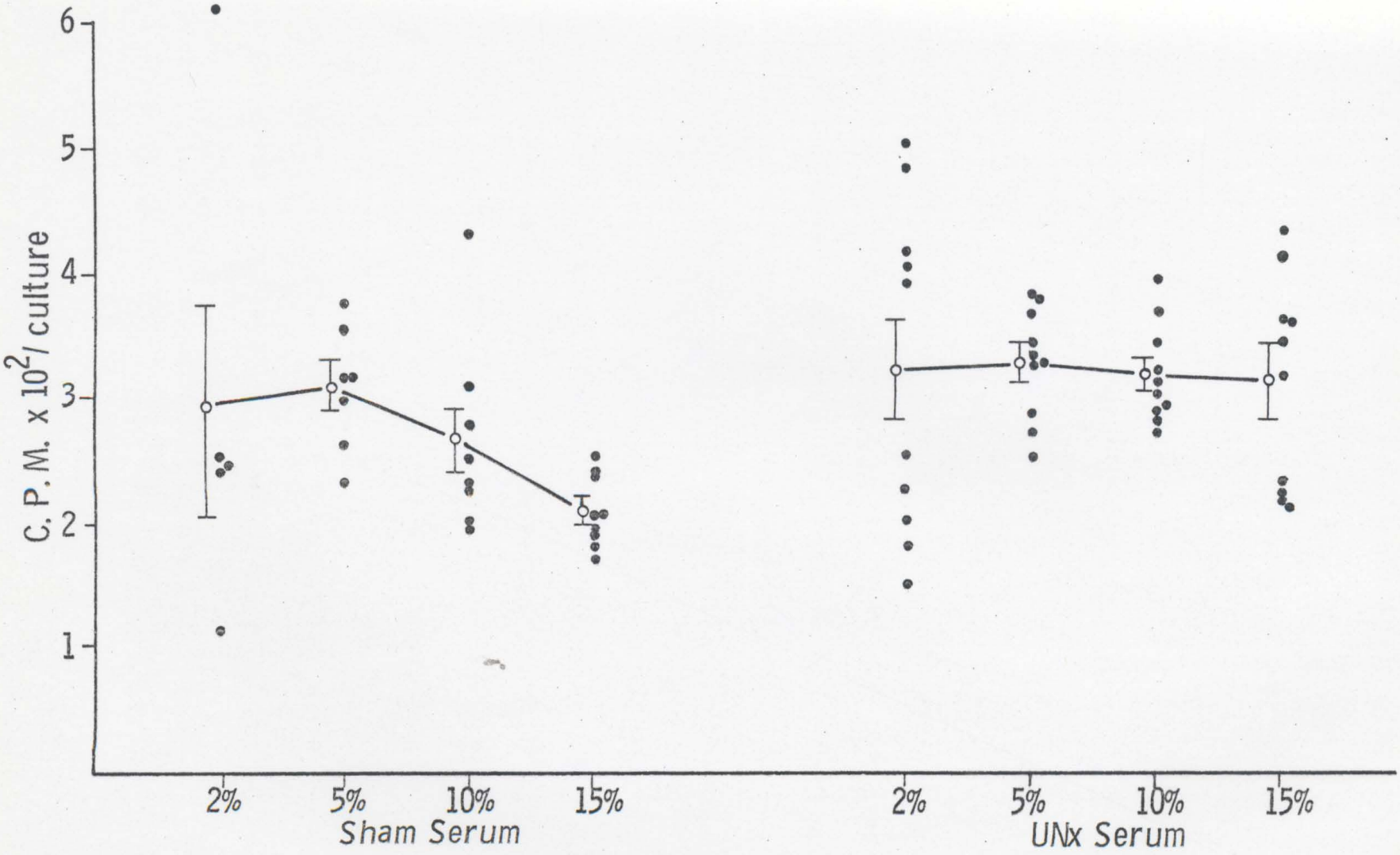

Figure 30. Comparison of dose-effects of 48-hour sham and UNx rat sera on uptake of tritiated thymidine by primary kidney cultures.

UNx. If comparisons are made between sham and UNx groups at each serum concentration, it may be argued that the UNx sera contain a stimulatory factor that overcomes a possible general inhibitory effect of high serum concentration (Lozzio et al., 1975). An alternative explanation is that there is a kidney-specific inhibitor (a chalone) present in the sham serum and not in the UNx serum. These two possibilities are not distinguishable in the present experiment.

These results, however, are equivocal, and their interpretation uncertain, because the number of cultures used per group is small. (Where there are less than ten data points in a group cultures had been discarded due to pH changes in the medium - a standard practice in all experiments reported in this thesis.) Ideally, there should be about 30 cultures per group, giving a total of 240 cultures.

The time required for manipulations of cultures (such as changing medium, gassing, adding isotope, harvesting), and therefore the variation among replicates, increase with the total number of cultures. It has been my experience that handling more than about 100 cultures may result in unacceptable variation. On the other hand, too few cultures give unreliable data. The plotting of means or variances of increasing numbers of replicate cultures (Finney, 1964; Morley, 1974) indicates that the minimum number of replicate cultures necessary for a reliable estimate of the mean is about 15 cultures, which means setting up somewhat more than 150 cultures in order to be certain of having 120 cultures in good condition on day five. This experiment can not be done in two parts, since there

is variation among the batches of cultures set up at different times, and comparisons can not be made from experiment to experiment. This necessity to include all groups in one experiment and the limitation on total number of cultures which can be handled in one experiment means that the largest feasible number of groups of 15 cultures each is five or six, allowing for discarding of cultures not in good condition.

It is important to carry out these dose-response experiments properly: however, this must await improvement in the culture methodology which reduces variation among replicates or allows larger numbers of cultures to be handled in an experiment.

The source of the mitotic stimulator found in serum is unknown. Ross et al. (1974) demonstrated that eviscerated rats maintained by parenteral alimentation were capable of compensatory renal growth comparable to that of non-eviscerated UNx controls. They measured kidney weight, and total RNA/DNA ratios two days after uninephrectomy. One can therefore conclude that the stomach, intestines, pancreas and spleen are not necessary for this growth, and none can be the source of the serum factor.

The growing contralateral kidney itself must be considered a potential source of the factor. Investigators have used serum from bilaterally nephrectomized donors in various experimental designs in order to test this hypothesis. Lytton et al. (1969) produced parabiotic pairs of rats by joining their skin. They reported that UNx in one rat resulted in increased uptake of tritiated thymidine

by the kidneys of its partner, while BiNx did not. The authors concluded that kidney tissue is necessary for the stimulation of DNA synthesis, but there are a number of reasons why this conclusion is unwarranted: (a) if the assumption is made that humoral factors are exchanged between parabiotic pairs (and the authors obviously do), then kidney tissue is present in the system; (b) the parabiosis technique used is insufficient for maintenance of homeostasis in the bilaterally nephrectomized animal, as stated by these authors and as shown by Sibley and Huggins (1946); (c) uremia developing in one animal may have deleterious effects on its partner, including inhibition of compensatory renal growth; (d) small numbers of animals were used, and no data concerning body weight changes were given. There is therefore a possibility that the lack of response to bilateral nephrectomy is spurious.

Vichi and Earle (1970) found equal stimulation of tritiated thymidine uptake by kidneys of intact rats injected with serum from rats who had received UNx 48 hours or BiNx 36 hours previously. The urea nitrogen level of the BiNx serum was elevated: 117 mg/100 ml.

Preuss et al. (1970), Goldin and Preuss (1974) and Preuss and Goldin (1975) used an *in vitro* technique, measuring labelled uridine and thymidine uptake by rat kidney slices incubated with sham, UNx or BiNx serum from rats operated 24 hours previously. Uptake of both precursors was stimulated by UNx serum, but not by BiNx serum, which resulted in the same uptake as sham of uridine and in a lower uptake of thymidine. These authors conclude that the presence of

kidney tissue is necessary to generate the postulated serum growth factor. The possible inhibitory effect of uremic serum from BiNx donors was not investigated.

No conclusion on the necessity of kidney tissue for the production of kidney-specific growth may be drawn from a survey of these reports. Data are sparse and conflicting. The influence of uremia on the assay methods used has not been investigated.

The results of my experiments indicate that 48-hour BiNx serum is highly inhibitory to tritiated thymidine uptake, since uptake was decreased to background levels in cultures so treated, as shown in Figure 26. Urea nitrogen in this serum was over ten times the normal value. No stimulation or inhibition of uptake by any type of serum is seen 24 hours after surgery. Thus, the inhibition of uremia obscures whatever effect BiNx serum might have by virtue of its containing a kidney-specific growth factor. Stimulation by BiNx serum injected into intact rats (Vichi and Earle, 1970) may have occurred in their system, and not in the others reviewed, because the recipient's renal function was able to cope with the relatively small amount of uremic serum injected, and the kidneys were thus able to respond to the injected growth factor.

The origin of the humoral growth factor involved in compensatory renal growth remains obscure. Its elucidation will not be easy, since the side-effects of removal of tissues and organs are difficult to control.

It is recognized that a wide variety of cell growth stimulators (Holley, 1975) and inhibitors (Lozzio et al., 1975) are present in the sera used in these culture experiments: many of these are non-specific (insulin, for example), while others, such as the somatomedins, may be tissue-specific, and might therefore be implicated in the mediation of compensatory renal growth. Experimental observations and theoretical considerations both indicate that the factor in question is kidney tissue-specific, and confirmation of this should be possible using my culture system.

G. Human serum experiments

The long-term goal of much basic biological research lies in its eventual application to the human. When humans can not be used for the research, various laboratory animals are employed as models. As long as the results obtained in this way are relevant to human biology, the use of animals offers some advantages, among them availability of replicate (homologous) animals and of strains with specialized genetic traits. When non-invasive techniques are insufficient for the research at hand, as in the study of compensatory renal growth, the use of laboratory animals is an ethical necessity. In this situation, it is desirable to corroborate animal findings in humans whenever there is a situation which allows it. Such an opportunity is found in human kidney donors for transplantation. That the phenomenon of compensatory renal growth occurs in humans has been established, using functional studies and radiological measurement of kidney size after uninephrectomy in healthy individuals (see Review chapter). The only report of the testing of human serum in an assay system for kidney-specific growth factor is one by Vichi and Earle (1970), who injected intact rats with serum from rats and humans and measured uptake of tritiated thymidine by the recipients' kidneys. While UNx rat serum stimulated uptake, that from humans did not. The authors tested serum from humans two and seven days after UNx in a patient with renal vascular hypertension; before and two days after a patient with chronic renal insufficiency, being treated by hemodialysis, received bilateral nephrectomy; and from a patient

with chronic renal insufficiency, with a high blood urea nitrogen level. The authors interpreted these results to mean that the growth factor is species-specific. Other authors, using a serum injection experimental design (Silk et al., 1967) or an *in vitro* method (Ogawa and Nowinski, 1958) have found that the factor is not species-specific. These investigators used mice, rats and dogs. (Further discussion and criticisms of these experiments have been presented in the Review chapter).

Human serum from healthy, uninephrectomized donors stimulates tritiated thymidine uptake in my culture system. Figure 27. shows an increase in uptake correlating with time after surgery for two patients. Serum from a normal, unoperated control produced no change in uptake over the time studied.

Only two human experiments were done when the kidney transplantation program was temporarily discontinued. Its resumption will allow further *in vitro* studies. The dearth of information about compensatory renal growth in humans makes this line of investigation worthwhile, and the obtaining of serial serum samples from one individual, each large enough to treat a group of replicate cultures, is a good experimental design alternative to the use of many small animals to provide serum pools.

SUMMARY OF EXPERIMENTAL WORK AND CONCLUSIONS

This thesis describes a method for primary culture of kidney epithelium derived from young adult Fischer rats. The growth characteristics of the cultures are described: the maximum uptake of tritiated thymidine by cultures occurred on day four, and confluency was reached on day six or seven. The morphology of the cells was compatible with their derivation from proximal tubular epithelium. Treatment of cultures with xanthopterin, known to stimulate mitosis of renal epithelium *in vivo*, did not result in increased isotope uptake *in vitro*. It has been suggested by others that injection of kidney, but not liver, homogenate fractions inhibits compensatory renal growth *in vivo*. Treatment of cultures with soluble fraction of either kidney or liver homogenate showed dose-response effects, with the higher doses tested inhibiting isotope uptake. An unexpected finding was that the smallest dose tested of liver homogenate fraction was stimulatory.

Five-day-old cultures of kidney epithelium were treated with various rat sera for 24 hours. The chief finding reported in this thesis is that treatment with serum from rats uninephrectomized 48 hours previously resulted in a consistent, significant increase in uptake of tritiated thymidine by cultures, when compared with control sera. Serum from rats uninephrectomized 18 - 36 hours previously was not consistently stimulatory. Serum of unoperated rats did not differ from that of sham-operated ones, and both of these control sera resulted in a higher uptake of tritiated thymidine than did fetal calf

serum. When tritiated thymidine uptake was assayed daily in cultures set up and maintained in the various serum types, the differential effect of UNx and control sera was expressed after day four, while the rate of DNA synthesis was decreasing. This may be interpreted to mean that the UNx serum contained a stimulatory factor, rather than sham serum an inhibitory one (a chalone). Serum from bilaterally nephrectomized rats was no different from controls when obtained 24 hours after surgery, and was inhibitory to isotope uptake in cultures when obtained at 48 hours, an effect which may have been correlated with a high urea nitrogen content. In preliminary work, serum obtained from human kidney transplant donors resulted in isotope uptake increasing with time after uninephrectomy, while serial samples of human control serum did not differ among themselves.

This culture system, especially if it can be modified to reduce culture-handling times and variation among replicates, will lend itself to characterization of the serum factor, using either laboratory animals or healthy human kidney donors as a source of serum. Experiments could then be done to investigate the stability, dialyzability, and species- and tissue- specificity of the factor. It would be worthwhile to continue investigation of the inhibitory effects of kidney and liver homogenates, and of the unexpected finding that low concentrations of liver homogenate are stimulatory.

Review of the literature on compensatory renal growth indicates the probable existence of a circulating, kidney-specific growth factor. It was seen that _in vitro_ methods could be profitably used for confirmation and characterization of the factor, since they allow

dissociation of its effects from the complex physiological changes *in vivo* which follow uninephrectomy and which may also play a role in mediation of the compensatory growth. The culture system described in this thesis is a suitable tool for such investigation in laboratory animals or humans.

A differential effect of sera from sham-operated and uninephrectomized rats on uptake of labelled thymidine by cultures has been demonstrated. The temporal characteristics of the factor being detected by this culture system make it unlikely to be the primary stimulus for compensatory renal growth, a conclusion corroborated by theoretical considerations. The complexity of the compensatory renal growth response makes its elucidation by any one experimental design unlikely: information will come from, and give insight to, many and diverse areas of investigation. An understanding of the control of kidney growth in particular, and of cellular growth in general, will not be reached in the near future. Some think that definitive elucidation of the mechanism and control of mitosis can not be expected until sometime in the twenty-first century (Crick, 1970). I hope that the work begun with this thesis will contribute to this understanding.

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