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The control of compensatory growth in liver and kidney has been studied with particular reference to the early events in the process. It has been shown that immediately after unilateral nephrectomy there is no significant change in the glomerular filtration rate of the surviving kidney, though its blood supply increases by about 25%. As might be anticipated, an animal which has thus suffered a loss of half its renal function shows accumulation of urea and creatinine in its plasma. Perhaps surprisingly, on the other hand, the operation appears to cause no serious metabolic upset since plasma amino acid levels were remarkably unaffected.

Changes in renal function concomitant with the kidney growth produced by feeding a high-gelatin diet for three days were compared with the changes accompanying compensatory renal hypertrophy. The high-gelatin diet caused a significant elevation of glomerular filtration rate but an even greater increase in kidney weight. Contrary to unilateral nephrectomy the high-gelatin diet did not result in an increase in renal blood flow.

A preliminary investigation was made of the biochemical events in the early stages of compensatory renal hypertrophy. Measurements of RNA synthesis in vivo, RNA polymerase, DNA polymerase and heterodisperse nucleoplasmic RNA metabolism showed no marked changes. The accumulation of RNA, detectable after 12 hours, is the first unequivocal sign that kidney growth is underway. This increase in RNA content can be abolished if the animal is kept anaesthetised from operation until sacrifice.

The possibility of the use of the enzyme ornithine decarboxylase as an early indicator of growth was investigated. It was found
to increase 90-fold in activity early during liver regeneration and was maintained at an elevated level for at least 48 hours. Similar but transient increases were also produced by 10% hepatectomy and sham operation. Unilateral nephrectomy resulted in a transient peak of ornithine decarboxylase activity in the remaining kidney 4 hours after operation but sham operation produced a similar but somewhat smaller response. The latter response to operative stress could be abolished by treatments affecting glucocorticoid secretion. Dexamethasone, a potent glucocorticoid, markedly stimulated kidney ornithine decarboxylase activity. However, in addition to the response to stress there was an added increase in enzyme activity at 4 hours, attributable to the process of compensatory growth, both in liver and kidney. Analysis of plasma corticosterone showed that unilateral nephrectomy and sham operation were equally stressing.

Ornithine decarboxylase was found to be much higher in the kidney than in all other tissues examined except prostate. In contrast the levels of the enzyme S-adenosylmethionine decarboxylase, which is thought to be coupled to polyamine biosynthesis, were found to be comparable in liver and kidney. However, no marked changes in S-adenosylmethionine decarboxylase could be demonstrated during the first 24 hours of compensatory renal hypertrophy.

The relationship of ornithine decarboxylase activity to growth was investigated in C13 cells in culture. The enzyme was high in exponentially growing cells but low in 'resting' cells. Stimulation of 'resting' cells to growth resulted in a sharp peak in ornithine decarboxylase activity.
Control of Liver and Kidney Growth

by

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**Abbreviations**

These are as laid down in the Biochemical Journal Instructions to Authors (Biochem. J., 1972 **126**, 1-19) with the following additions:-

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>BHK/C13</td>
<td>Baby hamster kidney cells, clone 13</td>
</tr>
<tr>
<td>EC1</td>
<td>Eagle's medium containing 1% (v/v) calf serum</td>
</tr>
<tr>
<td>EC10</td>
<td>Eagle's medium containing 10% (v/v) calf serum</td>
</tr>
<tr>
<td>ERPF</td>
<td>Effective renal plasma flow</td>
</tr>
<tr>
<td>ETC</td>
<td>Eagle's medium containing 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HnRNA</td>
<td>Heterodisperse nucleoplasmic RNA</td>
</tr>
<tr>
<td>PAH</td>
<td>p-aminohippuric acid</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>p.f.u.</td>
<td>Plaque-forming units</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SAMD</td>
<td>S-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TES</td>
<td>N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>Tm</td>
<td>Transport maximum</td>
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APPENDIX 2. Bibliography
1. **Adaptive growth**

The mechanism by which growth is controlled in animal tissues is one of the major outstanding problems of biology. It has an obvious relevance to the problems of differentiation and ageing and it has a practical significance in the possible treatment of cancer. As long ago as 1894 it was pointed out by Bizzozero that mammalian cells can be classified into three types according to their proliferative activity (Goss, 1964).

1) **Renewing populations**, as in epidermis and the haemopoietic tissues, proliferate continually to make good a continual cell loss.

2) **Static populations**, as in striated muscle and nerve, contain cells which never divide at all, once differentiation is complete. Growth, when it occurs, takes the form of an increase in cell size, not of cell number.

3) **Expanding populations**, as in liver and kidney, proliferate in the growing individual, but cell division in the adult is very infrequent and balances an equally infrequent cell loss.

Some of the cells in the third group although normally quiescent in the adult animal can be induced to grow and divide by an appropriate chemical or surgical stimulus. Such cell populations are well suited for the study of the control of growth. Among those commonly investigated are the uterus after injection of oestrogen; the parotid after isoprenaline injection; the liver regenerating after partial hepatectomy; and the kidney in compensatory growth after unilateral nephrectomy. The growth of organs such as the liver and kidney
following removal of part of their functional mass is known as compensatory or adaptive growth. It has also been shown to occur in other organs such as the pancreas, salivary glands, thyroid, adrenal cortex and ovary (Goss, 1964).

2. Compensatory Renal Growth.

2.1. Gross aspects of the phenomenon.

The discovery that the remaining kidney grows after removal of its partner is generally attributed to the German surgeon Gustav Simon who in 1869 became the first surgeon to perform successfully a unilateral nephrectomy on a human patient. During the course of his preparations for this operation he tried the operation on several dogs and noticed that the remaining kidney increased in size by about 50% after 20 days (Nowinski, 1969). Since then many investigators have looked at this phenomenon, looking initially at the morphological and histological differences between the hypertrophic kidney and the normal kidney. More recently emphasis has shifted towards the causes of this growth and the biochemical mechanism by which it is accomplished.

Compensatory renal growth has been shown to occur in many different species. Although the remaining kidney never actually doubles in size it is nonetheless capable of a marked compensatory response. In the rat, the animal most commonly used in such investigations, this amounts to an increase to 60 to 70% of original renal weight 30 to 40 days after the operation (Jackson & Levine, 1929; Addis & Lew, 1940). Similar changes have been observed to occur in the mouse (75-80% at 1 to 2 months, Straube & Patt, 1961; Malt & Lemaitre, 1968), in the hamster (70% in 2 weeks, Reiter, 1968), in the rabbit (66% after 106 to 126 days, Addis et al., 1924) and in the dog (61% after only 20 days, Astarabadi & Essex, 1953). Not surprisingly increases in renal weight are difficult to estimate in human subjects but the
weight of the remaining kidney undoubtedly increases (Bucher & Malt, 1971).

The phenomenon is not restricted to mammals. Even in *Xenopus laevis* larvae the phenomenon of kidney regeneration can be observed; the total increase in mass is equivalent to 95% of the extirpated pronephros (Chopra & Simnett, 1969). In *Ambystoma* larvae also, following unilateral pronephrectomy growth occurs to about 60% increase after 10 days (Fox, 1960). Similar results were obtained by Fox (1956; 1957) for several *Triturus* species. Of all species studied cats would appear to be unique in not undergoing compensatory renal hypertrophy at all (Janicki, 1969).

These changes in kidney weight reflect real growth and not simply increased water content as shown by the fact that dry weight, principally protein, is about 24% of the wet weight at all times during compensatory renal growth in the mouse (Malt & Lemaitre, 1968) and remains similarly constant in the hamster at about 22% (Reiter, 1968). Halliburton (1966) has also observed that, in the rat, changes in dry weight of the kidney appear to parallel changes in wet weight.

2.2. Effect of size of resection on kidney growth.

That the growth of the kidney remaining is a compensating response to loss of kidney mass or function is indicated by the experiments of McCreight & Sulkin (1962) and Goss (1964). These show that growth of the remaining renal tissue is very roughly proportional to the amount removed. For example, 50% nephrectomy resulted in a 6-fold mitotic increase, 75% in 18-fold and 87.5% in a 25-fold stimulation. Goss (1964) has shown that these increases reflect greater kidney regeneration at the time, and not an altered time scale of the response to renal resection. The increase in
mitotic count in two remaining half-kidneys after bilateral heminephrectomy was the same as in the remaining whole kidney after unilateral nephrectomy (McCreight & Sulkin, 1962). On the other hand Saetren (1970) has observed that following unilateral heminephrectomy a greater mitotic peak occurred in the half-kidney than in the whole kidney, indicating that some part of the response may be due to localised damage. This mitotic response was enhanced 1.5 times by removing a whole kidney and 15-fold after removal of 1½ kidneys.


3.1.1. Morphological and histological.

In the kidney the functional unit is the nephron (Pitts, 1968) and it has been reported by several workers (Arataki, 1926; Saphir, 1927; Moore, 1929) and shown most elegantly by Hiramoto et al. (1962) using a rabbit anti-rat-kidney antiserum that no new glomeruli (and consequently nephrons) are formed during the hypertrophic process. Instead the constituent parts of the nephron and especially the tubules increase in size. The proximal tubule of the hypertrophied rat kidney is 32-35% longer, 14-17% wider and of 76-92% greater volume than that of the normal kidney and corresponding figures for the distal tubule are 17%, 10% and 47%, indicating that growth is more extensive in the proximal tubule (Hayslett et al., 1968; Arrizurietta de Muchnik et al., 1969).

In line with these changes in structure, corresponding changes in renal function may be observed after unilateral nephrectomy. Thus in rats the glomerular filtration rate for the single kidney increases 76% two to three weeks after the operation (Katz & Epstein, 1967). In the dog glomerular filtration rate and renal plasma flow increase 50% in the first postoperative weeks (Bugger-
Ultrastructural changes in the growing kidney have been observed in the rat by Anderson (1967) and include dilatation of the cisternae of rough endoplasmic reticulum, proliferation of Golgi membranes and agranular reticulum and increase in the number of cytoplasmic ribosomes. In the mouse kidney a prominent feature is the numerous whorl-like membranous structures in the basal and medial cytoplasm of proximal tubular cells 48 hours after the operation. This may be related to membrane biosynthesis (Leak & Rosen, 1966). There is some controversy as to the changes in mitochondria during kidney growth. Anderson (1967) noted a decrease in the number of mitochondria, and prior to mitosis, disorientation of those remaining, all these changes being most pronounced in the proximal tubular cells. On the other hand Johnson has reported an increase in the number of mitochondria amounting to 60% after only 2 days (Johnson & Amendola, 1969; Johnson, 1969), corresponding to an increase in mitochondrial protein of 23% and mitochondrial DNA of 35% after 2 days (Devlin & Ch'ih, 1970). This however is not the only point on which Johnson's findings differ from those of other investigators (Weinbren et al., 1971).

3.1.2. Increase in cell size or cell number?

Disagreement has been rife about the extent to which compensatory renal growth is due to increase in cell number as opposed to cell size. Histological studies have shown a transient 3-fold to 7-fold increase in mitotic activity on the second day after operation. The increase is most pronounced in the proximal tubules with a somewhat smaller response in the distal tubule. There is no significant increase in the number of connective tissue mitoses (Williams, 1961). Subsequently a second but smaller mitotic peak has been observed by some workers, between 3 and 8 days after the operation (Goss, 1964). Measurements
of DNA synthesis show a postoperative peak at 36 hours and sometimes a second peak at 3 to 6 days in parallel with the histological observations (McCreight & Sulkin, 1962; Simpson, 1961; Reiter, 1965; Mayfield et al., 1967; Threlfall et al., 1967; Reiter, 1968). However, the rather modest increase in mitotic activity revealed by these observations must be regarded with some caution since it has been shown that similar small increases can be brought about by relatively trivial injury to the kidney (Argyris & Trimble, 1964; Argyris et al., 1969; Malamud et al., 1971).

Undoubtedly DNA synthesis does occur during compensatory renal growth and is followed by mitosis. The extent to which the consequent increase in cell number contributes to the increase in kidney size was investigated by Malt & Lemaitre (1968) who showed that, in mice, the remaining kidney 28 days after unilateral nephrectomy had increased by 76% in weight and protein content and 30% in RNA content but only a meagre 14% in DNA content. Halliburton & Thomson (1965) showed that the initial weight increase (30%) was associated with an increase in RNA (40%) and protein (30%) rather than in DNA (less than 10%). Similar findings have been reported by Johnson & Vera Roman (1966), Threlfall et al., (1967) and Kurnick & Lindsay (1968). Malt's group has shown that the greater part of the activity in synthesis of RNA, protein and DNA resides in the tubules, a finding which correlates well with the morphological changes noted earlier (Malt & Lemaitre, 1968).

That compensatory renal growth can in fact be independent of hyperplasia has been emphasised by the work of Janicki & Lingis (1970) who found unabated renal hypertrophy in unilaterally nephrectomised rats treated with hydroxyurea to inhibit DNA synthesis. Treatment with vinblastine sulphate (a mitotic inhibitor) is similarly singularly ineffective in inhibiting kidney growth (Cosgriff &
3.2. The chemistry of renal growth.

3.2.1. Sequence of biochemical events following unilateral nephrectomy.

Current knowledge on the pattern of growth in the remaining kidney can be outlined as follows.

The earliest known response to removal of the contralateral kidney appears to be the disappearance of heterodisperse nucleoplasmic RNA demonstrable possibly within 10 minutes and certainly within one hour of the operation and persisting for at least 7 days (Willems et al., 1969). However the significance of such a change and its relationship to growth are at present obscure. Other reports indicate 25 to 100% increases in labelling of ribosomal RNA within 4 hours of the operation (Halliburton & Thomson, 1966; Halliburton, 1969), a finding which Malt has been unable to repeat (1969). Johnson & Vera Roman (1966) have reported an increased uptake of cytidine into slices from kidneys removed within 3 hours of contralateral nephrectomy. An increase in total RNA content of 7% can be detected within 12 hours, rising to 33% by 48 hours (Halliburton & Thomson, 1965). This probably reflects an increase in ribosomal RNA (Malt & Stoddard, 1966; Malt, 1969). Total protein content is significantly elevated above control values by 14 hours (6.6%) (Coe & Korty, 1967) and continues to rise at least up till 96 hours (Halliburton & Thomson, 1965). Increased DNA synthesis is first detectable at 24 hours and reaches a peak at 36 hours of up to 10 times the preoperative value (Phillips & Leong, 1967). This is followed after a twelve hour interval by a corresponding increase in mitotic activity of 3 to 7-fold (Phillips & Leong, 1967; Goss, 1964; Saetren, 1970). Some enzymes involved in DNA synthesis, including
deoxythymidine kinase, deoxythymidine monophosphate kinase and deoxycytidine monophosphate deaminase, reach a peak just ahead of DNA synthesis but the relative increases are slight (30% to 150%) compared to the DNA synthetic peak (Mayfield et al., 1967).

3.2.2. Metabolism of hypertrophic kidney.

There is evidence for metabolic changes in the hypertrophic kidney. A 35% increased utilisation of palmitate has been reported (Ross & Goldman, 1970). The distribution of acid mucopolysaccharides alters, their concentration increasing in the cortex and decreasing in the medulla (Allalouf & Ber, 1969). The levels of several enzymes also change. Sodium and potassium stimulated ATPase increases about 55% (Katz & Epstein, 1967; Fanestil, 1968) and glutamic dehydrogenase and alkaline phosphatase are also elevated about 2-fold (Nowinski et al., 1968). Changes in phosphate-dependent glutaminase (Janicki & Argyris, 1969), ε-lysine acylase (Paik & Kim, 1966) and lysosomal acid phosphatase (Hegdekar, 1968) have also been reported. The two dehydrogenases of the pentose phosphate pathway are reported to show small increases in activity at 36 and 72 hours after operation (Farquar et al., 1968).

3.3. Effect of environmental and internal influences on kidney growth.

The compensatory growth of the kidney can be greatly influenced both by external factors such as diet and by internal factors such as endocrine activity.

3.3.1. Modifying influences.

Perhaps the most striking difference in the response to unilateral nephrectomy is that between young and old animals. In the immature animal compensatory renal growth occurs mainly through increase in cell number whereas in the adult it occurs mainly through increase in cell size (Phillips & Leong, 1967; Karp et al., 1971; Shirley, 1972).
Moreover the mitotic peak is larger and better marked in young animals (Phillips & Leong, 1967). There is a difference in response between kidneys of males and females (Argyris & Trimble, 1964). Diet (Halliburton & Thomson, 1967; Goldman, 1971), X-irradiation (Caldwell et al., 1970), food and water restriction (Reiter, 1965) and altitude (Braun & Sharipov, 1970) also influence the rate, magnitude or type of response to unilateral nephrectomy. Clearly such variables must be taken into account during experimental planning and comparison with other workers' results, and their neglect may be responsible for many of the contradictory results published in this field.

3.3.2. Endocrine influence on kidney growth.

The influence of endocrine glands on compensatory renal hypertrophy is confused. However it would appear on balance that the presence of the pituitary, adrenals, testes, ovaries, and thyroid are not essential for the process although the response may be modified in their absence (Reiter, 1969; Bucher & Malt, 1971). Androgens stimulate kidney growth and antidiuretic hormone and deoxycorticosterone stimulate mitosis in the kidney; whereas estrogens injected into a male rat depress kidney size and ACTH inhibits the mitotic response to contralateral nephrectomy (Bucher & Malt, 1971). Thyroidectomy and hypophysectomy are followed by renal atrophy.

3.4. Other means of stimulating kidney growth.

Renal hypertrophy and/or hyperplasia also occur in response to many other influences apart from unilateral nephrectomy and it has been hoped that an investigation into these comparable phenomena might throw some light on the mechanisms underlying the compensatory growth response (Goss & Dittmer, 1969). Cold exposure (Reiter, 1968) and feeding of a high protein diet (Halliburton, 1969) have both been found to cause renal enlargement and DNA synthesis, the effect due
to unilateral nephrectomy being additive in each case. Diets containing urea do not cause kidney growth; but diets containing sufficient ammonium chloride to induce acidosis produce as much growth as high protein diets. Growth during ammonium chloride acidosis however has different characteristics to that following unilateral nephrectomy (Bignall et al., 1968; Janicki & Argyris, 1969; Janicki, 1970); and this is also true of the feeding of high protein diets (Halliburton, 1969).

Renal growth also occurs in response to injection of agents such as folic acid and uranyl nitrate which cause hydronephrosis (Taylor et al., 1968). Similar changes are produced by hydronephrosis resulting from ipsilateral ureteral ligation (Paulson & Fraley, 1970). Mercuric chloride injection causes necrosis followed by renal regeneration (Cuppage & Tate, 1967; McCreight & Witkofski, 1969; Ash & Cuppage, 1970). Injection of isoprenaline results in similar changes possibly again due to necrosis (Malamud & Malt, 1971; Rona et al., 1959). Renal tubular poisoning by lead acetate also results in renal growth to which the effects of unilateral nephrectomy are additive (Choie & Richter, 1972).

It is apparent from the foregoing that kidney growth may be induced by a variety of treatments but since such effects are additive to compensatory renal growth it would appear that a common underlying mechanism is not operative.

4. Liver regeneration after partial hepatectomy.

4.1. The phenomenon of compensatory liver growth.

It is interesting at this stage to compare the response of the kidney following unilateral nephrectomy to the much better known and much more investigated problem of liver regeneration following partial hepatectomy. Liver regeneration occurs in many different species but
most of the information available has been gained through experiments
on rats.

Removal of approximately two-thirds of the liver results in an
immediate compensatory response which is highly specific for liver,
growth being generally undetectable in other tissues. This is
characterised by an increase in cell number rather than an increase
in cell size, contrary to the case in the kidney. This difference
may well be due to the much simpler structure of the liver where the
hepatocyte is the functional unit instead of the nephron (Goss, 1966).
The regeneration is exceedingly rapid compared to compensatory renal
hypertrophy, the remnant doubling in size after 2 days and being almost
as large as the original intact organ within a week (Brues et al.,
1936). As in the kidney the response to partial hepatectomy, as
indicated by the rate of DNA synthesis, is proportional to the amount
of liver excised. A 9% hepatectomy produces virtually no DNA
synthesis although it would appear that the deficit is made good
slowly over a long period of time (Bucher & Swaffield, 1964).
Experiments of this sort are more meaningful than the corresponding
experiments performed on the kidney because of the gross architecture
of the liver whose lobed structure means that varying proportions
can be removed without damage to the lobes remaining.

The first cells to divide during liver regeneration are the
hepatocytes. Division starts on the periphery of the lobule and
spreads inwards. This may be because the peripheral hepatocytes
are more exposed to humoral agents in the portal blood or alternatively
because they are better nourished and more responsive. Division of
ductal and littoral cells throughout the lobule follows about a day
later (Bucher & Malt, 1971).
4.2. Sequence of biochemical events.

Much more is known about the early biochemical events in liver regeneration than about the corresponding events in compensatory renal hypertrophy. Indeed investigation into the biochemistry of compensatory renal hypertrophy has to a great extent followed lines already explored in liver regeneration. The speed of the regenerative response to partial hepatectomy and the virtual synchrony of the first wave of mitosis make it a particularly useful system for the study of the biochemistry of the growth process. Presumably this must start with activation of specific segments of the genome which control the sequence of biochemical events leading ultimately to growth. However there is as yet no conclusive proof of this although much suggestive evidence exists (Lieberman et al., 1963; Baserga & Stein, 1971).

Such gene activation may be envisaged as being controlled by small RNA molecules (Britten & Davidson, 1969), histones (Stellwagen & Cole, 1969) or nuclear acidic proteins (Baserga & Stein, 1971).

Certainly there are marked biochemical changes early during liver regeneration which are probably concerned with its control. For example, an increase in the hybridisation efficiency of rapidly labelled nuclear RNA of over 100% can be detected within an hour of the operation (Church & McCarthy, 1967). At the same time the acetylation of arginine rich histones approximately doubles and reaches a peak at about 4 hours (Allfrey, 1969; Allfrey, 1970). It has been reported that hybridisation-saturation of chromosomal RNA to DNA is also maximal at 4 hours (Mayfield & Bonner, 1972). Synthesis of nuclear acidic proteins is first detectable after 3 hours and reaches a peak at 5 hours (Pogo et al., 1968). Between 3 and 6 hours postoperatively the CTP and UTP pools are almost doubled in size (Bucher & Swaffield, 1969; Bucher et al., 1969). The rate of RNA
synthesis is also nearly doubled at this time (Fujioka et al., 1963; Bucher & Swaffield, 1969) and a net increase in cellular RNA content (mainly ribosomal RNA) is detectable (Lieberman & Kane, 1965; Bucher et al., 1969). The rate of labelling of tRNA is increased by 6 hours (Fausto & Van Lancker, 1968) as is the rate of phosphorylation of some histone species (Gutierrez-Cernosek & Hnilica, 1971). Approximately 10 hours after the operation the "template activity" is highest, indicating the maximum number of active genes (Mayfield & Bonner, 1972).

Somewhat later in the sequence of biochemical events changes become apparent which appear characteristic of growth rather than preparation for growth. RNA polymerase activity is maximal after 12 to 24 hours (Ro & Busch, 1967) and synthesis of ribosomal RNA is maximally elevated (10-fold) after 12 hours (Fausto & Van Lancker, 1968). A net increase in protein content is detectable after 12 hours but the period of most rapid protein synthesis is between 12 and 36 hours, a time during which many specific proteins and enzyme activities reach a peak and decline (Bucher, 1963). Phosphorylation of histone FI increases just prior to, or coincidentally with, a 30-fold increase in DNA synthesis at about 18 to 24 hours (Ord & Stocken, 1968; Stevely & Stocken, 1968; Grisham, 1962). It has been suggested by Johnson (1969) that DNA replication is triggered when a growing cell reaches a certain size - the "critical mass" hypothesis first developed from experiments on unicellular organisms. However this view has recently been strongly contested on the basis of measurements of the sizes of cells undergoing DNA synthesis during liver regeneration (Weinbren et al., 1971). These authors have observed that cells of all sizes, not merely the largest, engage in DNA synthesis.

The replication of DNA is followed by cell division, the mitotic peak (30-fold) appearing 24 to 30 hours after the operation and 6 hours
after the peak of DNA synthesis. The whole cycle (24 hours) is much faster than that occurring in the remaining kidney (48 hours) and is repeated by some of the parenchymal cells at least once more in the course of the compensatory regeneration (Edwards & Koch, 1964).

4.3. **Endocrine and other influences.**

A variety of factors influence liver regeneration. Thus the mitotic peak occurs earlier and is higher and more clearly marked in young animals than in old (Bucher et al., 1964). This is parallel to the situation already described in kidney hypertrophy (see p.9). Fasting and irradiation both delay and perhaps diminish DNA synthesis in liver regeneration (Bucher, 1963) and here again there is a parallel with kidney hypertrophy (p.10). A variety of antimetabolites and other chemical agents have the same affect. Regeneration is also inhibited by the imposition of exceptional physical stress as for example during centrifugation at 4.7g (Feller et al., 1967) or by removal of a further part of the liver 3 hours after the original hepatectomy (Weinbren & Taghizadeh, 1965).

As is the case in kidney hypertrophy, the influence of endocrine glands on liver regeneration is somewhat obscure. Adrenalectomy does not interfere with DNA synthesis although restoration of liver mass is slower (Bucher, 1963). Hypophysectomy delays the rise in DNA synthesis without diminishing it (Rabes & Bründle, 1969). The effect of hypophysectomy can be reversed by administration of both growth hormone and cortisone but not by either alone (Bucher & Malt, 1971). Liver regeneration, unlike compensatory renal hypertrophy, is not affected by sex hormones.

Stress or administration of glucocorticoids and adrenaline during the premitotic phase of growth both tend to suppress or delay liver regeneration (Davis & Hyde, 1966). On the other hand many of the
early changes characteristic of regeneration such as accumulation of ribosomal RNA can be elicited by stressful stimuli such as celite injection (Tsukada et al., 1968). Such changes are prevented by adrenalectomy and may be restored by hydrocortisone injection (Lieberman, 1969). In this connection it is remarkable that the peak of DNA synthesis in liver regeneration occurs earlier if the animal has been subject to stress several hours before operation (Moolten et al., 1970). It may be therefore that the responses to stress and to partial hepatectomy have common features.

4.4. Summary.

Liver regeneration differs from compensatory renal hypertrophy in several important characteristics. Liver growth is much faster than kidney growth and eventually restores a greater proportion of the resected mass. Most important, liver regeneration is totally due to an increase in cell number whereas compensatory renal growth is predominantly due to an increase in cell size. In spite of these obvious differences, however, there are certainly many similarities between these two examples of adaptive growth. Although a much fuller picture is available in the case of liver regeneration it is quite apparent that the sequence of biochemical events is essentially the same in each case. An early rise in RNA synthesis and accumulation is followed somewhat later by an accumulation of protein and eventually by DNA synthesis and mitosis. In addition liver regeneration and compensatory renal hypertrophy are both modified by similar influences, such as surgical intervention and diet.

It is reasonable to suppose that the compensatory growth of liver and kidney have other common features, perhaps even similarities in the mechanisms responsible for their control. Certainly the response in each case is extraordinarily rapid, the sequence of biochemical events
being initiated in all probability within one hour of the operation.

5. **Control of liver regeneration.**

Two main theories have been put forward to account for liver regeneration, which we may term the haemodynamic and the humoral.

5.1. **The haemodynamic theory.**

The flow of blood through the liver is accounted for chiefly by portal blood which has already passed through spleen and gut. The volume of this flow is determined by the constriction and dilation of blood vessels in these viscera and is unaffected by the liver itself. Consequently after partial hepatectomy the remaining liver fragment has to accept as much portal blood flow as the original intact organ. It has been suggested that this increased blood flow and the consequent congestion in the liver fragment provide the stimulus for regeneration. To test this hypothesis, Thomson and his colleagues devised surgical methods to increase portal blood flow in normal dogs with or without concomitantly increasing oxygen tension in the liver. According to the hypothesis, these procedures should have caused liver growth. In fact no evidence of growth was detected (Alston & Thomson, 1963; Thomson & Clarke, 1965; Thomson, 1969).

Mizumoto and his colleagues have shown that in the dog partial hepatectomy is indeed followed by congestion and hypertension in the liver. This can be abolished if the hepatic artery is ligated at operation; but such ligation does not prevent regeneration from taking place (Mizumoto et al., 1970). This is additional evidence that increased portal pressure and congestion are not important in bringing about regeneration.

5.2. **Control of liver regeneration by humoral factors.**

The second theory of liver regeneration is that partial hepatectomy
results in some unspecified change in the composition of the circulating blood and this in turn is the stimulus for liver regeneration. The early attempts to demonstrate such a change took two main forms. In the first, a partial hepatectomy was performed in one partner in a parabiosis and evidence of growth was sought in the liver of the intact partner. In the second, substantial quantities of serum were transferred from normal to hepatectomised rats or vice versa and the liver of the recipients examined for evidence of growth. The results of these were confusing and contradictory (Bucher & Malt, 1971) and led to the invention of novel parabioses providing unprecedentedly rapid exchange of blood between the partners via extracorporeal cannulas (Alston & Thomson, 1963; Moolten & Bucher, 1967; Lieberman, 1969; Sakai, 1970). If one partner of such a parabiosis was partially hepatectomised there was generally evidence of growth in the liver of both partners, but, in spite of the rapidity of the exchange of blood, growth was much more pronounced in the partially hepatectomised animal than in its normal partner. This would seem to suggest that liver regeneration is under the control of a humoral factor with a very short half-life.

The most unequivocal evidence for the humoral theory was provided by experiments in which the median lobe of the liver was transplanted to a subcutaneous site. When such animals were subsequently subjected to partial hepatectomy the transplant as well as the remaining fragment of the liver proper showed clear evidence of DNA synthesis and mitosis (Grisham et al., 1964; Leong et al., 1964; Virolainen, 1964; Virolainen, 1967).

It has been suggested from time to time that the factor responsible for liver regeneration is to be found in portal rather than systemic blood and recent evidence shows that this could indeed be the case.
Experiments involving transplantation of auxiliary livers into rats and cross-circulation indicated the presence in portal blood of a growth-stimulating factor which is inactivated by the liver itself. It was suggested that this factor could regulate liver size by an imbalance between it and the number of hepatic cells present.

The operation of such a factor would also explain the different responses of the partially hepatectomised and normal livers in the earlier cross-circulation experiments. The liver remnant could inactivate most of the factor, growing in the process and allowing only a small surplus into the systemic circulation to be either transferred to the intact partner or repassaged through the same liver fragment. It is easy to imagine such circumstances leading to the results described by Alston & Thomson (1963), Moolten & Bucher (1967), Lieberman (1969) and Sakai (1970).

Even assuming that the humoral theory is true we do not know whether liver regeneration is due to the appearance in the bloodstream of a growth promoting factor or to the disappearance of a growth inhibitor. Theoretically it would be very desirable to have an in vitro assay of factors affecting liver growth by observing their effect on liver cells in culture. Unfortunately this approach is difficult because the cells that proliferate in liver explants are generally fibroblasts or other nonparenchymal cells (Bucher & Malt, 1971). Growth-promoting properties in serum from partially hepatectomised rats have however been reported despite these complications, although there is no evidence to suggest that the mechanisms involved in the in vitro and in vivo systems are identical. Recently it has been reported that primary cultures of differentiated liver cells (selected for parenchymal cells by omission of arginine...
and inclusion of ornithine in the medium) respond to serum from partially hepatectomised rats in a manner which suggests the operation of a positive control system operating through growth promoters rather than inhibitors (Paul et al., 1972; Leffert & Paul, 1972).

The contrary view, that normal liver is restrained from growth by an inhibitor in the blood which disappears after hepatectomy, has been put forward by Glinos (1967). It has been shown that plasmapheresis, which might be expected to remove the supposed inhibitor, stimulates growth in the normal liver; and that haemoconcentration, produced by fluid restriction, which might be expected to concentrate the inhibitor, diminishes liver growth after partial hepatectomy (Glinos, 1967; Virolainen, 1967). Liver breis have also been alleged to inhibit liver regeneration when given intraperitoneally. Mitotic inhibitors of this sort have been termed "chalones" by Bullough (1962; 1965) and several attempts have been made to extract a liver chalone. In perhaps the most successful such attempt it is claimed that such a growth inhibiting factor has been purified 450-fold from rabbit liver (Verly et al., 1971). This substance, a polypeptide of small molecular weight, inhibits DNA synthesis in liver slices from the remaining lobe of a subtotally hepatectomised rat. At least one attempt to repeat these observations has been unsuccessful (Jondorf, 1972, personal communication). In view of the work of Freed & Sorof (1966) showing inhibition of mitosis of cells in culture by a class of liver proteins, the supposition that any purified "chalone" is important in the control of liver regeneration must be treated with caution.

5.3. The work hypothesis.

The work hypothesis is an extension of the humoral theory and suggests that the liver remnant grows because the remaining hepatocytes have to perform the role originally performed by the whole liver. In
this case the humoral factor, rather than being a specific growth regulating substance with no other role, is merely a reflection of the inability of the liver remnant to maintain stable blood concentrations. The consequently greater amount of metabolic activity required of the liver fragment would then be the stimulus to growth. In this way the growth of the liver would be analogous to the growth of skeletal muscle in response to exercise.

Virtually no experimental evidence can be adduced to support the contention that the hyperfunction of any of the multifarious metabolic activities of the liver is in fact responsible for the subsequent growth. A possible exception is to be found in the experiments of Alston & Thomson (1968) who showed that intraperitoneal administration of a protein hydrolysate over a period of 48 hours resulted in changes in RNA, protein and mitotic activity similar to those occurring during liver regeneration. However these changes may be due to some extent to the removal of a liver biopsy at the start of the experiment, to the stress imposed on the animal by repeated injection, or to mechanical irritation of the liver. Stress has been shown to increase liver RNA and protein synthesis (Majumdar et al., 1967; Tsukada et al., 1968) and mechanical irritation of the liver leads to increased DNA synthesis (Weinbren et al., 1969).

5.4. Summary.

Despite all the effort expended to identify the factors responsible for liver regeneration, progress has been depressingly slow. At present it appears that haemodynamic changes have been ruled out of court. On the other hand there is a substantial body of evidence for a humoral factor of some sort. Its nature remains mysterious; it seems short-lived; it may be a specific hormone or a metabolic overload; we do not know whether it is a stimulus to growth which
appears after hepatectomy or an inhibitor which is depressed.

6. Control of compensatory renal hypertrophy.

As with liver regeneration the possible mechanisms controlling compensatory renal hypertrophy can be considered conveniently under three headings: haemodynamic factors, humoral factors and the "work hypothesis". As with other aspects of kidney growth its control has been much less investigated than has that of liver growth. Perhaps for this reason there is less to choose between these three approaches in the case of the kidney than in the case of the liver where the search has narrowed down to a humoral factor of some sort.


The kidney is the organ most lavishly supplied with blood per unit mass; between them the two kidneys normally accommodate about a quarter of the cardiac output of a resting animal. It is obvious that unilateral nephrectomy must constitute a major haemodynamic upset, and it is possible that this could be responsible for the growth of the contralateral kidney. The blood supply to the kidney is much simpler than that to the liver and one consequence of this is that the kidney to a large extent controls its own blood flow whereas the liver's blood flow is to a large extent governed by the amount of blood coming from the intestines.

Blood flow to the remaining kidney is known to increase after unilateral nephrectomy (Hartman & Bonfilio, 1959) but no mechanism by which this could influence renal size is at present known. It is however interesting in this connection to notice the relatively greater size of the right kidney of mice and rats (Johnson & Amendola, 1968; Halliburton, 1966) a fact which, it has been suggested, is due to local haemodynamic factors which tend to give the right kidney a greater blood supply than the left.
Increased blood flow could result in the increased supply of growth promoting substances or metabolites to the kidney but the most likely mechanism by which it could cause growth is by increasing the work of the kidney and this will be considered in section 6.3.


Compared with liver regeneration there is virtually no evidence to support the view that a humoral factor controls compensatory renal hypertrophy. As with liver regeneration, serum transfer from nephrectomised to normal animals has yielded discordant results. Negative findings (Williams, 1962; Goss, 1963; Kurnick & Lindsay, 1967) are balanced by a similar number of positive results (Lowenstein & Stern, 1963; Silk et al., 1967; Vicki & Earle, 1970). These authors variously report factors of renal or extrarenal origin. Ogawa & Nowinski (1958) have shown that serum from blood withdrawn from rats on the second day after unilateral nephrectomy is capable of stimulating mitosis in rat kidney medulla cultured in plasma clots. Lowenstein & Lozner (1966) have reported stimulation of incorporation of radioactive thymidine into DNA in renal cortex slices incubated with serum from unilaterally nephrectomised rats. More recently it has been claimed that plasma from unilaterally nephrectomised rats is 50% more effective than plasma from sham-operated rats in stimulating DNA synthesis in renal cortical slices. (Preuss et al., 1970; Terry et al., 1970).

Several attempts have been made to demonstrate a humoral factor by performing a unilateral nephrectomy on a pregnant animal and looking for signs of growth in the foetal kidneys; but these have all failed to show stimulation (Rollason, 1961; Goss, 1963; Goss, 1964; Malt & Lemaitre, 1969; Skreb et al., 1971).

Parabiotic experiments have also been performed but have given
rise to contradictory results. Thompson & Lytton (1967) have reported that in parabiotic rats there is no increase in weight of the kidneys of the normal animal 3 weeks after unilateral nephrectomy in its partner. On the other hand Lytton et al., (1969) have reported that in a similar experiment unilateral nephrectomy caused a 71% increase in mitotic activity in the kidneys of the intact partner, whereas bilateral nephrectomy had no effect. Kurnick & Lindsay (1968) have reported that in parabiotic mice unilateral nephrectomy causes kidney hypertrophy in the intact partner. Bilateral nephrectomy produces a larger response and removal of 3 out of the 4 kidneys provoked a larger response still in the one remaining. On the other hand Johnson & Vera Roman (1968) in a cross-circulation experiment found that unilateral nephrectomy in one partner did not increase the number of cells synthesising DNA in the kidneys of the other.

As in liver there is uncertainty whether the supposed humoral factor stimulates growth or inhibits it. Injection of kidney breis has been reported to inhibit mitosis after unilateral nephrectomy, suggesting the presence of an organ-specific inhibitor of mitosis in kidney (Saetren, 1956; Dicker, 1971a,b). Goss (1963) however has found that such effects are not tissue specific and intraperitoneal injections of fresh egg albumin inhibits mitosis in the kidney as effectively as injection of kidney macerates. Williams (1962) found that rats injected with macerates did not eat and that starvation inhibited the mitotic peak in the same way as did the kidney macerates. Roels (1969) in an extensive investigation obtained many anomalous results and found that mitotic activity was very variable during compensatory renal growth. Using explants of Xenopus laevis pronephri Simnett & Chopra (1969) obtained an inhibition
of mitosis by adding the supernatant of adult mesonephros homogenate to the culture medium.

If a humoral factor exists its activity is unaffected by renomesenteric venous shunt indicating that if it is of renal origin it survives passage through the liver (Bump & Malt, 1970).

The case for a humoral factor in the mediation of the growth response to unilateral nephrectomy must be regarded at present as unproven.

6.3. The work hypothesis.

Just as partial hepatectomy leaves the remaining liver fragment to perform as best it can the function of the intact liver, so unilateral nephrectomy leaves a single kidney to perform the functions normally discharged by two. It seems not unreasonable to suppose that the remaining kidney might meet this situation by performing its normal excretory function at a more rapid rate than normal and one might expect that such an overworked kidney would be stimulated to grow in much the same way that a skeletal muscle grows in response to vigorous exercise.

There is however a complication in the case of the kidney. The function of kidney like the function of muscle (but unlike the function of liver) has an equivalent amount of thermodynamic work associated with it. This takes the form of reabsorption of virtually all the sodium ion filtered through the glomeruli. The work of the kidney, therefore, is rigidly linked (save in exceptional circumstances) to the glomerular filtration rate (GFR) and the excretory function of the kidney can only be increased by means of a corresponding increase in GFR (Pitts, 1968).

This limitation must be borne in mind in interpreting the results of experiments designed to increase the excretory load on the kidneys.
of intact animals. These have commonly taken the form of administration of diets high in protein or supplemented with large amounts of urea or a variety of salts. In general high protein diets and diets containing sufficient ammonium chloride to produce acidosis produce varying degrees of kidney hypertrophy; whereas urea, sodium chloride and potassium citrate have no effect (Halliburton, 1969).

A more radical approach on essentially the same lines is to divide one ureter and allow it to drain into the peritoneal cavity, or into the gut, in the expectation that the urine issuing from its cut end would be reabsorbed; the excretory function of the kidney in which it is elaborated would thus be nullified. Experiments of this sort, though theoretically attractive, are difficult to execute since there is grave danger, on the one hand, of blockage of the cut ureter and consequent hydronephrosis, and, on the other, of peritonitis resulting from the irritant action of the urine (Thomson, unpublished results, 1968).

It is, of course, entirely possible that the functional overload which induces the remaining kidney to hypertrophy after removal of its partner has little or nothing to do with excretion. The kidney is well-known to be an important site of gluconeogenesis, which may be related to the formation of ammonia necessary to maintain acid base balance (Krebs, 1963; Smith & Long, 1971; Goorno et al., 1967). It is conceivable that the overloading of this function after unilateral nephrectomy might be the stimulus to growth. Such a supposition would accord well with the fact that the feeding of high protein diets and the administration of ammonium chloride to produce acidosis, both of which increase renal gluconeogenesis, also, as we have seen above, induce kidney growth (Joseph & Subrahmanyam, 1970; Halliburton, 1969; Janicki, 1970).
On the other hand it is true that, of the various dietary proteins tested, gelatin is far and away the most effective in causing renal hypertrophy when fed in large amounts; and it has been well known for many years that gelatin also produces a marked increase in GFR. This in turn would be expected to result in an increase in the reabsorptive work done by the kidney. As Johnson (1969) has pointed out, the only way of increasing the workload of the kidney (in the thermodynamic sense) is by an increase in glomerular filtration rate, thus necessitating increased reabsorption. Peters (1963) did find an increase in glomerular filtration rate occurring early after unilateral nephrectomy, a finding which Johnson (1969) has used as confirmation of his ideas that kidney growth is due to work hypertrophy. Johnson also found that there was an increase in the number of mitochondria coincident with the increase in glomerular filtration rate (Johnson & Amendola, 1969; Johnson, 1969). It is precisely by this mechanism that haemodynamic changes could result in kidney growth (see 6.1.). If after unilateral nephrectomy the increased blood flow through the remaining kidney resulted in increased GFR, it would follow that, to prevent loss of sodium ions, reabsorption would have to increase.

7. **The situation in 1969.**

The situation in 1969 could be summarised by saying that compensatory renal hypertrophy was reasonably well characterised though not in as much detail as liver regeneration. The mechanism which brought it about however remained uncertain. There was evidence, but not conclusive evidence, that kidney growth was related to changes in the chemical composition of the plasma; but it was not certain whether this change took the form of variations in major solutes such as urea, amino acids or proteins, or whether it was a change in a special humoral or hormonal agent analogous to ACTH.
There was evidence equally that some sort of functional overload brought about kidney growth; but the mechanism by which this was brought about was by no means clear. In particular it was uncertain how far the kidney responded to such functional overloads by increasing its GFR and hence its thermodynamic work. Comparison of kidney hypertrophy and liver regeneration had not provided any answers to these questions though some of the techniques devised to tackle the liver problem, such as the cross-circulations, seemed to be applicable also to the kidney.

The work described in this thesis was aimed at three related objectives. The section entitled "Renal Physiology" is an attempt to establish what changes unilateral nephrectomy produces in the organism and to decide which of these changes might stimulate the remaining kidney to hypertrophy. The section entitled "Nucleic Acid Metabolism" stems from attempts to devise an assay for a supposed humoral agent controlling kidney growth. This led to a study of the early events in the process of hypertrophy and in particular to the preparations for nucleic acid synthesis; and this in turn led to the work which is the subject of the section "Polyamine Metabolism".
RENAL PHYSIOLOGY
Introduction

There are two alternative ways in which the organism might be expected to react to removal of one kidney:

(a) It is possible that the remaining kidney might continue to function very much as before. In that case the organism would be faced with the equivalent of a partial failure of renal function and we might expect that some of the biochemical symptoms of such failure might manifest themselves. Thus there might be an accumulation of compounds normally cleared by the kidneys such as urea and creatinine, and possibly changes in other compounds such as amino acids which are normally metabolised in the kidneys, and one might reasonably suppose that one of these alterations might be the stimulus which caused the remaining kidney to grow.

(b) Alternatively after unilateral nephrectomy the sort of changes in blood chemistry which would result from loss of half the kidney mass might be prevented by the remaining kidney "functioning" at twice its normal rate, and it is quite possible that such "hyperfunction" might be the stimulus to kidney growth in the same way that vigorous exertion stimulates growth in skeletal muscles.

To distinguish between these two possibilities it was clearly necessary to look:

(1) for changes in the composition of the blood after unilateral nephrectomy; and
(2) for changes in kidney function.
2. Changes in composition of the blood.

2.1. Plasma creatinine

Surprisingly little is known of the effect of unilateral nephrectomy on the organism as a whole. The operation is certainly well tolerated and, although some days or weeks elapse before compensatory hypertrophy approaches completion, the animal shows no obvious ill-effects other than a slight transient weight loss. A similar weight loss occurs in sham-operated rats. Halliburton (1966) looked for changes in haematocrit and plasma sodium after the operation but found none.

An attempt to compare all the constituents of plasma before and after unilateral nephrectomy would be a monumental task. For present purposes it seemed sufficient initially to look at serum creatinine. Since creatinine passes the glomerular membrane freely and is not reabsorbed or secreted to an appreciable extent, it will be removed from the bloodstream at a rate equal to the product of its concentration in the blood and the glomerular filtration rate. If, therefore, removal of one kidney halves the filtration rate for the animal as a whole, one would expect plasma creatinine to rise to about twice the normal level. If, however, the remaining kidney can increase its filtration rate to compensate for the absence of its partner, the increase in the plasma creatinine will be diminished to a corresponding extent. Unfortunately, although creatinine is routinely estimated in clinical laboratories, the standard estimation (the Jaffé reaction) is not very specific, and human serum at least is known to contain compounds other than creatinine which give a significant reaction. There is moreover some doubt whether the tubular reabsorption and secretion of creatinine is really so small that it can be safely neglected. There is some evidence for the view that errors from
this source are more serious in man and the rat than in the dog (Pitts, 1968). Nevertheless the ease with which the necessary estimations could be carried out made estimation of plasma creatinine levels an attractive first step in the present investigation.

As a preliminary, plasma creatinine was measured in a rat before, and 90 minutes after, bilateral nephrectomy. The figures obtained were:

<table>
<thead>
<tr>
<th>Type</th>
<th>Creatinine (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>0.47</td>
</tr>
<tr>
<td>Postoperative</td>
<td>0.79</td>
</tr>
</tbody>
</table>

An increase of 68% in 90 minutes. One could therefore feel confident that plasma creatinine levels would respond rapidly to variations in renal function.

Table 1 shows the serum creatinine levels in normal, unilaterally nephrectomised and sham-operated rats. Sham operation had no effect. Unilateral nephrectomy, on the other hand, produced an increase of approximately 50% (P<0.01) both at 24 hours and 48 hours after the operation. Clearly, therefore, the remaining kidney has not been able to completely compensate for the absence of its partner by increasing its own filtration rate, though some increase may have taken place.

Table 2 shows a not entirely successful attempt to extend this approach by measuring serum creatinine before and after operation in each animal. The preoperative blood samples were taken from the jugular vein and the postoperative samples from the inferior vena cava. This means, unfortunately, that all the postoperative samples, consisting as they did to a large extent of blood which had just passed through the kidneys, would tend to have lower creatinine concentration than corresponding jugular vein samples. This effect was greater than had been anticipated. In the control
Table 1.

Serum creatinine levels in normal, unilaterally nephrectomised and sham operated rats weighing between 180 and 200g.

<table>
<thead>
<tr>
<th>Condition of animal</th>
<th>Serum creatinine concentration (mg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours after operation</td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td>0.76 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
</tr>
<tr>
<td>Sham operation</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
</tr>
<tr>
<td>Normal</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
</tr>
</tbody>
</table>

All values given are means ± S.E.M.

Number of animals given in parenthesis.

* Significantly different from sham operation, P<0.01 (2-way analysis of variance).
Table 2.

Comparison of plasma creatinine levels before operation and at sacrifice in unilaterally nephrectomised and sham operated rats weighing between 170 and 200g.

<table>
<thead>
<tr>
<th>Condition of animal</th>
<th>Sacrifice occurring at</th>
<th>Creatinine concentration at sacrifice</th>
<th>Creatinine concentration before operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 hours after operation</td>
<td>24 hours after operation</td>
<td>8 days after operation</td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td>1.03 ± 0.12*</td>
<td>1.13 ± 0.12*</td>
<td>0.97 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>Sham operation</td>
<td>0.80 ± 0.05</td>
<td>0.66 ± 0.07</td>
<td>0.76 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

All values given as mean ± S.E.M.

Number of animals given in parenthesis.

* Significantly different from values for sham operated rats P<0.01.
animals the postoperative caval blood samples were on average only 72% as high as the preoperative jugular samples. If one assumes that the kidneys contribute about one half of the caval blood, this would indicate a remarkably high creatinine clearance in the kidneys (about 50%). The results at any rate were internally consistent. It is quite clear that, compared to sham-operated rats, unilaterally nephrectomised rats show a consistent 40% elevation of postoperative serum creatinine at 3 hours, 24 hours or 8 days after the operation (compared with the 50% increase found previously). There is no evidence in Table 2 for any significant difference between the three time intervals examined. The remaining kidney in each case seems unable totally to compensate for the absence of its partner.

2.2. Plasma amino acids.

Because of the resemblance between the hypertrophy after unilateral nephrectomy and the hypertrophy produced by high protein diets (Halliburton, 1969), it seemed of particular interest to find out what happened to plasma amino acid levels after unilateral nephrectomy. Fortunately modern methods of chromatography make it possible to analyse all the major amino acids of plasma in a single operation. Table 3 shows the result of such an analysis. There is remarkably little difference between the normal and unilaterally nephrectomised animals. The only significant difference is in proline, which is lower in the nephrectomised animal. The significance of this observation is qualified, however, by the fact that there is a similar depression in proline concentration in sham-operated animals. One is tempted to speculate that the proline change is nothing to do with unilateral nephrectomy but reflects the requirement for proline in collagen synthesis in repair of the operation wound.
Table 3.

Analysis of plasma amino acid levels in normal rats and rats 24 hours after unilateral nephrectomy or sham operation weighing between 180 and 210g.

Estimations were performed on a Jeolco amino acid analyser using sodium buffers.

<table>
<thead>
<tr>
<th>Plasma concentration nmol/ml plasma.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
</tbody>
</table>

Values for normal and unilaterally nephrectomised animals are means ± S.E.M. There were 4 animals in each normal group and 3 animals in each unilaterally nephrectomised group. Values for sham operated animals represent single animals.

* Significantly different from the corresponding value for normal rats with P<0.025.
Table 4.

Analysis of plasma amino acid levels in normal rats and rats 24 hours after unilateral nephrectomy or sham-operation.

Rats each weighed 200g.

Estimations were performed on a Lockarte amino acid analyser using lithium buffers.

<table>
<thead>
<tr>
<th>Amino Acids nmol/ml plasma</th>
<th>Normal (6)</th>
<th>Unilateral nephrectomy (6)</th>
<th>Sham-operation (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoserine</td>
<td>22.0 ± 5.3</td>
<td>38.1 ± 15.7</td>
<td>23.7 ± 6.6</td>
</tr>
<tr>
<td>Taurine</td>
<td>190 ± 11</td>
<td>202 ± 27</td>
<td>250 ± 40</td>
</tr>
<tr>
<td>Urea</td>
<td>5020 ± 421</td>
<td>7460 ± 650§</td>
<td>4752 ± 326</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>35.0 ± 1.3</td>
<td>36.6 ± 2.2</td>
<td>31.0 ± 4.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>169 ± 7</td>
<td>163 ± 22*</td>
<td>226 ± 14†</td>
</tr>
<tr>
<td>Serine</td>
<td>270 ± 24</td>
<td>237 ± 28</td>
<td>309 ± 30</td>
</tr>
<tr>
<td>Asparagine</td>
<td>92.9 ± 14.1</td>
<td>69.4 ± 4.1</td>
<td>88.8 ± 13.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>154 ± 7</td>
<td>137 ± 13</td>
<td>152 ± 12</td>
</tr>
<tr>
<td>Glutamine</td>
<td>505 ± 27</td>
<td>483 ± 37</td>
<td>583 ± 53</td>
</tr>
<tr>
<td>Proline</td>
<td>198 ± 19</td>
<td>158 ± 32</td>
<td>146 ± 28</td>
</tr>
<tr>
<td>Glycine</td>
<td>404 ± 26</td>
<td>490 ± 58</td>
<td>449 ± 28</td>
</tr>
<tr>
<td>Alanine</td>
<td>569 ± 43</td>
<td>500 ± 53</td>
<td>533 ± 50</td>
</tr>
<tr>
<td>Cystine</td>
<td>63.9 ± 7.5</td>
<td>46.2 ± 7.9*</td>
<td>65.6 ± 5.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>44.3 ± 3.1</td>
<td>47.4 ± 2.6</td>
<td>50.6 ± 4.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>59.9 ± 3.8</td>
<td>58.8 ± 4.8</td>
<td>63.8 ± 2.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>97.0 ± 5.9</td>
<td>106 ± 6</td>
<td>101 ± 10</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>56.6 ± 5.5</td>
<td>54.2 ± 3.9</td>
<td>56.8 ± 3.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>49.6 ± 1.7</td>
<td>56.6 ± 3.1§</td>
<td>48.8 ± 3.0</td>
</tr>
<tr>
<td>Trytophan</td>
<td>47.7 ± 17</td>
<td>58.2 ± 18.4</td>
<td>58.4 ± 20.2</td>
</tr>
<tr>
<td>Ornithine</td>
<td>51.7 ± 4.8</td>
<td>42.5 ± 3.9</td>
<td>43.6 ± 3.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>350 ± 24</td>
<td>371 ± 33</td>
<td>441 ± 22</td>
</tr>
<tr>
<td>Histidine</td>
<td>61.0 ± 5.1</td>
<td>54.2 ± 5.2</td>
<td>62.2 ± 4.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>153 ± 13</td>
<td>149 ± 15</td>
<td>173 ± 10</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.

* Differs from sham operation P<0.05
† Differs from normal P<0.005
§ Differs from normal P<0.05
¶ Differs from sham operation P<0.005 and from normal P<0.01
Table 4 shows the results of a similar but more extensive analysis using a different chromatographic system. Here again the most striking finding is that there are few differences between normal, sham-operated and unilaterally nephrectomised animals. Proline is again depressed after both sham operation and unilateral nephrectomy. Though the differences are not significant by themselves, they are significant if considered in conjunction with the previous experiment. There is a curious and inexplicable elevation in threonine after sham operation (but not after unilateral nephrectomy). There is a significant but small elevation in phenylalanine after unilateral nephrectomy. Otherwise the general impression is that the removal of one kidney makes extraordinarily little difference to plasma amino acid levels. In one sense this is to be expected. Amino acids filtered in the glomerulus are normally totally reabsorbed by the kidney. One would not expect unilateral nephrectomy to alter this state of affairs. The kidney however is also involved in glutamine formation and gluconeogenesis, and here one would have expected unilateral nephrectomy to produce some change either in the levels of glutamate and glutamine or of amino acids in general. It is clear that no such change takes place. The one large effect apparent is the 50% increase in urea concentration shown in Table 4. This is exactly in line with the 50% increase in creatinine previously noted and confirms the fact that the remaining kidney cannot completely compensate for the absence of its partner.


The evidence, described in the preceding sections, that after unilateral nephrectomy creatinine and urea accumulate in plasma made it all the more desirable to have some accurate means of determining
at least some aspects of kidney function before and after unilateral nephrectomy. The principal parameter one would wish to measure would be glomerular filtration rate since it is upon this that all tubular processes depend. The classical technique for doing this is by measurement of inulin clearance and the main problem was to adapt this method to an animal as small as the rat. Ideally one would look for a simple procedure which interfered as little as possible with the animal, preferably did not require anaesthesia and did not necessitate collection of urine.

These requirements seemed to be most nearly met by the method of Harza et al., (1967). This involves the intraperitoneal injection of about 10 ml of polyvinylpyrrolidone (PVP) solution containing a known concentration of inulin. At the same time a priming dose of inulin is given intravenously. Thereafter, concentrations of inulin in the blood and peritoneal fluid are measured at convenient intervals. The theory is that the volume of fluid in the peritoneal cavity will remain constant because the colloid-osmotic pressure exerted by the PVP is equivalent to that of the blood. Inulin will, however, diffuse from the peritoneal fluid into the blood, and will be removed from the blood by the kidneys. If the concentrations of inulin are correctly adjusted, a steady state will be achieved in which the diffusion of inulin from the peritoneal cavity to the blood equals clearance of inulin from the blood by the kidneys.

The rate of disappearance of inulin from the fluid in the peritoneum can be measured by taking serial samples at suitable time intervals. If this quantity is divided by the blood inulin concentration an estimate of inulin clearance is obtained. In practice, to spare the animal pain and distress, it is necessary
to carry out the procedure under anaesthesia. For this purpose pentobarbital was found to be convenient. It had the additional advantage that as a barbiturate it was less likely to depress inulin clearance than some other anaesthetics (Harza et al., 1967).

Figure 1 shows the results of a successful experiment of this type. There is a steady fall in the inulin concentration in the peritoneal fluid throughout the 2½ hours of the experiment. Since the concentration of inulin in the blood remained fairly constant from the 50th to the 120th minute, it can be assumed a steady state had been established and that the disappearance of inulin from the peritoneum equalled the rate of its removal from the blood by the kidneys. The results of a series of experiments using this method are shown in Table 5. Somewhat surprisingly these indicate that, while unilateral nephrectomy depressed glomerular filtration rate to about 60% that of its normal value, sham operation produced an almost equal depression. There was moreover a disappointing variation from experiment to experiment. This was partly attributable to the difficulty in fitting the correct straight line to the points obtained when the peritoneal inulin concentration was plotted against time. The variation in the blood inulin concentration was another probable source of error. There seemed to be a marked variation between animals in the rates at which inulin passed from the peritoneum into the blood and this was reflected in marked variations from animal to animal in the blood inulin level at which steady state conditions were reached. The method was moreover technically difficult. Successful withdrawal of samples from the peritoneum required a large needle, since the bevel of a small needle was easily occluded by mesentery or intestine. On the other hand, the puncture which such a needle made in the abdominal wall was so large that the PVP...
Estimation of inulin clearance in a 200g male rat by the method of Harza, Mályusz and Szócs.

The animal was anaesthetised with Nembutal (0.5ml of a 1/5 dilution in physiological saline) injected intramuscularly. 10ml of 6% (w/v) inulin in PVP solution was injected intraperitoneally and 0.75ml 4% (w/v) inulin in physiological saline was injected intravenously at 0 time.

The steady fall in inulin concentration in the peritoneal fluid is used to calculate the rate of absorption of inulin from the peritoneum (0.80mg/min). Since the blood inulin concentration remains approximately constant, this rate of absorption must equal the rate of clearance by the kidneys. Division of this figure by the blood inulin concentration (0.409mg/ml) gives the inulin clearance of 0.98ml blood/min/100g body weight.
Figure 1

Inulin in peritoneal fluid (mg/mL)

Inulin in blood (mg/mL)

min since intraperitoneal injection
Table 5.

Inulin clearance determined by the method of Harza, Mályusz and Szőcs (1967) on rats weighing between 175 and 215g.

<table>
<thead>
<tr>
<th></th>
<th>Inulin clearance (ml blood/100g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>1.52 ± 0.25 (11)</td>
</tr>
<tr>
<td>Unilateral nephrectomy (24h)</td>
<td>0.938 ± 0.13 (4)</td>
</tr>
<tr>
<td>Sham operation (24h)</td>
<td>1.05 ± 0.11 (4)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.
inulin solution tended to leak out. For all these reasons it seemed that the method was unsatisfactory as it stood and that it was incapable of being refined to the degree of accuracy and reproducibility which was required. Accordingly it was abandoned.

3.2. Single injection clearances.

One of the main problems in estimating renal clearance of any compound lies in maintaining the plasma concentration at a constant level. This can be circumvented by the so-called "single injection method". If a suitable quantity of a compound cleared only by the kidney is administered as a single intravenous injection, its disappearance from the plasma can in general be resolved into two components, one attributable to diffusion from the plasma to any other fluid compartments into which the compound has access, and the other to excretion by the kidney. These components can in theory be resolved mathematically. The method, therefore, is experimentally simple but mathematically complex. Its accuracy depends on the precise measurement of the fall in the plasma level of the compound over a 10 or 100-fold concentration range. This is greatly facilitated if the compound can be obtained with a radioactive label in a sufficiently high state of radiochemical purity.

Unfortunately commercially available samples of $^{14}$C and $^3$H labelled inulin are reported to be commonly contaminated with polysaccharide material of low molecular weight (Cohen, 1969). Since these may be, unlike inulin, reabsorbed in the tubule, their presence would throw doubt on the validity of the entire procedure. Figure 2 shows the results obtained when a commercial batch of radioactive inulin was subjected to gel chromatography on Sephadex G-25. The presence of a low molecular weight impurity is quite obvious. Re-chromatography of the main peak showed that this would be a suitable
Figure 2.

Chromatography of \(^{3}H\)inulin on Sephadex G-25 compared with re-chromatography of the earlier fractions.
FIGURE 2

FRACTIONS TAKEN
FOR RE-CHROMATOGRAPHY

Fraction number

RE-CHROMATOGRAPHY
method of purification. However for the preliminary investigation of the single injection method it seemed preferable to use some compound which is obtainable in such a degree of radiochemical purity that further purification would not be necessary. \[^{3}\text{H}]\text{PAH}\) (p-aminohippuric acid), which is almost completely cleared by a single passage through the kidney and is not significantly removed by any other tissue, was obtainable in the necessary degree of radiochemical purity.

Figure 3 shows the results of an experiment in which a single dose was given by intravenous injection and the level in the plasma estimated at suitable time intervals up to 60 minutes thereafter. It will be apparent that the experimental points plotted semi-logarithmically form a biphasic curve. The early and steep part of this represents the diffusion of PAH from the plasma to other fluid compartments. The later and slower phase of the curve represents chiefly removal of PAH by the kidney. The early part of the steep phase and the later part of the slow phase are roughly rectilinear. The composite curve can be analysed by extrapolating the later part of the slow phase back to time zero. From this extrapolation it is possible to read off an imaginary concentration corresponding to each time interval at which the earlier blood samples were taken. Subtraction of these imaginary figures from the actual concentrations gives a new series of figures which can be plotted semi-logarithmically as a straight line. From the slopes and intercepts of the two straight lines a clearance value can be calculated as shown in Figure 3.

The value of 2.35 ml plasma/minute/100g body weight found in this experiment is in good agreement with the figure of 2.11 ml plasma/minute/100g body weight reported for PAH by other workers (Blaufax et al., 1967).
Estimation of PAH clearance in a 195g male rat by the single injection method.

The animal was anaesthetised by an intraperitoneal injection of 0.5ml 1 in 5 Nembutal in physiological saline. 14μCi[2-3H]PAH was injected into the right jugular vein at time 0 and blood samples were withdrawn in heparinised syringes at suitable intervals thereafter from the left jugular vein.

The actual plasma PAH levels are shown by circles. Extrapolation of the terminal portion of the curve back to the axis makes it possible to read off an imaginary concentration corresponding to each time at which the blood samples were taken. Subtraction of the imaginary figures from the actual concentrations gives a new series of figures which can be plotted semi-logarithmically as a straight line. The slopes of the two straight lines (calculated as \( \frac{0.693}{t_1} \), \( b_1 \) and \( b_2 \), and the intercepts with the vertical axis, \( A \) and \( B \), are used in the calculation of the PAH clearance using the formula

\[
\text{Plasma clearance} = \frac{\text{Dose} \times b_1 \times b_2}{Ab_2 + Bb_1}
\]

In this animal the PAH clearance was 2.35ml/min/100g body weight.
**Figure 3**

D.P.M. / 50μL PLASMA

- **B** = 36000
- **A** = 6780
- **T_{1/2}** = 23.5 MIN
- **B_{1}** = 0.0295
- **T_{1/2}** = 2.1 MIN
- **B_{2}** = 0.330

MIN AFTER PAH INJECTION
Nonetheless the necessity of trying to fit a straight line to the terminal part of a curve and then using this to calculate a further set of points to which a second straight line must be fitted means that there is a good deal of subjective judgement and consequent uncertainty in the calculation. (See above). Accordingly this method also was abandoned.

3.3. **Standard clearance method.**

There seemed therefore to be no alternative to measuring renal clearance of inulin or anything else by the standard method of continuous infusion to achieve a steady state in which the plasma concentration would be constant and in which the rate of removal by the kidney could be assumed to be equal to the rate of infusion. Table 6 shows the results of a preliminary experiment which demonstrated that a steady state was quite rapidly reached and maintained and that, under the conditions employed, the blood was cleared of inulin at the rate of about 1 ml/100g/min (equivalent to about 0.5 ml plasma). About 90% of the infused inulin was recovered in the bladder washings, a quite acceptable figure in view of the losses to which the process of washing out the bladder was liable.

Table 7 shows a similar experiment on the clearance of PAH. In this case a priming dose was given in an attempt to hasten attainment of a steady state. PAH is removed from the blood both by glomerular filtration and by secretion in the renal tubule. The combined effect of these processes is to clear PAH almost completely from the blood in a single passage through the kidney. Its clearance, therefore, is a convenient measure of effective renal blood flow. The results in Table 7 show that a steady state was attained without difficulty with a clearance of approximately 1.76 ml plasma/100g/min
Table 6.

Attainment of equilibrium during infusion of 10% inulin in 0.9% saline into a normal rat weighing 175g.
The infusion rate was 17mg/20min.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inulin excreted by the kidneys mg/20min</th>
<th>Mid point blood concentration mg/ml</th>
<th>Clearance ml/blood/100g/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>7.7</td>
<td>0.386</td>
<td>1.32</td>
</tr>
<tr>
<td>20-40</td>
<td>17.8</td>
<td>0.386</td>
<td>1.08</td>
</tr>
<tr>
<td>40-60</td>
<td>15.6</td>
<td>0.410</td>
<td>1.08</td>
</tr>
<tr>
<td>60-80</td>
<td>10.8</td>
<td>0.438</td>
<td>0.70</td>
</tr>
<tr>
<td>80-100</td>
<td>16.8</td>
<td>0.458</td>
<td>1.04</td>
</tr>
<tr>
<td>100-120</td>
<td>14.9</td>
<td>0.468</td>
<td>0.91</td>
</tr>
<tr>
<td>Average of last 5</td>
<td>15.2</td>
<td>0.432</td>
<td>1.01</td>
</tr>
</tbody>
</table>

The animal was kept anaesthetised with intraperitoneal injections of nembutal.

Inulin was infused into the right jugular vein.

The amount of inulin excreted per 20 minute period was obtained by cannulating the bladder and flushing with 3 ml of 0.9% saline.

At the middle of each 20 min collection period a blood sample was withdrawn from the left jugular vein.
Table 7.

Attainment of equilibrium during infusion of 0.77% PAH in 0.9% saline into a normal rat weighing 245g.
The infusion rate was 1.54mg/20min.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>PAH excreted by the kidneys mg/20min</th>
<th>Plasma mid point concentration µg/ml</th>
<th>Clearance ml/plasma/100g/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-30</td>
<td>0.71</td>
<td>21.2</td>
<td>0.681</td>
</tr>
<tr>
<td>30-50</td>
<td>1.84</td>
<td>17.2</td>
<td>2.18</td>
</tr>
<tr>
<td>50-70</td>
<td>1.14</td>
<td>16.8</td>
<td>1.38</td>
</tr>
<tr>
<td>Mean</td>
<td>1.23</td>
<td>18.6</td>
<td>1.41</td>
</tr>
</tbody>
</table>

The animal was kept anaesthetised with intraperitoneal injections of nembutal.
After a priming dose of 500µl of infusion solution (3.85mg PAH),
PAH was infused into the right jugular vein.
The amount of PAH excreted per 20 minute period was obtained by
cannulating the bladder and flushing with 3 ml of 0.9% saline.
At the middle of each 20 min collection period a blood sample
was withdrawn from the left jugular vein.
and that about 80% of the infused PAH was recovered by washing out the bladder. Again this recovery is acceptable in view of the difficulty of the procedure and the losses it involved. The clearance obtained is in good agreement with that found by the single injection method.

While these results were encouraging it was necessary to obtain additional background information before adopting the method and some check that inulin and PAH were not being cleared outside the kidneys was obviously desirable. Table 8 shows the results of an experiment in which inulin and PAH were given as a single injection into bilaterally nephrectomised rats. The plasma concentrations of each were measured at suitable time intervals and used to calculate the apparent volumes through which the inulin and PAH had diffused. In the case of inulin, equilibrium was reached after about 30 minutes with the inulin apparently dispersed through a total volume of 17 ml/100g body weight. This is what one would expect if inulin diffuses, as is commonly supposed, throughout the extracellular water. There is no change after 30 minutes, confirming that inulin is not cleared by organs other than the kidney. PAH by contrast diffuses throughout a much larger volume (70 ml/100g body wt.) equivalent virtually to all the water in the body. One consequence of this distribution of PAH is that it enters into red blood cells and can be extracted from them during passage through the kidney. This results in a 9% overestimate of true renal blood flow by using plasma samples in the rat (Wedeen & Weiner, 1969). Equilibrium is not attained until 50 minutes but thereafter there is no further change, thus confirming that PAH likewise is not removed by tissues other than kidney.

It is important when employing PAH clearance as a measurement of
Table 8.

Times of equilibration of inulin and PAH in 3 bilaterally nephrectomised rats weighing between 190 and 235g.

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Time after injection (min)</th>
<th>Volume of distribution ml/100g body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inulin</td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>15.6</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td>27.0</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>55.0</td>
<td>17.6</td>
</tr>
<tr>
<td>3</td>
<td>60.0</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>70.0</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>80.0</td>
<td>17.0</td>
</tr>
</tbody>
</table>

At time 0 an intravenous injection of 0.25ml of a solution containing 0.76% (w/v) PAH and 7.6% (w/v) inulin in 0.9% (w/v) NaCl was given to each animal via the left jugular vein. Blood samples were withdrawn from the right jugular vein and the volume of apparent distribution calculated.
renal blood flow to ensure that the plasma concentration does not exceed the transport maximum (Pitts, 1968). If this happens the plasma will no longer be completely cleared of PAH in a single passage through the kidney and PAH clearance will no longer equal renal blood flow. The plasma PAH concentrations in our experiments were very low (less than 20μg/ml) and variations from animal to animal did not appear to produce corresponding variations in clearance. It seems, therefore, that the transport maximum had not been exceeded. In addition, the PAH clearances obtained in normal animals of 2.28 ± 0.12ml/100g/min (Table 9) are comparable with the figure given by the single injection method 2.35ml/100g/min) using only trace amounts of radioactively labelled PAH (Figure 3). Blaufox et al. (1967) also found PAH clearances of 2.11 ± 0.15ml/100g/min using the single injection clearance method and Bonjour & Malvin (1969) found PAH clearances of 2.50 ± 0.14ml/100g/min in anaesthetised rats using standard methods.

The main disadvantage of continuous infusion clearance determination technique is that it must be performed on anaesthetised animals. This is especially worrying since the purpose of these methods was to investigate the possible role of changes in GFR in the initiation of compensatory renal growth. As will be shown in Chapter 3 this growth does not appear to take place in anaesthetised rats. This suggests that anaesthesia may abolish the stimulus to growth. Pentobarbital (Nembutal) anaesthesia was chosen for the experiments because it has little effect on the GFR (Goodman & Gilman, 1965). However, barbiturate anaesthesia has a generally depressive effect on the circulation (Conway & Ellis, 1969) and although the kidney generally controls its own blood flow within
the autoregulatory range of 80-120 mmHg (Rothe et al., 1971) or 40-24 mmHg (Shipley & Study, 1951) a depression of renal blood flow measured by PAH clearance has been observed in rats under pentobarbital anaesthesia (Bonjour & Malvin, 1969). These authors however found no depression of GFR in the anaesthetised rats compared with unanaesthetised animals.

Pentobarbital anaesthesia has however the countervailing advantage of minimising the variation between animals. Bonjour & Malvin (1969) found much smaller standard deviations in anaesthetised than in unanaesthetised rats. The more an animal is disturbed the more clearance values are depressed, and so it is reasonable to suppose that the most satisfactory method is that giving the highest clearance value and hence the approximate maximal function (Lippman, 1948). The inulin clearances obtained in our experiments on normal rats of 0.64 ± 0.04 ml/min/100g body weight (Table 9) were if anything higher than those obtained on unanaesthetised rats by Kleinman et al. (1965) of 0.59 ± 0.03 ml/min/100g body weight. There would appear to be some variability in inulin clearance determinations however, since in another experiment performed 6 months later we obtained clearances of 0.872 ± 0.057 ml/100g/min (see Table 12). Inulin clearances of 0.569 ± 0.145 ml blood/min/100g body weight (Harza et al., 1967) under hexobarbital anaesthesia have been reported as have values of 0.82 ± 0.10 under pentobarbital anaesthesia (Katz & Epstein, 1967) and 0.716 ± 0.053 under anaesthesia with Inactin, another barbiturate (Hayslett et al., 1968). In one experiment chloral hydrate was employed as anaesthetic because it does not decrease blood pressure so much and essentially the same PAH clearance was obtained (2.02 ml/min/100g body wt.) This compares with the reported value for PAH clearance (2.11 ± 0.15)
under chloral hydrate anaesthesia by the single injection clearance method (Blaufox et al., 1967).

It seemed therefore that our techniques for measuring PAH and inulin clearance by continuous infusion under barbiturate anaesthesia should yield reliable estimates of effective renal plasma flow (ERPF) and glomerular filtration rate (GFR).

4. Inulin and PAH clearances after unilateral nephrectomy and high protein diet.

Tables 9, 10 and 11 show the inulin and PAH clearances in normal, sham-operated and unilaterally nephrectomised rats. The filtration fraction is the percentage of the renal blood flow which is filtered (i.e. the ratio of inulin clearance to PAH clearance). The value found for normal rats in Table 9 is similar to the figure of 0.28 ± 0.03 found for unanaesthetised rats by Bonjour & Malvin, (1969) but showed less variability. It was much less variable than the figure (about 0.47 with a standard deviation of 0.42) reported by Peters (1963) for unanaesthetised rats. Table 10 shows that 1 hour after sham operation the inulin clearance, PAH clearance and filtration fraction were indistinguishable from the values for normal animals; however 1 hour after unilateral nephrectomy there was a 22% increase (P<0.025) in the PAH clearance of the remaining kidney. The change is even more significant when PAH clearance is expressed per gram of kidney rather than per 100g body weight (P<0.01). Despite this increased renal blood flow the inulin clearance in the remaining kidney was unchanged whether expressed per g of kidney or per 100g body weight. Consequently there was an 18% depression of the filtration fraction (P<0.0025).

Table 11 shows that 24 hours after either sham operation or unilateral nephrectomy there were increases in both inulin and
Table 9.

Inulin and PAH clearances in normal rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>198 ± 8.3</td>
</tr>
<tr>
<td>Inulin clearance (ml/100g/min/kidney)</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>PAH clearance (ml/100g/min/kidney)</td>
<td>1.14 ± 0.06</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.275 ± 0.016</td>
</tr>
</tbody>
</table>

Filtration fraction = total inulin clearance / total PAH clearance

All values given ± standard error of the mean for 7 animals
Table 10.

Inulin and PAH clearances 1 hour after right unilateral nephrectomy or sham operation.

<table>
<thead>
<tr>
<th></th>
<th>Sham operation</th>
<th>Unilateral nephrectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>189 ± 2.6 (8)</td>
<td>192 ± 5.5 (8)</td>
</tr>
<tr>
<td>Inulin clearance (ml/100g/min/kidney)</td>
<td>0.32 ± 0.02 (8)</td>
<td>0.33 ± 0.02 (7)</td>
</tr>
<tr>
<td>PAH clearance (ml/100g/min/kidney)</td>
<td>1.17 ± 0.05 (8)</td>
<td>1.42 ± 0.10 (8)*</td>
</tr>
<tr>
<td>Left kidney weight (g)</td>
<td>0.606 ± 0.019 (8)</td>
<td>0.620 ± 0.095 (7)</td>
</tr>
<tr>
<td>Right kidney weight (g)</td>
<td>0.645 ± 0.020 (8)</td>
<td>0.631 ± 0.020 (8)</td>
</tr>
<tr>
<td>Inulin clearance/kidney weight (ml/min/g)</td>
<td>0.97 ± 0.04 (8)</td>
<td>1.02 ± 0.02 (6)</td>
</tr>
<tr>
<td>PAH clearance/kidney weight (ml/min/g)</td>
<td>3.55 ± 0.19 (8)</td>
<td>4.46 ± 0.26 (7)†</td>
</tr>
<tr>
<td>Wt. of left kidney /wt. of right kidney</td>
<td>0.94 ± 0.02 (8)</td>
<td>0.98 ± 0.02 (7)</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.279 ± 0.014 (8)</td>
<td>0.228 ± 0.008 (7)§</td>
</tr>
</tbody>
</table>

Filtration fraction = total inulin clearance / total PAH clearance

All values given ± standard error of the mean

Number of experiments given in parenthesis

* Significantly different from sham-operated rats P<0.025

† Significantly different from sham-operated rats P<0.01

§ Significantly different from sham-operated rats P<0.0025
Table 11.

Inulin and PAH clearances 24 hours after right unilateral nephrectomy or sham operation.

<table>
<thead>
<tr>
<th></th>
<th>Sham operation</th>
<th>Unilateral nephrectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>204 ± 5.2      (7)</td>
<td>198 ± 5.7 (8)</td>
</tr>
<tr>
<td>Inulin clearance (ml/100g/min/kidney)</td>
<td>0.37 ± 0.02 (7)</td>
<td>0.42 ± 0.03 (8)</td>
</tr>
<tr>
<td>PAH clearance (ml/100g/min/kidney)</td>
<td>1.40 ± 0.05 (7)</td>
<td>1.68 ± 0.04 (8)*</td>
</tr>
<tr>
<td>Left kidney weight (g)</td>
<td>0.716 ± 0.053 (7)</td>
<td>0.756 ± 0.038 (8)</td>
</tr>
<tr>
<td>Right kidney weight (g)</td>
<td>0.727 ± 0.053 (7)</td>
<td>0.634 ± 0.027 (7)</td>
</tr>
<tr>
<td>Inulin clearance /kidney weight (ml/min/g)</td>
<td>1.05 ± 0.06 (7)</td>
<td>1.11 ± 0.10 (7)</td>
</tr>
<tr>
<td>PAH clearance /kidney weight (ml/min/g)</td>
<td>4.03 ± 0.19 (7)</td>
<td>4.44 ± 0.18 (8)</td>
</tr>
<tr>
<td>Wt. of left kidney /wt. of right kidney</td>
<td>0.99 ± 0.02 (7)</td>
<td>1.21 ± 0.04 (7)*</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.261 ± 0.009 (7)</td>
<td>0.252 ± 0.019 (8)</td>
</tr>
</tbody>
</table>

Filtration fraction = total inulin clearance / total PAH clearance

All values given as mean ± S.E.M. for number of animals in parenthesis.

* Significantly different from sham-operated rats P<0.0005
PAH clearances and perhaps a slight fall in filtration fraction. These changes are presumably a response to the stress of anaesthesia and operation. There was remarkably little difference between the unilaterally nephrectomised and sham-operated animals. Although the remaining kidney in the former had increased in size, the only other difference was its blood flow had increased in proportion ($P<0.0005$).

Taking Tables 10 and 11 together one may conclude that, apart from a small increase in blood flow immediately after the operation, unilateral nephrectomy produces remarkably little change in the blood flow and filtration rate of the remaining kidney. Such changes as are apparent at 24 hours appear to accompany, rather than precede, the process of compensatory growth.

Table 13 shows the results of a parallel experiment to find out whether the kidney growth produced by feeding a high protein diet, which in its extent and rapidity resembles compensatory renal hypertrophy (Halliburton, 1969), is accompanied by any dramatic change in function. In this experiment rats which had been previously maintained on the standard "rat cubes" diet used in the animal house were transferred for three days to one or other of two semisynthetic isocaloric diets containing either 15% casein or 15% casein plus 30% gelatin. Both diets caused a slight loss of body weight but the gelatin-containing diet also produced, as expected (Halliburton, 1969), a 33% increase in kidney weight ($P<0.0005$) although the liver weight was unaffected. This striking increase in kidney size was not accompanied by a proportional change in inulin clearance (which increased by only 23%, $P<0.05$) or in PAH clearance. Indeed if these quantities are related to kidney weight instead of body weight it is apparent that PAH clearance per g of kidney, if anything, falls ($P<0.05$). Here again, therefore, there can be no suggestion that
### Table 12.

Inulin and PAH clearances in 13 normal rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>189 ± 4.4</td>
</tr>
<tr>
<td>Inulin clearance (ml/100g/min/kidney)</td>
<td>0.872 ± 0.057</td>
</tr>
<tr>
<td>PAH clearance (ml/100g/min/kidney)</td>
<td>2.78 ± 0.15</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.319 ± 0.022</td>
</tr>
</tbody>
</table>

\[
\text{Filtration fraction} = \frac{\text{total inulin clearance}}{\text{total PAH clearance}}
\]

All values given ± standard error of the mean.
Table 13.

Inulin and PAH clearances in rats maintained for 3 days on a high gelatin or a control diet.

<table>
<thead>
<tr>
<th></th>
<th>15% Casein</th>
<th>15% Casein 30% Gelatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of body weight (g)</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Total kidney weight (g)</td>
<td>1.19 ± 0.03</td>
<td>1.58 ± 0.08 §</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>6.82 ± 0.21</td>
<td>7.03 ± 0.15</td>
</tr>
<tr>
<td>Inulin clearance (ml/100g/min)</td>
<td>0.93 ± 0.08</td>
<td>1.14 ± 0.06 *</td>
</tr>
<tr>
<td>PAH clearance (ml/100g/min)</td>
<td>2.76 ± 0.28</td>
<td>2.98 ± 0.18</td>
</tr>
<tr>
<td>Inulin clearance /kidney weight (ml/min/g)</td>
<td>1.50 ± 0.11</td>
<td>1.38 ± 0.08</td>
</tr>
<tr>
<td>PAH clearance /kidney weight (ml/min/g)</td>
<td>4.44 ± 0.39</td>
<td>3.63 ± 0.21 *</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.342 ± 0.010</td>
<td>0.387 ± 0.027</td>
</tr>
</tbody>
</table>

All values are means ± S.E.M. for groups of 6 rats weighing between 195 and 205g

* Different from control P<0.05

§ Different from control P<0.0005
kidney growth is in any sense a consequence of increased kidney function. The well-known effect of gelatin in increasing GFR (Vogl, 1953), is presumably secondary to its effect in causing kidney growth and not vice versa.

5. Conclusions.

The significance of the results described in this section is perfectly clear. Immediately after unilateral nephrectomy the remaining kidney is the recipient of a slightly augmented (20-25%) blood flow, but the volume of glomerular filtrate does not increase. There is therefore no reason to suppose that it compensates for its absent partner by increasing its own functional capacity. On the contrary it would appear that compounds such as urea and creatinine, which are normally excreted by the kidney because they pass through the glomerular membrane and are not actively absorbed, tend to accumulate in the plasma. This accumulation is apparently well tolerated; the animals appear healthy and the blood amino acid levels, which might be altered by any serious metabolic upset, show no significant change. Nonetheless it is quite possible that the accumulation of some such metabolite might provide the impulse which causes the remaining kidney to grow.
NUCLEIC ACID METABOLISM
NUCLEIC ACID METABOLISM.

1. Introduction.

The previous chapter showed that after unilateral nephrectomy there is little change in the function of the remaining kidney. The compensatory renal hypertrophy is therefore not the result of it operating at more than its normal capacity. On the contrary the animal suffers temporarily from a degree of renal insufficiency as demonstrated by the increases in plasma creatinine and urea.

It is a reasonable assumption that it is some aspect of this renal insufficiency which stimulates the remaining kidney to grow and it would obviously be desirable to demonstrate positively the operation of such a humoral agent. Analogy with liver regeneration would suggest that the transplantation and cross-circulation experiments might be adapted to yield just such evidence.

There are, however, three formidable difficulties to contend with.

i.) Liver regeneration is signalised by a spectacular outburst of DNA synthesis and mitotic activity, the intensity of which can be regarded as a reliable estimate of the rate of growth. Kidney hypertrophy cannot be estimated with the same ease and confidence. It involves only a modest increase in cell number; indeed there may be no cell division at all. Consequently DNA synthesis and mitosis are quite useless as measures of growth. It is necessary instead to rely on changes in RNA metabolism and these, as will be shown below, are not always easy to demonstrate, or, when demonstrated, capable of unambiguous interpretation.

ii.) The structure of the liver in the rat allows the
experimenter considerable freedom in deciding how much tissue to
excise. He can easily take 10%, 67% or 87%, and intermediate
quantities are quite possible. In each case, damage to the
surviving remnant is minimal. No such range of options is
available in the case of the kidney. One kidney or both can
be removed. Any attempt to remove a fraction of either kidney
results in a mitotic response to the damage, both locally and
in the contralateral kidney (Saetren, 1970; Argyris & Trimble,
1964; Argyris et al., 1969; Malamud et al., 1971).

iii.) In the case of liver regeneration, the cross-circulation
experiments have indicated that the supposed humoral agent is
exceedingly short-lived and difficult to demonstrate even in
the most favourable circumstances. In the case of the kidney
the circumstances are, as we have just seen, certainly not
favourable.

It is therefore not surprising that few attempts have been made
to investigate kidney hypertrophy by cross-circulation and that these
have yielded negative results of uncertain significance (Johnson &
Vera Roman, 1968). The technique does however hold out the hope of
providing an unambiguous and conclusive test of the humoral theory,
if only some means could be found whereby the hypertrophy process
could be detected with certainty, and preferably early in its course.
The obvious means of achieving this would seem to lie in a knowledge
of the changes in RNA metabolism which occur in the remaining kidney
in the first 12 hours or so after unilateral nephrectomy.

Of the changes which might be of use, the increase in RNA per
cell (i.e. RNA/DNA ratio) is the most obvious. The increase of 7%
within 12 hours is just sufficient to be reliably measured if the
determination is carried out with great care and is adequately
controlled (Halliburton & Thomson, 1965). Since the half-life of kidney RNA does not alter during compensatory renal hypertrophy (Malt & Lemaitre, 1968), its accumulation must be due to increased synthesis and this should be demonstrable using isotopically labelled precursors. Malt and Stoddard (1966) have shown increased labelling of ribosomes with radioactive uridine after unilateral nephrectomy. Information on early changes in synthesis of ribosomal RNA is rather scanty. Halliburton and Thomson (1966) and Halliburton (1969) have reported substantial increases (25 to 100%) in the incorporation of orotic acid 20 minutes to 4 hours after unilateral nephrectomy, but this conclusion was based on indirect arguments and must be regarded with some reserve.

The earliest and most dramatic change in RNA metabolism is the marked reduction in labelled RNA extracted from the nucleoplasm which migrates slower than 28S RNA on polyacrylamide gels, perhaps due to faster processing (Willems et al., 1969). This decrease was certainly apparent within 1 hour of the operation and was perhaps detectable after only 10 minutes. It was still evident after 7 days.

When the present investigation was begun it seemed that the changes in heterodisperse nucleoplasmic RNA (HnRNA) offered the best prospect of detecting and measuring kidney hypertrophy early in its course, and therefore of making cross-circulation experiments feasible. An attempt was therefore made to repeat the work of Willems et al. (1969).

2. Extraction of HnRNA.

The method of demonstrating the labelling of HnRNA reported by Willems et al. (1969) consisted of injecting mice with 250μCi of tritiated uridine and slaughtering them 10 minutes or 1 hour later. The kidneys were rapidly removed and homogenised and the nuclei
Acrylamide gel electrophoresis of HnRNA prepared by the method of Willems et al. (1969).

Figure 4a shows an example of the results obtained on normal mice from the departmental colony.

Figure 4b, redrawn from Willems et al. (1969), shows the labelling pattern obtained for kidney HnRNA from sham-operated Charles' River mice.

In each case animals were injected with 250μCi[5-3H]uridine 1 hour prior to sacrifice. Purification of nuclei and extraction of HnRNA were as described by Willems et al. (1969). RNA was electrophoresed for 5 hours at 5mA/gel on 2.8% polyacrylamide gels with ethylene diacrylate as cross-linker.
Distance run (cm). Migration from left to right
isolated. The nucleoplasmic RNA was then extracted and subjected to electrophoresis on polyacrylamide gels. In the present study the published method was followed religiously. A typical pattern for normal mice is shown in Figure 4 alongside a copy of one of the patterns obtained by Willems et al. (1969). The absence of labelled HnRNA (>28S) is obvious. The abundance of labelled low molecular weight RNA suggests that the HnRNA had been degraded during the course of the isolation. Attempts were made to avoid this by adopting a rapid method of isolating nuclei and by the use of a variety of ribonuclease inhibitors. Neither of these expedients proved successful. Nuclei were obtained in good yield and in a high degree of purity; and some of the ribonuclease inhibitors gave improved recovery of 28S RNA, but the electrophoretic patterns reported by Malt's group were never approximated, let alone repeated.

3. **Measurement of rate of RNA synthesis in vivo.**

The attempt to repeat Malt's experiments having failed, it seemed worth while to investigate the possibility of using other and less elaborate methods of demonstrating changes in RNA metabolism after unilateral nephrectomy. An attempt was made to detect increased rates of RNA synthesis by measuring incorporation of a specific precursor ([5-3H] uridine) into kidney RNA.

As a preliminary the isotope was injected either intravenously or intraperitoneally into rats of the same body weight and the changes in plasma radioactivity with time were observed. Figure 5 shows that the volume of distribution of [5-3H]uridine was apparently about 70-85 ml/100g (roughly equal to total body water) and that with injection by either route a constant plasma concentration was soon established (neglecting possible metabolism).

Table 14 shows the relative rates of incorporation into TCA
Figure 5.

Plasm radioactivity following intravenous and intraperitoneal injection of [5-\(^3\text{H}\)]uridine into rats weighing between 165 and 170g.

The animals were anaesthetised with an intraperitoneal injection of 0.5ml 1 in 5 Nembutal in physiological saline.

Approximately 50µCi[5-\(^3\text{H}\)]uridine (i.e. 111 x 10^6d.p.m.) was injected into each animal at time 0.

Intravenous injection was via the left jugular vein. Blood samples were withdrawn from the right jugular vein.

The apparent volume of distribution of [5-\(^3\text{H}\)]uridine is approximately 70-85ml/100g body weight.
Figure 5

Intraperitoneal injection

Intravenous injection

Time after injection (min)
Table 14.

The incorporation of \( [5-^{3}\text{H}] \)uridine into acid-insoluble material from different organs of a normal rat weighing 185g.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Weight taken (g)</th>
<th>d.p.m./g of tissue x 10^{-3}</th>
<th>Acid-insoluble</th>
<th>Acid-soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left kidney</td>
<td>0.60</td>
<td>154 ± 15 (4)</td>
<td>137 ± 7 (4)</td>
<td></td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.60</td>
<td>612 ± 28 (4)</td>
<td>1810 ± 200 (4)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.65</td>
<td>30.4 ± 4.7 (4)</td>
<td>1380 ± 33 (4)</td>
<td></td>
</tr>
</tbody>
</table>

The isotope (125\(\mu\)Ci) was injected intraperitoneally 1 hour before the animal was killed.

Values are means ± S.E.M. with number of determinations in parenthesis.
precipitable material in kidneys, liver and heart of a normal rat. The liver has a much higher incorporation (about 4-fold) than the kidneys on a weight basis. This perhaps reflects the fact that the RNA/DNA ratio in the liver is 4 times that in the kidney (Thomson, 1953). The kidney in turn appears to be about 4-fold more active in RNA synthesis than the heart. These estimates are not corrected for the specific activity of precursor pools but the acid-soluble radioactivity is very similar in liver and heart. Since this method appears capable of demonstrating different rates of RNA synthesis in different organs, it might well be equally capable of demonstrating changes in RNA synthesis during compensatory renal hypertrophy.

Tables 15 and 16 both indicate, however, that the rate of uridine incorporation is unlikely to be increased much, if at all, 12 to 18 hours after unilateral nephrectomy, at least as far as can be judged by this method. This is especially evident when the acid-soluble radioactivity, which is probably mainly attributable to UTP (Bucher & Swaffield, 1965; 1966; Kochakian & Hill, 1966), is taken into account. Since changes in the specific activity of the UTP pool in the kidney after unilateral nephrectomy are parallel to those after sham operation (Malt, 1969), it would appear that measurement of acid-soluble radioactivity might be a fair estimate of the specific activity of the UTP precursor pool.

Tables 17 and 18 show the results of similar experiments in which $[^{32}\text{P}]$orthophosphate was injected intraperitoneally or intravenously. Again there is no significant difference between unilaterally nephrectomised and sham-operated rats, although in Table 18 there is clearly greater incorporation in the liver than in the kidney.
Table 15.

Incorporation of $[5^{-3}H]$uridine into kidney acid-insoluble material after right unilateral nephrectomy or sham operation.

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Acid insoluble</th>
<th>Acid soluble</th>
<th>Acid insoluble x 10$^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>left kidney</td>
<td>0.55</td>
<td>80.5 ± 4.7 (5)</td>
<td>3740 ± 70 (4)</td>
</tr>
<tr>
<td>Sham operation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>left kidney</td>
<td>0.59</td>
<td>58.3 ± 6.8 (5)</td>
<td>2900 ± 80 (4)</td>
</tr>
<tr>
<td>right kidney</td>
<td>0.57</td>
<td>60.6 ± 3.7 (5)</td>
<td>3000 ± 70 (4)</td>
</tr>
</tbody>
</table>

The isotope (125μCi) was injected intraperitoneally 11 hours after operation and 2 hours before the animals were killed.

Both rats weighed 170g.

Values are means ± S.E.M. for the number of determinations in parenthesis.
Table 16.

Incorporation of [5-\textsuperscript{3}H]uridine into kidney acid insoluble material after right unilateral nephrectomy or sham operation in rats weighing 165g.

<table>
<thead>
<tr>
<th>Unilateral nephrectomy</th>
<th>Weight</th>
<th>Acid insoluble</th>
<th>Acid soluble</th>
<th>Acid insoluble x 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left kidney</td>
<td>0.59</td>
<td>92.3 ± 6.0</td>
<td>843 ± 46</td>
<td>1.09</td>
</tr>
<tr>
<td>Sham operation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.54</td>
<td>82.8 ± 2.3</td>
<td>691 ± 21</td>
<td>1.20</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.57</td>
<td>81.9 ± 10.6</td>
<td>727 ± 43</td>
<td>1.12</td>
</tr>
</tbody>
</table>

The isotope (125\textmu Ci) was injected intraperitoneally 17 hours after operation and 1 hour prior to sacrifice.

Values are means ± S.E.M. for number of determinations in parenthesis.
Table 17.

Incorporation of $[^{32}\text{P}]$orthophosphate into kidney acid insoluble material after right unilateral nephrectomy or sham operation in rats weighing between 160 and 165g.

<table>
<thead>
<tr>
<th></th>
<th>$[^{32}\text{P}]$ incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>left kidney c.p.m./kidney</td>
</tr>
<tr>
<td></td>
<td>weight (g)</td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td>0.64</td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td>0.56</td>
</tr>
<tr>
<td>Sham operation</td>
<td>0.52</td>
</tr>
<tr>
<td>Sham operation</td>
<td>0.49</td>
</tr>
</tbody>
</table>

The isotope (100μCi) was injected intraperitoneally 18 hours after operation and 2 hours before sacrifice.

Values are means ± S.E.M. for 4 determinations.
Table 18.
Incorporation of $[^{32}\text{P}]$ orthophosphate into acid insoluble material in kidneys of unilaterally nephrectomised and sham-operated rats weighing between 160 and 165g.

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Acid insoluble</th>
<th>Acid soluble</th>
<th>Insoluble Soluble $\times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unilateral nephrectomy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.54</td>
<td>280 ± 19</td>
<td>1960 ± 0</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.62</td>
<td>244 ± 18</td>
<td>1670 ± 40</td>
</tr>
<tr>
<td><strong>Sham operation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.58</td>
<td>252 ± 23</td>
<td>1770 ± 50</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.62</td>
<td>264 ± 12</td>
<td>2140 ± 30</td>
</tr>
<tr>
<td>Liver</td>
<td>0.68</td>
<td>1850 ± 15</td>
<td>2530 ± 70</td>
</tr>
</tbody>
</table>

The isotope (100µCi) was injected into the tail vein 13 hours after operation and 1 hour prior to sacrifice.

Values are means ± S.E.M. for number of determinations in parenthesis.
Clearly, then, neither uridine nor \(^{32}\)P orthophosphate incorporation can be used as a simple indication that hypertrophy is in progress.

4. **Measurement of accumulation of RNA.**

The attempt to demonstrate RNA synthesis by incorporation of radioactive precursors having failed, consideration was given to the possibility of using RNA accumulation as an indicator of hypertrophy. A small but reliable change could be of more value as an indicator of growth than one which was larger but more variable. RNA accumulation can be measured quite accurately because the two kidneys of a normal rat have exactly the same chemical composition. Consequently the excised kidney acts as an internal control for its partner (Halliburton and Thomson, 1965). Since the DNA content of the kidney does not change for several hours after unilateral nephrectomy, unlike protein content and wet and dry weights, the change in RNA/DNA ratio affords the best index of the rate of accumulation of RNA (Halliburton, 1966).

Accordingly groups of 4 rats were either unilaterally nephrectomised or sham-operated and killed 20 hours later. Table 19 shows that after this time there was a significant increase in kidney weight of 13% (\(P<0.05\)) but no significant change in total RNA and protein. The only significant chemical change was a decrease in the DNA concentration of 9% (\(P<0.025\)) due to the hypertrophy without DNA synthesis which is a characteristic of the system.

Table 20 shows the results of an experiment in which the kidney removed at operation (right kidney) acted as internal control for the changes in the remaining kidney after 24 hours. In this experiment after unilateral nephrectomy there were no significant increases in kidney weight, and protein content, but a significant increase in RNA content of 13% (\(P<0.05\)) compared with the right kidneys of the same group. As in the last experiment a significant change after unilateral
Table 19.

The effect of unilateral nephrectomy and sham operation on the composition of the left kidney after 20 hours. There were 4 rats weighing between 160 and 165g in each group.

<table>
<thead>
<tr>
<th></th>
<th>Sham operation</th>
<th>Unilateral nephrectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight change (g)</td>
<td>-6.3 ± 3.0</td>
<td>-5.0 ± 2.1</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.525 ± 0.030</td>
<td>0.595 ± 0.019 *</td>
</tr>
<tr>
<td>Protein per kidney (mg)</td>
<td>76.9 ± 7.1</td>
<td>82.2 ± 3.9</td>
</tr>
<tr>
<td>RNA per kidney (mg)</td>
<td>3.40 ± 0.18</td>
<td>3.73 ± 0.04</td>
</tr>
<tr>
<td>DNA per kidney (mg)</td>
<td>2.76 ± 0.17</td>
<td>2.85 ± 0.10</td>
</tr>
<tr>
<td>Protein/kidney weight (mg/g)</td>
<td>146 ± 9</td>
<td>139 ± 8</td>
</tr>
<tr>
<td>RNA/kidney weight (mg/g)</td>
<td>6.48 ± 0.10</td>
<td>6.28 ± 0.20</td>
</tr>
<tr>
<td>DNA/kidney weight (mg/g)</td>
<td>5.24 ± 0.10</td>
<td>4.77 ± 0.15 †</td>
</tr>
<tr>
<td>RNA/protein (x10²)</td>
<td>45.0 ± 3.6</td>
<td>45.7 ± 2.3</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>27.8 ± 1.3</td>
<td>29.1 ± 2.3</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>1.23 ± 0.03</td>
<td>1.31 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.
* Significantly different from sham-operated rats P<0.05
† Significantly different from sham-operated rats P<0.025
Table 20.
Chemical changes in the kidney 24 hours after unilateral nephrectomy and sham operation.

<table>
<thead>
<tr>
<th></th>
<th>Unilateral nephrectomy</th>
<th>Sham operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right kidney</td>
<td>Left kidney</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.53 ± 0.02</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>70.9 ± 6.9</td>
<td>81.4 ± 10.0</td>
</tr>
<tr>
<td>RNA (mg)</td>
<td>3.37 ± 0.10</td>
<td>3.79 ± 0.18  *</td>
</tr>
<tr>
<td>DNA (mg)</td>
<td>2.91 ± 0.05</td>
<td>2.68 ± 0.14</td>
</tr>
<tr>
<td>Protein/kidney weight (mg/g)</td>
<td>127 ± 11</td>
<td>139 ± 11</td>
</tr>
<tr>
<td>RNA/kidney weight (mg/g)</td>
<td>6.36 ± 0.18</td>
<td>6.54 ± 0.20</td>
</tr>
<tr>
<td>DNA/kidney weight (mg/g)</td>
<td>5.49 ± 0.13</td>
<td>4.61 ± 0.12  †</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>24.4 ± 2.1</td>
<td>30.2 ± 2.5</td>
</tr>
<tr>
<td>RNA/protein x 10</td>
<td>49.2 ± 6.0</td>
<td>48.1 ± 4.8</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>1.16 ± 0.02</td>
<td>1.42 ± 0.01  §</td>
</tr>
</tbody>
</table>

| Left kidney RNA/DNA  | 1.23 ± 0.02  § | 1.01 ± 0.03  |
| Right kidney RNA/DNA |            |               |

Values are means ± S.E.M. for 4 rats weighing between 160 and 165g.

* Significantly different from right kidney P<0.05.
† Significantly different from right kidney P<0.0025.
§ Significantly different from right kidney P<0.00025.
‖ Significantly different from sham-operated rats P<0.0025.
nephrectomy is the decrease of 16% in the DNA concentration (P<0.0025) compared to the kidney removed earlier. Again, though, the DNA concentration in the kidneys of the sham-operated rats might suggest some growth. The RNA/DNA ratio is 23% higher in the growing kidney than in its partner removed earlier (P<0.0005) as is indicated by the left kidney / right kidney ratio which is 22% higher than that in sham-operated rats (P<0.0025). The increase in RNA/DNA ratio obviously affords the most sensitive indication of growth but it would appear to be only of value when compared with the kidney removed earlier from the same rat. This is probably a result of the biological variation encountered and is unfortunate from the point of view of the intended cross-circulation experiments. Although it should be very easy to detect a diminution of growth as measured by RNA/DNA ratio, in a unilaterally nephrectomised rat cross-circulated with a normal rat, a large number of experiments would be required to detect a growth response in the intact cross-circulated partner. However even with this limitation it was considered that valuable information could be obtained.

Table 21 shows the results of experiments intended to give more information about the timing of the increase in RNA. In one of them (15 hours) hooded rats were used instead of the normal albino (originally Wistar) rats from the departmental colony, in the hope that the more inbred strain would have less variable RNA/DNA ratios in the kidneys. Unfortunately this was not the case and the same scatter was observed. Increases in RNA/DNA ratio of 9% after 12 hours, 14% after 15 hours, 17% after 19 hours and 23% after 24 hours were obtained after unilateral nephrectomy. In the choice of a suitable time over which to run subsequent experiments a compromise was reached between short time interval and magnitude of response, and a period of 16 hours was selected.
Table 21.

Effect of unilateral nephrectomy and sham operation on kidney RNA/DNA ratio.

<table>
<thead>
<tr>
<th>Time after operation (h)</th>
<th>Weight of rats (g)</th>
<th>RNA/DNA ratio in left kidney</th>
<th>RNA/DNA ratio in right kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unilateral nephrectomy</td>
<td>Sham operation</td>
</tr>
<tr>
<td>12</td>
<td>160-165 (Albino rats)</td>
<td>1.11</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.07</td>
<td>1.00</td>
</tr>
<tr>
<td>15</td>
<td>195-200 (Hooded rats)</td>
<td>1.15</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.12</td>
<td>1.01</td>
</tr>
<tr>
<td>19</td>
<td>160-165 (Albino rats)</td>
<td>1.24</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.10</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Table 22 shows that when unilaterally nephrectomised rats were cross-circulated for between 8 and 16 hours with a normal partner, no growth, as evidenced by a change in RNA/DNA ratio, was apparent in the remaining kidney 16 hours after the operation. The scatter of RNA/DNA ratios in the kidneys of different rats shows that it is hopeless to attempt to obtain evidence of stimulation in the intact partner. The failure of the remaining kidneys in the unilaterally nephrectomised partners to show signs of growth might be attributable to the animal being anaesthetised throughout the 16 hours of the experiment. Phenobarbital was selected as anaesthetic because it is a suitably long-acting agent and barbiturates have a minimal effect on renal function (see last chapter). However, as is shown in Table 23, phenobarbital alone at the level administered (200 mg/kg) was capable of inhibiting the accumulation of RNA in the remaining kidney 16 hours after unilateral nephrectomy. Indeed even sub-anaesthetic doses of phenobarbital (150 mg/kg) were capable of inhibiting the response. Pentobarbital (Nembutal) at 150 mg/kg had the same effect. Chloral hydrate, though not a barbiturate, also inhibited the accumulation of RNA at anaesthetic doses (1.5g/kg) but not to the same extent as did the barbiturates. A 9% increase was observed whereas a 15% increase might have been expected but much variation between animals was apparent. Since barbiturates are the most convenient anaesthetics, and even chloral hydrate inhibits compensatory renal hypertrophy, it was decided that the cross-circulation experiments would have to be postponed until a more sensitive and preferably earlier method of detection of kidney growth was developed.

It has recently been reported that vinblastine, a dimeric alkaloid extracted from the periwinkle plant, enhances the growth due to unilateral nephrectomy despite its effect in inhibiting both DNA and RNA synthesis (Cosgriff & Sudarsanam, 1970). This may suggest that the anaesthetic
Table 22.

Effect of cross-circulation on change in kidney mean cell RNA content 16 hours after unilateral nephrectomy.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time of cross-circulation</th>
<th>Wt. of rats (g)</th>
<th>Left/Right DNA Unilaterally nephrectomised animal</th>
<th>Intact partner</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-16 hours</td>
<td>190</td>
<td>1.00</td>
<td>1.06</td>
</tr>
<tr>
<td>2</td>
<td>0-8 hours</td>
<td>215</td>
<td>1.01</td>
<td>1.06</td>
</tr>
<tr>
<td>3</td>
<td>0-8 hours</td>
<td>225</td>
<td>1.01</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>0-16 hours</td>
<td>155</td>
<td>1.00</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Mean of cross-circulated unilaterally nephrectomised rats = 1.00 ± 0.00 (S.E.M.)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RNA/DNA in left and right kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unilateral Nephrectomy</td>
</tr>
<tr>
<td>1</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>1.47</td>
</tr>
<tr>
<td>2</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>1.51</td>
</tr>
<tr>
<td>3</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>1.54</td>
</tr>
<tr>
<td>4</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>1.29</td>
</tr>
</tbody>
</table>
Table 23.

Effect of anaesthetics on kidney growth after unilateral nephrectomy as measured by mean cell RNA content 16 hours after operation.

<table>
<thead>
<tr>
<th>Phenobarbital</th>
<th>Phenobarbital</th>
<th>Chloral hydrate</th>
<th>Pentobarbital</th>
</tr>
</thead>
<tbody>
<tr>
<td>(200mg/kg)</td>
<td>(150mg/kg)</td>
<td>(1.5g/kg)</td>
<td>(150mg/kg)</td>
</tr>
<tr>
<td>Right kidney</td>
<td>Left kidney</td>
<td>Right kidney</td>
<td>Left kidney</td>
</tr>
<tr>
<td>No. of animals</td>
<td>in each group</td>
<td>No. of animals</td>
<td>in each group</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. of animals</td>
<td>in each group</td>
</tr>
<tr>
<td>(6)</td>
<td>(4)</td>
<td>(3)</td>
<td>(1)</td>
</tr>
<tr>
<td>Body wt. (g)</td>
<td>185</td>
<td>175</td>
<td>180-195</td>
</tr>
<tr>
<td>Kidney wt. (mg)</td>
<td>623 ± 19</td>
<td>618 ± 184</td>
<td>575 ± 9</td>
</tr>
<tr>
<td>mg RNA/g</td>
<td>3.74 ± 0.11</td>
<td>3.77 ± 0.10</td>
<td>1.80 ± 0.12</td>
</tr>
<tr>
<td>mg DNA/g</td>
<td>2.86 ± 0.07</td>
<td>2.85 ± 0.06</td>
<td>1.49 ± 0.17</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>1.30 ± 0.05</td>
<td>1.32 ± 0.04</td>
<td>1.23 ± 0.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Left RNA/DNA</th>
<th>Right RNA/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.02 ± 0.03</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>1.09 ± 0.03</td>
<td>1.04</td>
</tr>
</tbody>
</table>

All animals were subjected to right unilateral nephrectomy.

Values are means ± S.E.M.

Anaesthetics were administered by intraperitoneal injection.
Table 24.
Effect of vinblastine on compensatory renal growth as measured by mean cell content of RNA 16 hours after unilateral nephrectomy.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Vinblastine</th>
<th>Unilateral nephrectomy</th>
<th>Unilateral + vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right kidney</td>
<td>Left kidney</td>
<td>Right kidney</td>
<td>Left kidney</td>
</tr>
<tr>
<td>Body wt. (g)</td>
<td>189 ± 3</td>
<td>188 ± 3</td>
<td>199 ± 3</td>
<td>191 ± 4</td>
</tr>
<tr>
<td>Kidney wt. (mg)</td>
<td>657 ± 21</td>
<td>627 ± 19</td>
<td>668 ± 16</td>
<td>645 ± 19</td>
</tr>
<tr>
<td>RNA (mg)</td>
<td>3.11 ± 0.07</td>
<td>2.89 ± 0.09</td>
<td>3.17 ± 0.11</td>
<td>3.01 ± 0.15</td>
</tr>
<tr>
<td>mg RNA/g</td>
<td>4.75 ± 0.14</td>
<td>4.62 ± 0.16</td>
<td>4.74 ± 0.09</td>
<td>4.66 ± 0.14</td>
</tr>
<tr>
<td>DNA (mg)</td>
<td>2.41 ± 0.09</td>
<td>2.35 ± 0.12</td>
<td>2.41 ± 0.12</td>
<td>2.37 ± 0.11</td>
</tr>
<tr>
<td>mg DNA/g</td>
<td>3.67 ± 0.11</td>
<td>3.74 ± 0.12</td>
<td>3.60 ± 0.11</td>
<td>3.66 ± 0.07</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>1.30 ± 0.03</td>
<td>1.24 ± 0.07</td>
<td>1.32 ± 0.04</td>
<td>1.28 ± 0.05</td>
</tr>
<tr>
<td>Left RNA/DNA</td>
<td>0.96 ± 0.04</td>
<td>0.97 ± 0.04</td>
<td>1.10 ± 0.02</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>Right RNA/DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for groups of 6 rats.
inhibition of compensatory renal hypertrophy occurs at a level other than simply RNA synthesis. It is interesting that the barbiturates did not cause a fall in renal RNA content which might be expected to occur if RNA synthesis was inhibited and normal turnover continued over the 16 hour period. At any rate vinblastine was tested for its ability to influence the rise in renal RNA content after unilateral nephrectomy, either by enhancing or inhibiting it. Table 24 shows that it did neither. The RNA accumulation amounted to 15% whether or not the drug was used. This incidentally demonstrates the hypertrophic nature of compensatory kidney growth. Vinblastine is a powerful mitotic inhibitor but does not depress the immediate RNA increase or the later increase in weight of the kidney.

5. DNA polymerase.

No sufficiently marked and early changes could be observed in the rates of RNA and DNA synthesis but it seemed possible that increases in the enzymes responsible might be a more sensitive indication of growth. DNA polymerase activity was measured in nuclei and supernatant using native and denatured DNA as primer by the method of Lindsay et al. (1970). Most of the activity was found in the supernatant, possibly due to leakage of enzyme from the nuclei during preparation. With both nuclear and supernatant enzymes the capacity to incorporate deoxyribonucleoside triphosphates, using both native and denatured DNA as primer, was roughly equivalent although the supernatant enzyme had a slight preference for native DNA. From Figure 6 it can be seen that no marked change could be detected in any of the DNA polymerase activities after unilateral nephrectomy compared with sham-operated control rats. Certainly there is no change sufficiently marked or early enough to act as an early indicator of compensatory renal growth. This is probably attributable both to the accessory role of the enzyme in DNA synthesis and the
DNA polymerase activity in the left kidney of male rats weighing between 165 and 175g following unilateral nephrectomy and sham operation.

Each point represents the value for the left kidney of a single animal. All animals were killed at 10.00 hours. Operations were conducted at the appropriate times prior to this. DNA polymerase activity was measured as described in "Materials and Methods".
**Figure 6**

Supernatant enzyme, native DNA

- Nephrectomy
- Sham

Supernatant enzyme, denatured DNA

- Nephrectomy
- Sham

Nephrectomy versus Sham

DNA polymerase: Incorporation of $[^3]$HdTTP: d.p.m./mg protein

Hours after operation
unimportance of DNA synthesis during the growth process. Mayfield et al. (1967) have shown only small increases in deoxythymidine kinase, deoxythymidine monophosphate kinase and deoxycytidine monophosphate deaminase 48 hours after unilateral nephrectomy, whereas another enzyme in the deoxyribonucleoside triphosphate biosynthetic pathway, deoxycytidine monophosphate kinase, and deoxythymidine monophosphate phosphatase showed no consistent changes. These results are in keeping with the DNA polymerase results reported here.

6. RNA polymerase.

In contrast to the late appearance and low rate of DNA synthesis in the growing kidney, the increase in RNA can be easily detected within 12 hours of the operation, as shown in Table 21. Consequently, the possibility of detecting increased RNA polymerase activity in the first few hours after unilateral nephrectomy was investigated. It has been shown by Widnell and Tata (1966) that in crude preparations of mammalian nuclei at least two types of DNA-dependent RNA polymerase activity can be distinguished, one of which is activated by Mg²⁺ at low ionic strength, and the other by Mn²⁺ at high ionic strength. These activities are functions of two enzymes which are separable by "DEAE-Sephadex" chromatography (Roeder & Rutter, 1969). "Polymerase I" (low salt) has since been shown to reside in the nucleolus, while "polymerase II" (high salt) is found in the nucleoplasm (Roeder & Rutter, 1970). It would appear, then, that the high salt activated enzyme could be responsible for synthesis of heterodisperse nucleoplasmic RNA, whereas the low salt Mg²⁺ activated enzyme could be involved in the synthesis of ribosomal precursor RNA.

The method employed here for preparation of crude nuclei resulted in 44% recovery of nuclei as judged by recovery of DNA. Each stage in the purification procedure was checked by phase contrast microscopy.
A nuclear preparation from kidneys of normal male rats weighing 200g was used as the enzyme source.

The composition of low salt Mg$^{2+}$ activated RNA polymerase and high salt Mn$^{2+}$ activated RNA polymerase incubation mixtures was as described in "Materials and Methods".
Incorporation of $[^3\text{H}]\text{ATP}$ d.p.m.

Min of incubation
Table 25.

The effect of α-amanitin on Mg\(^{2+}\) and Mn\(^{2+}\) activated RNA polymerase activity in nuclei from normal rat kidneys.

<table>
<thead>
<tr>
<th>Conditions of assay</th>
<th>- Amanitin</th>
<th>+ Amanitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg(^{2+})</td>
<td>0.027</td>
<td>0.024</td>
</tr>
<tr>
<td>Mn(^{2+})/((\text{NH}_4)\text{SO}_4)</td>
<td>0.288</td>
<td>0.084</td>
</tr>
</tbody>
</table>

Nuclei were incubated in the presence or absence of 2µg/ml α-amanitin. Otherwise the assay procedure was as described in "Materials and Methods".
RNA polymerase activity in the kidneys of normal, unilaterally nephrectomised and sham-operated rats.

<table>
<thead>
<tr>
<th>Time after operation (h)</th>
<th>Incorporation of ATP into acid-insoluble material (nmol ATP/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nephrectomy</td>
</tr>
<tr>
<td>4</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>8</td>
<td>0.003</td>
</tr>
<tr>
<td>16</td>
<td>0.017</td>
</tr>
<tr>
<td>24</td>
<td>0.04</td>
</tr>
<tr>
<td>48</td>
<td>0.04</td>
</tr>
</tbody>
</table>

RNA polymerase activity was measured as described in "Materials and Methods". Assays and zero time blanks were performed in triplicate and corrected for the DNA content of the incubation mixture.
Figure 7 shows the kinetics of both the high salt and low salt activated enzymes, agreeing with the results of Widnell & Tata (1966) using rat liver nuclei. In all kidney preparations there was considerably more activity with the high salt activated enzyme than with low salt in agreement with the work in liver. Table 25 shows that in confirmation of the supposed identity of the high salt enzyme to "polymerase II", and the low salt enzyme to "polymerase I", in the kidney, α-amanitin (the toxic bicyclic octapeptide from the mushroom Amanita phalloides) selectively inhibited the high salt activity. This is the same as found for liver nuclei by Stirpe & Fiume (1967).

Table 26 shows that after unilateral nephrectomy no pronounced change was found in either of the two RNA polymerase activities in the remaining kidney after times ranging from 4 to 48 hours.

7. Discussion.

One of the more surprising findings of this section is that the simple incorporation experiments gave no evidence of greater RNA synthesis after unilateral nephrectomy than after sham operation. It is known that RNA synthesis in the liver increases after either sham operation or partial hepatectomy (Bucher & Swaffield, 1969). However increased RNA synthesis in the kidney after sham operation is not likely to be the explanation for the results obtained here since the measurements of RNA polymerase activity failed to detect any significant differences between unilaterally nephrectomised, sham-operated and normal rats at 4, 8, 16, 24 or 48 hours after operation.

This failure to detect increased RNA synthesis during compensatory renal hypertrophy is probably related to the fact that RNA synthesis is normally proceeding at a fairly high rate in the kidney. Even in liver regeneration, where the accumulation of RNA is faster than during compensatory renal growth, great difficulty has been experienced in
attempting to demonstrate increased rates of synthesis (Bucher et al., 1969). Fujioka et al. (1963) have shown that the rate of RNA labelling doubled during the first 6 hours after partial hepatectomy and remained at the same level for a further 6 hours, but this is subject to nutritional alteration (Ove et al., 1966).

With such small changes in RNA synthesis occurring even in liver regeneration it is perhaps not surprising that no detectable changes were found during compensatory renal hypertrophy. On the other hand as can be seen in Table 20 there is a 23% increase in RNA content during the first 24 hours. Malt & Lemaitre (1968) have shown that in both unilaterally nephrectomised and sham-operated mice the half-life of ribosomal RNA was 4.1 days, thus indicating that the increase must be due to increased synthesis. Assuming that the half-life of rat kidney ribosomal RNA is the same, this means that normally about 12% of kidney RNA is synthesised in 24 hours. If the 23% increase in RNA is largely accounted for by rRNA it would necessitate an increase in rRNA synthesis of almost 3-fold in the first 24 hours after unilateral nephrectomy. This ought to be easy to detect. The fact that it is not easily detectable may be due to the fact that whereas only stable RNA molecules such as rRNA and tRNA contribute to the accumulation, the techniques of measuring total RNA synthesis also detect synthesis of RNA molecules such as the heterodisperse nucleoplasmic RNA (HnRNA) which turn over very rapidly. Cooper (1969) has shown that in nongrowing lymphocytes only 2 to 4% of the RNA synthesis is ribosomal. If the lymphocytes are stimulated to grow by phytohaemagglutinin this figure increases to 18%. Using kidney cortex cells in culture Lee et al. (1970) have shown that no increase in synthesis of HnRNA occurs during the pre-DNA synthetic phase of growth.

These results indicate that in all probability the rRNA synthesis
in the kidney is a small part of the total RNA synthesis and that such a small change necessary to account for the observed accumulation of RNA might easily pass undetected by measurement of total RNA synthesis. However Cooke _et al._ (1971) have found that estimation of RNA polymerase activity in nuclei provides an accurate, simple and rapid means for determining alterations in RNA synthesis in growing and non-growing cells. Since the Mg\(^{2+}\) low salt activated enzyme is probably responsible for the synthesis of ribosomal RNA it might be expected that an increase in its activity would be found during compensatory renal hypertrophy whereas an increase in the Mn\(^{2+}\) high salt activated enzyme would not be expected. In actual fact there was no significant increase in either so the paradox of RNA accumulation without increased RNA synthesis remains.

The other important finding in this part of the work is that barbiturate, and to a lesser extent chloral hydrate, anaesthesia suppresses the increase in RNA in the remaining kidney after unilateral nephrectomy. Phenobarbital at 200mg/kg spread over the 16 hours maintained the animals at the level of surgical anaesthesia, but prevented the increase in RNA/DNA ratio characteristic of compensatory renal growth. The questions arise whether this effect is due to anaesthesia as such or to the particular anaesthetic agent employed, and whether it is only the accumulation of RNA which is inhibited or growth in general.

A sub-anaesthetic dose of phenobarbital (150mg/kg over 16 hours) also inhibited the rise in RNA/DNA ratio (Table 23) indicating that perhaps the effect is due to the anaesthetic rather than the anaesthesia. On the other hand phenobarbital, pentobarbital and chloral hydrate all inhibited the increase, indicating that this may be a general property of hypnotic drugs. It is of course difficult to distinguish between direct and indirect effects of drugs.
A similar effect is found in liver regeneration. Phenobarbital at 100mg/kg delays the DNA synthesis occurring during liver regeneration by several hours (Schindler & Bürki, 1971) and this may suggest that it is growth in general which is inhibited and not simply the rise in RNA. However it could equally well be agreed that the drug is inhibiting RNA synthesis directly and this inhibition is subsequently interfering with the sequence of events leading to growth. Pentobarbital also inhibits RNA synthesis in Ehrlich ascites tumor cells in vivo and in vitro (Rovera & Baserga, 1970). However not only RNA synthesis is affected. Pentobarbital (60 mg/kg) also inhibited DNA synthesis under similar conditions although phosphorylation of thymidine to its mono-, di- and triphosphates was not affected (Baserga & Weiss, 1967).

Such considerations suggest that barbiturates are not suitable anaesthetics in studies of nucleic acid synthesis during growth. However even chloral hydrate inhibited the RNA increase to some extent and this may suggest that the anaesthetic inhibition is independent of the action of barbiturates as inhibitors of RNA synthesis. In this connection it is interesting that there was never any decrease in RNA content over the 16 hour period as might be expected if the anaesthetic agents shut off RNA synthesis completely. Instead they may allow normal rates of RNA synthesis but simply prevent any increase due to growth. In other words the barbiturates may inhibit the growth response rather than, or as well as RNA synthesis. This unfortunately means that there is virtually no chance of detection of a humoral factor if cross-circulations are performed over such a long time as 16 hours. Earlier events of growth however might not be influenced by anaesthesia.

The investigations reported here show that there is little likelihood of finding any changes in nucleic acid metabolism which fulfil the criteria for an early indicator of growth. What is required is a large
change which occurs within a few hours, is easy to measure, and can be shown to be an intrinsic part (or better still an essential part) of the growth process. The search for such a change led to the work described in the next chapter entitled "Polyamine Metabolism".
POLYAMINE METABOLISM
1. Introduction.

As can be seen from the last chapter, changes in nucleic acid metabolism occurring during compensatory renal hypertrophy are too small or too late to be of any use as early indicators of growth. However an early change of some magnitude, and one which can be shown to be related to the growth process, is essential for the study of the events responsible for initiation of the growth. In the case of liver regeneration the earliest such change is the increase in the enzyme ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17).

This was first shown by Jänne & Raina (1968) who found a 40-fold increase only 8 hours after partial hepatectomy. This observation followed from the finding that the rate of synthesis of putrescine from radioactively labelled ornithine is about 5-fold increased 3 hours after partial hepatectomy (Jänne, 1967). Russell and Snyder (1968) have shown that ornithine decarboxylase activity in the liver remnant is perceptibly increased only 1 hour after partial hepatectomy and that it rises to a maximum equivalent to 22 times the normal value at 16 hours and then falls gradually but is still elevated at 96 hours. In contrast there was found to be no increase after 4, 16 or 96 hours in sham-operated rats and ornithine decarboxylase activity was unaltered in pancreas, spleen, stomach, adrenals and kidneys after 16 hours. Other workers have found even larger increases (60-70-fold) in this liver enzyme after partial hepatectomy (Schrock et al., 1970; Fausto, 1969). Figure 8 illustrates diagrammatically the early onset and remarkable magnitude in the change in ornithine decarboxylase compared to the other chemical changes characteristic of regeneration.

Liver ornithine decarboxylase is remarkable also in that, when
Sequence of biochemical changes in regenerating liver emphasising the early onset and remarkable magnitude of the rise in ornithine decarboxylase.

Information on ornithine decarboxylase is compiled from Schrock et al. (1970), Russell & Snyder (1968) and results obtained during the course of this work.

Information on other changes was obtained from Bucher & Malt (1971).
Figure 8

TIME after PARTIAL HEPATECTOMY (Hours)

% INCREASE

7,500

5,000

2,500

0

ORNITHINE DECARBOXYLASE

DNA SYNTHESIS

CDP REDUCTASE

MITOSIS

RNA SYNTHESIS

SYNTHESIS OF RIBOSOMAL RNA

dTMP kinase

FAT CONTENT

0 10 20 30 40 50
protein synthesis is stopped by cycloheximide or puromycin administration, it disappears from the liver (whether normal or regenerating) with a half-life of only 11 minutes (Russell & Snyder, 1969). This is shorter than the known half-lives for other mammalian enzymes which generally are in the range 12 hours to several days (Schimke & Doyle, 1970). There are however objections to using inhibitors of protein synthesis in attempting to estimate the turnover rate of mammalian enzymes since the rate of degradation may be altered as well as the rate of synthesis (Schimke & Doyle, 1970). However the half-life of liver ornithine decarboxylase can also be estimated by making use of growth hormone which on injection produces a transient increase in the enzyme between 2 and 6 hours later. The half-life calculated from the declining limb of the curve is about 24 minutes (Russell et al., 1970). Since some synthesis is probably still occurring at this stage this is in reasonable agreement with the half-life obtained with cycloheximide or puromycin. This extremely fast turnover rate means that the high levels in liver regeneration must reflect sustained high rates of synthesis.

Increases in ornithine decarboxylase activity have also been found in parallel with growth in other systems such as developing embryos of the rat, the chick and *Xenopus laevis*, and in several tumors (Snyder et al., 1970). Stastny & Cohen (1970) have reported a 40-fold increase in the enzyme activity 4 hours after stimulation of cultures of chick embryo epidermis with epidermal growth factor.

Ornithine decarboxylase is the first, and possibly the rate limiting, step in the pathway of polyamine biosynthesis shown in Figure 9 (Jänne, 1967; Raina et al., 1970). This pathway, originally demonstrated in bacteria (Tabor & Tabor, 1964) has since been shown in mammalian systems (Pegg & Williams-Ashman, 1968; 1969) although there is some doubt whether
Pathway of polyamine biosynthesis.
Figure 9

\[
\begin{align*}
\text{Ornithine} & \quad \text{S-adenosylmethionine} \\
\text{Putrescine} & \quad \text{Methylthiadenosine} \\
\text{S-adenosylmethionine} & \quad \text{Putrescine} \\
\text{Spermidine} & \quad \text{Spermine}
\end{align*}
\]
S-adenosylmethionine decarboxylation is coupled to spermidine (or spermine) synthesis (Feldman et al., 1971; 1972; Jänne et al., 1971; Raina & Hannonen, 1971; Hannonen et al., 1972).

The polyamines, spermidine and spermine, are present in all cells in substantial concentration (2 to 10 mM) and may help to organise structure in cells, organelles, and individual polymers (Cohen, 1971). A correlation has been drawn between polyamine and RNA levels (Raina & Jänne, 1970; Stevens, 1970). Raina & Telaranta (1967) have found that in rat liver the intracellular distribution of spermidine closely parallels that of RNA, and Dykstra & Herbst (1965) and Raina et al. (1966) have shown that the increase in spermidine concentration parallels the rise in RNA in regenerating liver. Again in the developing chick embryo the peak of polyamine concentration coincides with the peak of RNA synthesis (Caldarera et al., 1965). It has even been found that in rat liver 4 hours after injection of growth hormone the sharp peak in ornithine decarboxylase activity is coincident with a smaller rise in RNA polymerase activity (Raina & Jänne, 1970). Cohen (1971) has pointed out that this last observation may be partly due to increased polyamine levels in the crude preparation stimulating the RNA polymerase activity in vitro.

Spermidine is known to stimulate RNA synthesis in isolated nucleoli (Raina & Jänne, 1970) and markedly activates purified Micrococcus lysodeikticus RNA polymerase (Gumport, 1970).

There is also evidence of a relationship between polyamines and RNA in bacteria. In Escherichia coli the accumulation of ribosomal RNA under a wide variety of conditions parallels the spermidine content, and exogenous spermidine can relax RNA synthesis in amino acid-starved cultures (Raina et al., 1967). In both animals and bacteria polyamines are associated with ribosomes (Khawaja, 1972; Stevens & Morrison, 1968), which they appear to stabilise (Hardy & Turnock, 1971). Recently
Russell (1971) has shown that in anucleolate mutants of *Xenopus laevis* the increases in ornithine decarboxylase and S-adenosylmethionone decarboxylase which occur during normal development are prevented. These are the only known enzyme deficiencies in these mutants and this suggests that polyamine biosynthesis is related to ribosomal RNA synthesis. There is therefore abundant evidence to connect ornithine decarboxylase with RNA and protein synthesis and it may be assumed with some confidence that the increased enzyme levels in liver regeneration are an essential part of the regenerative process. If a similar change could be shown in compensatory renal hypertrophy it would be a useful early indicator of the hypertrophic process. As a preliminary it was obviously advantageous to experiment first with the liver enzyme.

2. Development of assay for ornithine decarboxylase.

The most suitable assay for ornithine decarboxylase seemed to be the second method described by Russell & Snyder (1968). This involves incubation of tissue extract with DL-[5-\(^{14}\text{C}\)]ornithine and extraction of the labelled putrescine formed into alkaline butanol. This method was never found to work reliably: blanks were high and variable. The radioactive substrate was checked for impurities but it was found that virtually all the radioactivity co-chromatographed with authentic ornithine, although two other small peaks of impurities were also found. The trouble appeared to be due to failure to achieve efficient separation of putrescine from ornithine in the butanol extraction. Table 27 shows the results of an experiment designed to improve this separation. The Russell & Snyder method of adding 1 ml of 0.1 M-NaOH and saturating with sodium chloride did not give complete extraction of putrescine and allowed a large carry-over of radioactive ornithine. Of all the methods tried (including saturation with 7:1 Na\(_2\)SO\(_4\)-Na\(_3\)PO\(_4\) salt mixture as described by Snyder & Russell, 1970) it can be seen that simply raising the sodium
Table 27.
Comparison of different extraction and washing procedures on recovery of putrescine and ornithine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Extraction of putrescine (%)</th>
<th>Extraction of ornithine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraction in butanol (7ml) with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1M-NaOH, NaCl saturated</td>
<td>20.7</td>
<td>3.7</td>
</tr>
<tr>
<td>0.1M-NaOH, Na₂SO₄/Na₃PO₄ saturated</td>
<td>100</td>
<td>22.5</td>
</tr>
<tr>
<td>1M-NaOH, NaCl saturated</td>
<td>100</td>
<td>0.0654</td>
</tr>
<tr>
<td>1M-NaOH, Na₂SO₄/Na₃PO₄ saturated</td>
<td>100</td>
<td>10.3</td>
</tr>
<tr>
<td>0.1M-NaOH Dowex 1X8 (OH)</td>
<td>63.5</td>
<td>1.07</td>
</tr>
<tr>
<td>1M-NaOH Dowex 1X8 (OH)</td>
<td>69.0</td>
<td>0.306</td>
</tr>
<tr>
<td>2. Butanol washing with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1M-NaOH</td>
<td>96</td>
<td>66.4</td>
</tr>
<tr>
<td>1M-NaOH</td>
<td>95</td>
<td>14.0</td>
</tr>
<tr>
<td>0.1M-NaOH, NaCl saturated</td>
<td>88</td>
<td>4.74</td>
</tr>
<tr>
<td>1M-NaOH, NaCl saturated</td>
<td>100</td>
<td>5.41</td>
</tr>
<tr>
<td>0.1M-NaOH, Na₂SO₄/Na₃PO₄ saturated</td>
<td>100</td>
<td>50.2</td>
</tr>
<tr>
<td>1M-NaOH, Na₂SO₄/Na₃PO₄ saturated</td>
<td>100</td>
<td>29.2</td>
</tr>
</tbody>
</table>

1. Tubes were set up containing terminated assay mixtures without ornithine. 0.5μCi[5-³H]ornithine or 0.05μCi[1,4-¹³C₂]putrescine were added to individual tubes and extraction in 7ml butanol was as described.
2. 0.5μCi[5-³H]ornithine or 0.05μCi[1,4-¹³C₂]putrescine were added to 7ml butanol and washing carried out as described.

In each case 1ml of the butanol phase was counted as described in "Materials and Methods".
hydroxide concentration to 1M was the most effective. Similarly the best washing of the butanol phase was obtained using 1M-NaOH saturated with NaCl.

Even with this modified method high blank values could obscure all but the highest ornithine decarboxylase activities. A quite different possible source of error was that putrescine might be destroyed as it was formed, for example by diamine oxidase in the tissue extract. This was found not to be the case, since radioactively labelled putrescine added prior to incubation could be completely recovered.

At this stage it was decided to try the other method reported by Russell & Snyder (1968) involving absorption of \(^{14}\)CO\(_2\) released from carboxyl-labelled substrate. The CO\(_2\) trapping solution specified by them was found to be able, in only 8 minutes, to trap between 96 and 100\% of the gas liberated by addition of acid to 2 ml buffered solution containing 5 mM-sodium bicarbonate. Complete absorption was attained well within 30 minutes as measured by standard manometric techniques. These quantities of CO\(_2\) are much larger than would be released in an ornithine decarboxylase assay and, therefore, to check absorption at the other extreme the ability to absorb small quantities of \(^{14}\)CO\(_2\) released from labelled sodium carbonate was established. Again over 90\% was absorbed within 10 minutes with 100\% absorption within 30 minutes.

Using this assay system it was found in one experiment that with 1 ml of a tissue extract 18.4 pmol were released with a 25\(\mu\)M L-ornithine concentration and 68.5 pmol with a 100\(\mu\)M concentration. Corresponding values for 1.5 ml supernatant were 28.2 pmol and 113.5 pmol indicating linearity of reaction with enzyme added and that the substrate concentration used (50\(\mu\)M as L-ornithine) was nonsaturating and probably below the Km of the enzyme. The Km for ornithine decarboxylase for L-ornithine
in crude prostatic preparations is reported to be 190\mu M (Pegg & Williams-Ashman, 1968).

It was found in several further experiments that the time course of incubation was perceptibly curved in contradiction to the claim of Russell & Snyder (1968) that it was linear over the 30 minute incubation period. A further experiment was designed to determine whether or not the addition of mercaptoethanol to 2.5mM (Pegg & Williams-Ashman, 1968) would result in a linear time course but it did not (Figure 10). However mercaptoethanol stimulated enzyme activity, in contradiction to the findings of Schrock et al. (1970) and was consequently employed routinely in ornithine decarboxylase assays.

Because of the low ornithine decarboxylase levels in normal liver the above experiments were conducted with supernatant preparations of livers from animals injected with thioacetamide (150mg/kg) 24 hours previously. This treatment is known to result in increased ornithine decarboxylase levels (Fausto, 1970) and in confirmation Figure 11 shows the time courses of the activity in supernatant preparations from a normal and a thioacetamide injected rat. It is interesting that if the time course for the normal liver is drawn on an expanded scale the curvature is almost identical to that for the thioacetamide preparation, thus indicating that the non-linear curve is not an intrinsic property of the assay such as substrate utilisation and depletion. In fact after 40 minutes incubation only 0.03\% of the available L-ornithine has been used indicating both the low activity in liver and the great sensitivity of the assay. This sensitivity arises from the very low and constant blank value compared with the high and variable blank obtained using the first method described here.

Although the stimulation of liver ornithine decarboxylase by thioacetamide injection has been used here simply as an easy way of
The enzyme source was the supernatant from a liver of a rat (200g) injected with thioacetamide 24 hours previously, prepared as described in "Materials and Methods". The assay was conducted, using the same supernatant, in the presence and absence of 2.5mM mercaptoethanol, as described in "Materials and Methods".
Figure 10

- Mercaptoethanol: + 2.5 mM
- No mercaptoethanol

DPM
I$_{4}CO_{2}$
Collected
100 mgLiver

Time (min) of incubation

3000 2000 1000
Stimulation in vivo of ornithine decarboxylase activity by injection of thioacetamide.

Thioacetamide (150mg/kg) was injected intraperitoneally (in 0.5ml physiological saline) into a male rat of 200g body weight 24 hours prior to sacrifice.

The livers from the thioacetamide treated rat and a normal control rat (200g body weight) were prepared and assayed for ornithine decarboxylase as described in "Materials and Methods" (PO₄ buffer) in the presence of 2.5mM-mercaptoethanol.
obtaining high activity preparations it is nonetheless an interesting phenomenon in itself. Thioacetamide injection (200mg/kg) into rats itself causes liver proliferation as well as a marked enlargement of nucleoli (Ekelund, 1971). This last observation may be related to an observed lesion in the processing of ribosomal precursor RNA in ascites cells after treatment with the drug (Burdon, 1967). Fausto (1970) has reported that the rise in liver ornithine decarboxylase activity after injection of thioacetamide is accompanied by increased RNA synthesis, a finding which is consistent with the association of ornithine decarboxylase activity and RNA synthesis mentioned earlier.

3. Ornithine decarboxylase activity during liver regeneration.

Turning now to the ornithine decarboxylase changes during liver regeneration a preliminary experiment was performed in which one group of 4 rats was partially hepatectomised and another group of 4 rats was sham-operated. The animals were killed 4 hours after the operation and liver supernatant prepared for enzyme assay using a 30 minute incubation. Surprisingly, the enzyme activities for the 67% hepatectomised group were 2287, 7561, 2601 and 1646 d.p.m./30min/0.1g liver, whereas those for the sham-operated group were 821, 2161, 2317 and 735 d.p.m./30min/0.1g liver. Obviously there is no marked increase during liver regeneration compared to sham-operated control rats. However when compared to the value for the normal rat in the thioacetamide experiment (143 d.p.m./30min/0.1g liver) it can be seen that the ornithine decarboxylase activity has in fact increased in both 67% hepatectomised (10 to 50-fold) and sham-operated rats (5 to 20-fold).

Further experiments were carried out to clarify this situation since these results were apparently contradictory to those reported by Russell and Snyder (1968) who found no increase 4 hours after sham operation. Since the time course was known to be curved, and because this curvature
varied between individual supernatant, all the subsequent assays were performed by following the time course of the reaction. Figure 12 shows typical examples of time courses for liver supernatants from 67% heptatectomised, 10% heptatectomised, and normal rats. In addition two types of sham operation were performed, one a simple laparotomy in which the liver lobes were not delivered nor interfered with in any way, and the other an operation in which the median and left lateral lobes were delivered through the wound and were physically manipulated in stimulation of the operative procedure of 67% heptatectomy. Although the curvature of the time courses varied markedly it bore no obvious relationship to the enzyme activity or to the treatment which the animal had received. The results were expressed in the form of initial rates of reaction extrapolated from the individual time curves. Table 28 shows that a slight but significant elevation of ornithine decarboxylase activity was evident even after the simplest and presumably least stressful sham operation (P<0.01) compared with normal rats. 67% heptatectomy resulted in a massive increase in enzyme level of 30-fold in agreement with the results of other workers, but there was a great deal of variation (over a 10-fold range) in activities from this group. Surprisingly 10% heptatectomy produced changes not significantly different from 67% heptatectomy. A 10% heptatectomy does not lead to the intense regeneration which follows 67% heptatectomy; instead the deficit appears to be made good over a longer period (Bucher & Swaffield, 1964). Perhaps even more surprisingly, handling of the median and left lateral lobes of the liver resulted in levels of ornithine decarboxylase in the caudate and right lateral lobes not significantly different from the levels found after 67% heptatectomy. It is true that manipulation of the liver of this sort is said to increase the incorporation of labelled thymidine into DNA but this
Figure 12.

Example of time courses of ornithine decarboxylase from livers of normal, laparotomised, sham hepatectomised, 10% hepatectomised and 67% hepatectomised rats.

Each animal weighed 200g.

Ornithine decarboxylase was measured using PO₄ buffer with 2.5mM-mercaptoethanol in the assay.
Figure 12

Ornithine Decarboxylase: D.P.M., $^{14}$CO$_2$ Collected / 100 mg Liver

- 10% Hepatectomy
- 67% Hepatectomy
- Sham Hepatectomy
- Normal
- Laparotomy

Min of Incubation
Table 28.

Liver ornithine decarboxylase activity 4 hours after operation on rats weighing between 190 and 200g.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>d.p.m. collected/10min/5000000 d.p.m. of L-[1-14C]ornithine in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>67% Hepatectomy</td>
<td>3116 ± 964 (9) * §</td>
</tr>
<tr>
<td>10% Hepatectomy</td>
<td>1133 ± 330 (3) * ‡</td>
</tr>
<tr>
<td>Sham hepatectomy</td>
<td>2010 ± 520 (3) † ¥</td>
</tr>
<tr>
<td>Laparotomy</td>
<td>248 ± 27 (3) ‡</td>
</tr>
<tr>
<td>Normal</td>
<td>105 ± 19 (2)</td>
</tr>
</tbody>
</table>

Each assay was performed by a time course in the presence of 2.5mM-mercaptoethanol. Enzyme activities are expressed as initial rate extrapolated to 10min.

0.5ml of a 1/5 homogenate supernatant was used as the enzyme source in each case.

Values are means ± S.E.M. for number of animals in parenthesis.

* Significantly different from laparotomised rats P<0.05
† Significantly different from laparotomised rats P<0.025
§ Significantly different from normal rats P<0.05
¶ Significantly different from normal rats P<0.0125
≈ Significantly different from normal rats P<0.01
‡ Significantly different from normal rats P<0.0025
effect is restricted to the handled lobes (Weinbren et al., 1969).

These results cast doubt on the conclusions of Russell & Snyder (1968) that ornithine decarboxylase activity is related to growth.

Schrock et al. (1970) have also found recently that the rise in enzyme activity is the same (about 60 to 70-fold) 4 hours after 67% hepatectomy or 10% hepatectomy. In addition a smaller (9-fold) rise was found after sham operation yet neither sham operation nor 10% hepatectomy produce any appreciable increase in DNA synthesis.

Figure 13 shows the results of an experiment to compare the effects on liver ornithine decarboxylase activity of 67% hepatectomy and 10% hepatectomy. It is immediately apparent that the two procedures produce similar effects at 4 hours and 10 hours, but that with 10% hepatectomy there is an abrupt return to normal enzyme levels shortly thereafter whereas with 67% hepatectomy the activity is still high 48 hours after the operation.

The results in Table 28 and Figure 13 might suggest that quite apart from any association with growth, liver ornithine decarboxylase activity increases in response to stress. Similar increases though not so marked and occurring later have been observed in glyoxalase activity after partial hepatectomy and sham hepatectomy (Alexander & Boyer, 1971).

In a previous section it was shown that the increase in RNA per cell which is normally one of the first phenomena in compensatory renal hypertrophy is abolished if the animal is maintained continuously under anaesthesia. It was clearly of interest therefore to see what the effect of anaesthetics would be on the rise in liver ornithine decarboxylase produced by hepatectomy or laparotomy. The results in Table 29 show that to varying degrees they depressed the expected increase in enzyme activity. So also did decerebration. A common factor in these effects of anaesthesia and decerebration could well
Figure 13.

Effect of 67% hepatectomy and 10% hepatectomy on liver ornithine decarboxylase at various times after the operation.

Each rat weighed 200g.

Assays were conducted as described in "Materials and Methods" in the presence of 2.5mM-mercaptoethanol and PO₄ buffer. 0.5ml of the supernatant from a 1/5 homogenate in a total volume of 2ml was used for each assay time point.

All operations were conducted between 09.00 and 12.00 hours.
Figure 13

Ornithine Decarboxylase: D.P.M., $^{14}$CO$_2$ Collected / 100mg of Liver

MIN OF INCUBATION

0 10 20 30 40
Table 29.

Effect of anaesthesia and decerebration on the ornithine decarboxylase rise after 67% hepatectomy or laparotomy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of sacrifice (hours)</th>
<th>Ornithine decarboxylase activity. d.p.m. $^{14}$CO$_2$ collected/10min/500000 d.p.m. of L-[$^{14}$C]ornithine in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>67% hepatectomy</td>
<td>4</td>
<td>3116 ± 964</td>
</tr>
<tr>
<td>+ pentobarbital anaesthesia</td>
<td>4</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>78</td>
</tr>
<tr>
<td>+ chloral hydrate anaesthesia</td>
<td>4</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>156</td>
</tr>
<tr>
<td>+ decerebration</td>
<td>4</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>77</td>
</tr>
<tr>
<td>Laparotomy</td>
<td>4</td>
<td>248 ± 27</td>
</tr>
<tr>
<td>+ pentobarbital anaesthesia</td>
<td>4</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>156</td>
</tr>
</tbody>
</table>

Rats weighing between 195 and 205g were kept anaesthetised where necessary by intravenous injection of 10% (w/v) chloral hydrate or 1/5 Nembutal in physiological saline via a cannula inserted into a tail vein.

All assays were performed by time course and the initial rate was extrapolated to 10 minutes. 0.5ml of a 1/5 homogenate supernatant was used in each case.
be the prevention of release of pituitary hormones. Decerebration could easily interfere with pituitary blood supply and function and may also interfere at the level of the hypothalamus. Anaesthetics are also known to interfere with pituitary function. Prolactin secretion is blocked by a single injection of pentobarbital in the rat (35mg/kg; Yokoyama et al., 1971) and Knobil & Meyer (1968) have shown that a single injection of pentobarbital (30mg/kg) into a Rhesus monkey stabilises the "base line" plasma concentration of growth hormone and maintains it at a depressed level for the duration of deep anaesthesia. Russell & Snyder (1969) have shown that if a 67% hepatectomy is performed in hypophysectomised rats the increase in ornithine decarboxylase is delayed for several hours. The normal timing of the response is however largely restored by administration of growth hormone. We have already seen that growth hormone by itself elevates ornithine decarboxylase activity in rat liver, but only transiently (Jänne & Raina, 1969; Russell et al., 1970). Growth hormone release from the pituitary does seem to occur after hepatectomy (Echave Llanos et al., 1971). The possibility arises then that growth hormone may either be the agent causing the early response of ornithine decarboxylase after 67% hepatectomy or may be required permissively for the growth response to occur. Figure 14 shows that a preparation of human growth hormone did indeed lead to increased liver ornithine decarboxylase levels when injected intraperitoneally. It can be seen that a large dose (10mg/kg) is needed to produce an effect.

If growth hormone has a permissive role in the early events in liver regeneration, which is only delayed in its absence, then injection of low doses of growth hormone into anaesthetised rats might allow the growth response after partial hepatectomy to manifest itself in the form of a rise in ornithine decarboxylase activity. Table 30 shows
Figure 14.

**Stimulation of ornithine decarboxylase activity by injection of growth hormone.**

Growth hormone was injected intraperitoneally in 0.5 ml physiological saline into rats weighing 200g. The animals were sacrificed 4 hours later and liver ornithine decarboxylase measured as described in "Materials and Methods" in the presence of 2.5mM-mercaptoethanol.
Table 30.

Growth hormone reverse of anaesthetic inhibition of ornithine decarboxylase rise 4 hours after partial hepatectomy.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Operation</th>
<th>Growth hormone (mg/kg)</th>
<th>Anaesthetic (ml)</th>
<th>Ornithine decarboxylase activity d.p.m. $^{14}$CO$_2$ collected/10min/500000 d.p.m. of L-$[1-^{14}$C]ornithine in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67% hepatectomy</td>
<td>1</td>
<td>0.6</td>
<td>2380</td>
</tr>
<tr>
<td></td>
<td>67% hepatectomy</td>
<td>1</td>
<td>0.6</td>
<td>6440</td>
</tr>
<tr>
<td></td>
<td>laparotomy</td>
<td>1</td>
<td>1.0</td>
<td>576</td>
</tr>
<tr>
<td>2</td>
<td>67% hepatectomy</td>
<td>0.5</td>
<td>0.6</td>
<td>1392</td>
</tr>
<tr>
<td></td>
<td>laparotomy</td>
<td>0.5</td>
<td>1.5</td>
<td>116</td>
</tr>
</tbody>
</table>

Rats weighing 195-205g were kept anaesthetised with intravenous injection of 1/5 (v/v) Nembutal in physiological saline via a cannula inserted into a tail vein.

Growth hormone was injected intraperitoneally dissolved in 0.5ml physiological saline.
the results of just such experiments. It is quite clear that levels of growth hormone which would have no effect on ornithine decarboxylase by themselves restore the response to hepatectomy when it has been abolished by anaesthesia. (Note that the sham-operated animals required more anaesthetic than hepatectomised. This presumably was due to detoxification of the anaesthetic in the liver.) These results suggest that inhibition of the ornithine decarboxylase rise after 67% hepatectomy by anaesthesia is due to blockade of growth hormone secretion. They also suggest that the differences in response between 67% hepatectomised and sham-operated rats are not simply due to different levels of stress and consequently greater growth hormone secretion in the former case. Instead there is support for the conclusion that the increased ornithine decarboxylase levels in the liver fragment early after 67% hepatectomy are indeed related to the growth process although stress, perhaps via growth hormone secretion, can mimic the response occurring during growth.

The results in Tables 28-30 and Figures 11-14 provided a suitable basis from which to extend the study of ornithine decarboxylase to hypertrophic kidney. Because of the rapid increase in RNA content during compensatory renal hypertrophy, and because of the relationship of ornithine decarboxylase activity to RNA synthesis, a rise in the activity of the enzyme would be expected. Table 31 shows the results of an experiment designed to test this. The enzyme activity was uniformly higher in the kidney after unilateral nephrectomy than after sham operation and there was a significant difference at 8 hours (P<0.01). Great variations were found in ornithine decarboxylase levels in kidney as they had been in liver. The most striking feature of this set of results, however, is that between 4 and 12 hours after both operations the activity appears to decline precipitously. The half-life of the
Table 31.

Ornithine decarboxylase activity in the left kidney after right unilateral nephrectomy and sham operation in rats weighing between 180 and 200g.

<table>
<thead>
<tr>
<th>Time after operation (h)</th>
<th>Unilateral nephrectomy</th>
<th>Sham operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.p.m. $^{14}$CO$_2$ collected/10min/500,000 d.p.m. of L-$[1-^{14}$C$]$ornithine in assay</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10430 ± 4910</td>
<td>3463 ± 728</td>
</tr>
<tr>
<td>8</td>
<td>3957 ± 639 *</td>
<td>779 ± 270</td>
</tr>
<tr>
<td>12</td>
<td>788 ± 438</td>
<td>138 ± 18</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for 3 animals in each group.

Assays were performed by time course and the initial rate was extrapolated to 10 minutes.

* Significantly different from sham-operated rats P<0.01
decay is of the order of 2 hours.


At this stage in the work a paper was published by Jönne & Williams-Ashman (1971) reporting that the activity and stability of ornithine decarboxylase from rat ventral prostrate are markedly increased by several thiol compounds of which dithiothreitol is the most effective. The enzyme is unstable in the absence of thiol compounds probably because of polymerisation to form catalytically inert dimers. This polymerisation process is apparently prevented but not reversed by the presence of dithiothreitol.

Accordingly attempts were made to change the assay used and to obtain higher activities and a more linear time course. In addition the methods used up till now had resulted in very labile enzyme preparations which could not be stored. Equal aliquots of the same liver from a rat which had been 10% hepatectomised 4 hours previously were used as enzyme source and were homogenised in the presence of either 5mM dithiothreitol (Jönne & Williams-Ashman, 1971) or 2.5mM mercaptoethanol as used previously. These thiol compounds were present throughout the preparation of the supernatant and the assay. Figure 15 shows that in the presence of dithiothreitol activities were enhanced and the time course was much more nearly a straight line. It appeared that even the initial rate of reaction was higher.

The experiment was repeated with almost identical results. It would appear, then, that using the older assay method of Russell & Snyder (1968) and even with addition of mercaptoethanol a large inactivation of the enzyme during preparation of the supernatant had occurred. The difference between assays with and without dithiothreitol was somewhat variable and this may suggest that the enzyme in the preparations without dithiothreitol had been decaying at different
Effect of dithiothreitol on ornithine decarboxylase from the liver of a 200g rat 10% hepatectomised 4 hours previously.

2g samples of the same liver were assayed for ornithine decarboxylase in the presence of either 5mM-dithiothreitol or 2.5mM-mercaptoethanol. Both sets of assays were conducted with 50mM-PO₄ buffer, pH 7.2.
**Figure 15**

D.P.M./ASSAY vs. MIN of INCUBATION

- **5 mM Dithiothreitol**
- **2.5 mM Mercaptoethanol**
rates. This could be a large source of error in the earlier work carried out here and in the work of all other investigators in the field at the time, such as the Finnish group (Raina) and various American workers (Bucher; Russell; Fausto). Jänne & Williams-Ashman (1971) also reported that a glycylglycine buffer pH7.2 was superior to other buffers tried and that sodium phosphate buffer pH7.2 (100mM) was strongly inhibitory to the rat prostatic enzyme. The Russell & Snyder (1968) method used in the experiments described above employs 50mM-sodium phosphate buffer pH7.2. It was therefore decided to screen several buffers. Figure 16 shows that, in the presence of 5mM-dithiothreitol, 100mM-HEPES pH7.2 (tested because it had been used in an assay for ornithine decarboxylase of E.coli by Morris et al., 1970) gave the highest activities. In another experiment it was found that whereas 100mM-HEPES + 5mM-dithiothreitol gave slightly higher activities than 50mM-HEPES + 5mM-dithiothreitol, 100mM-HEPES + 2.5mM-dithiothreitol gave a higher activity still and 100mM-HEPES + 10mM-dithiothreitol gave only about 60% of the last. This suggests that 100mM-HEPES is superior to 50mM-HEPES (all buffers pH7.2) and that dithiothreitol is in fact inhibitory at high concentrations.

Several experiments were performed to find the optimum concentration of dithiothreitol. It was found in one of these (Figure 17) that if dithiothreitol was added to the homogenisation medium, and not merely to the incubation mixture, up to 5 times more activity was obtained. In agreement with the work of Jänne & Williams-Ashman (1971) this suggests that ornithine decarboxylase rapidly becomes inactivated in the absence of dithiothreitol since the time from killing the rat to the end of the assay incubation was only 90 minutes. In other words this amounts to over 80% loss of activity in only 60 minutes at 0 to 4°C; a very unstable enzyme. Since dithiothreitol was present in the assay
Figure 16.

Effects of different buffers on ornithine decarboxylase activity.

2g samples of the same liver (from a 200g rat 10% hepatectomised 4 hours previously) were homogenised and assayed in each of the 3 buffers containing 5mM-dithiothreitol.
**Figure 16**

D.P.M./ASSAY vs MIN OF INCUBATION

- 100mM HEPES
- 100mM GLYCYLGLYCINE
- 50mM NaPO₄
Effect of presence of 1mM-dithiothreitol on stability of ornithine decarboxylase from the liver of a 200g rat 10% hepatectomised 4 hours previously.

2g samples of the same liver were prepared for ornithine decarboxylase assay in the presence or absence of 1mM-dithiothreitol. Assays were conducted using 100mM-HEPES, pH 7.2 over a range of dithiothreitol concentrations.
Figure 17

Supernatant prepared in presence of 1mM dithiothreitol

Supernatant prepared in absence of dithiothreitol

Dithiothreitol concentration in assay (mM)
the inactivation was apparently not reversible, and this finding is also in agreement with Jänne & Williams-Ashman (1971). A composite graph of all experiments on dithiothreitol optimum concentration is shown in Figure 18. 1mM-dithiothreitol was the highest concentration which was non-inhibitory. In another series of experiments on pyridoxal phosphate concentration it was found that between 10 and 100μM there was no difference in enzyme activity. Consequently the concentration used previously of 50μM was retained.

In yet another set of experiments the pH of the assay was varied and it can be seen from the composite graph in Figure 19 that the pH optimum of liver ornithine decarboxylase was 7.2 as used previously and in agreement with other workers.

At about this time a paper appeared in which a new assay using 50mM-TES and 0.1mM-EDTA was reported (Panko & Kenney, 1971). In the presence of 1mM-dithiothreitol from homogenisation to assay, 50mM-TES pH7.2 was found to be equally as good as 100mM-HEPES pH7.2 but no better. Addition of EDTA to either buffer was without effect.

In the process of modification of this assay many parameters were changed and therefore a final experiment was performed to ensure that release of CO₂ was stoichiometric with putrescine formation. By the method of release of ¹⁴CO₂ from [1⁻¹⁴C]ornithine the activity of a supernatant preparation was found to be 3.42nmol/30min incubation; and by the method of extraction of labelled putrescine arising from [5⁻³H]ornithine, the activity of the same sample was found to be 3.22nmol/30min incubation; thus confirming the stoichiometry of the modified assay.

With this improved assay higher activities and less variation were observed and these are considerable advantages in the experiments to follow.
Change in ornithine decarboxylase activity with varying dithiothreitol concentration in assay. Composite graph of 4 experiments.

In each experiment the supernatant was prepared in 100mM-HEPES pH 7.2 without added dithiothreitol.

Livers from 200g rats, 10% hepatectomised 4 hours previously were used as the source of the enzyme.
Supernatants were prepared (from the livers of 200g rats injected with 150mg/kg of thioacetamide 24 hours previously) in 100mM-HEPES buffer, pH 7.2 with 1mM-dithiothreitol. Assay mixtures were adjusted to the appropriate pH with 2M-HCl or 2M-NaOH.
**Figure 19**

Ornithine Decarboxylase % of Value at pH 7.2

![Graph showing ornithine decarboxylase activity across different pH values.](graph.png)
5. **Ornithine decarboxylase during compensatory renal hypertrophy.**

Table 32 shows the level of activity in a variety of normal tissues. Prostate and kidney have enormously higher activities than any of the other tissues examined. The kidney activity even using the modified assay varied greatly from animal to animal although within each animal the activities of the right kidney and left kidney were very similar. The mean ornithine decarboxylase in the male kidneys of 35.2 ± 8.6 nmol/30min/g of tissue, shown in Table 32, includes individual values ranging from 10 to 100 nmol/30min/g.

The mean kidney enzyme level is about 60 times higher than the level in the liver. This is perhaps rather surprising since both tissues consist of essentially "resting" populations of cells, although it is true that almost double the number of mitoses can be seen in kidney than in liver (Goss, 1964). As found in Chapter 3 however the rate of RNA synthesis in the liver probably exceeds that in the kidney by about 4-fold and the same difference is found in total RNA content. This would suggest that at least in these two organs RNA synthesis is not directly related to ornithine decarboxylase activity. Another interesting point is that the heart has perhaps even slightly higher ornithine decarboxylase activity per g of tissue than does the liver although as was found in Chapter 3 RNA synthesis in the liver probably exceeds that in the heart by about 20-fold. Again there appears to be a lack of proportionality between ornithine decarboxylase and RNA synthesis.

When a rat is subjected to unilateral nephrectomy the already high ornithine decarboxylase activity increases further reaching a peak 4 hours after the operation (Figure 20). It then falls precipitously until by 24 hours after the operation it is significantly below normal levels. This is quite different from the response in the
Table 32.

Ornithine decarboxylase activities in rat tissues.

Ornithine decarboxylase activities (nmol/30min/g of tissue) for different tissues in male and female rats are presented below.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ornithine decarboxylase (nmol/30min/g of tissue)</th>
<th>Number of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>35.2 ± 8.6 (11)</td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>49.4 (1)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.623 ± 0.314 (3)</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.744 ± 0.040 (2)</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.526 (1)</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.375 (1)</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.368 (1)</td>
<td></td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>1.31 (1)</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>49.7 ± 16.8 (2)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.
Changes in kidney ornithine decarboxylase in the left kidney after right unilateral nephrectomy or sham operation.

Male rats weighed between 160 and 200g.
The normal group contained 6 rats.
Other groups contained 3 rats.
Each point and vertical bar represent mean ± S.E.M.
Eigu

300

Unilateral nephrectomy

Ornithine Decarboxylase
nmol/30min/
g of kidney

200

Sham operation

100

0

Hours

10

20
liver after partial hepatectomy where the enzyme level increases about 60-fold and remains elevated for at least 48 hours. Just as after sham hepatectomy there was an increase in the liver enzyme, so after sham unilateral nephrectomy there was an increase in the kidney enzyme level. This increase closely paralleled the increase after unilateral nephrectomy although it was significantly smaller, and the subsequent fall in enzyme level reached lower levels than after unilateral nephrectomy. These results are essentially in agreement with those in Table 31 obtained using the old assay system. There were also other differences between these two responses in the kidney. By 12 hours after sham operation the enzyme level had declined to only 1.35 ± 0.21nmol/30min/g of tissue. The low standard error reflects the very consistent value obtained in individual animals at this time. Indeed this low level is comparable with that of other tissues such as spleen, liver, pancreas, brain etc. It can be calculated that the fall in activity from 160nmol/30min/g at 5 hours to 1.35nmol/30min/g at 12 hours reflects passage of at least 7 half-lives of the enzyme in the space of 7 hours. It would appear from this that the enzyme in kidney turns over at a very fast rate as is the case with the liver enzyme.

The responses after unilateral nephrectomy and sham operation are remarkably similar and at a cursory glance it would appear that the response to unilateral nephrectomy might only reflect an enhanced response to stress such as is caused by sham operation. However there are significant differences between the two curves, at 4 hours (P<0.01) and at 12 hours (P<0.0005) where the difference is 40-fold. Indeed the ornithine decarboxylase level in the kidney after unilateral nephrectomy appears never to reach the very low value found 12 hours after sham operation.
Table 33 shows the results of an experiment to find out whether the effects on kidney ornithine decarboxylase of unilateral nephrectomy or sham unilateral nephrectomy could be modified by a variety of agencies which would affect the degree of stress which the animal experienced. Pentobarbital anaesthesia, which has already been shown to suppress the accumulation of kidney RNA after unilateral nephrectomy, and also, the increase in liver ornithine decarboxylase after partial hepatectomy and sham hepatectomy, not surprisingly, also prevents any change in kidney ornithine decarboxylase after either unilateral nephrectomy or sham unilateral nephrectomy. The enzyme levels in both cases remain at the preoperative level. Prior adrenalectomy also depressed the postoperative enzyme levels but in this case while the level in unilaterally nephrectomised rats is approximately in the normal preoperative range the level in the sham unilaterally nephrectomised rats is well below this. Metyrapone, which inhibits 11β-hydroxylation of steroids and thus the biosynthesis of corticosterone and other adrenal steroids (Goodman & Gilman, 1965), if given in sufficient quantity, mimics the effects of adrenalectomy. A third demonstration of the effect of stress, mediated again presumably by adrenal steroids, is provided when the animals are pre-stressed by subjecting them to sham operation 8 hours before unilateral nephrectomy or sham unilateral nephrectomy. The effect is to depress, though only moderately, the response to each operation without abolishing the difference between the two. Finally administration of cycloheximide (25mg/kg) at the time of operation totally prevents the ornithine decarboxylase elevation after both unilateral nephrectomy and sham operation (Table 33). This shows that both responses are probably due to enzyme synthesis rather than simply activation of existing precursors. In fact 4 hours after cycloheximide administration
Table 33.

Modification of renal ornithine decarboxylase response 4 hours after unilateral nephrectomy or sham operation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Unilateral nephrectomy</th>
<th>Sham operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>231 ± 18 (7)</td>
<td>103 ± 18 (7)</td>
</tr>
<tr>
<td>Pentobarbital anaesthesia</td>
<td>34.3 ± 30.2 (2)</td>
<td>29 ± 3.0 (2)</td>
</tr>
<tr>
<td>Metyrapone 375mg/kg</td>
<td>40.0</td>
<td>7.4</td>
</tr>
<tr>
<td>50mg/kg</td>
<td>127</td>
<td>22.5</td>
</tr>
<tr>
<td>5mg/kg</td>
<td>194</td>
<td>184</td>
</tr>
<tr>
<td>Metyrapone 125mg/kg (over 4 injections)</td>
<td>43.6 ± 16.2 (3)</td>
<td>1.65 ± 0.49 (3)</td>
</tr>
<tr>
<td>Pre-stressing 8 hours prior to operation</td>
<td>72.5 ± 22.3 (3)</td>
<td>41.1 ± 17.9 (3)</td>
</tr>
<tr>
<td>Adrenalectomy</td>
<td>40.5 ± 5.4 (3)</td>
<td>16.2 ± 2.1 (3)</td>
</tr>
<tr>
<td>Cycloheximide 25mg/kg</td>
<td>0.024</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.

Cycloheximide and metyrapone were injected intraperitoneally. Pentobarbital anaesthesia was maintained by injection of 1/5 (v/v) Nembutal in physiological saline via a cannula inserted into a tail vein.
ornithine decarboxylase levels were markedly reduced. Assuming an initial value of 35nmol/30min/g of kidney this would correspond to the passage of at least 7 half-lives in 4 hours or a half-life of less than 35 minutes. This indicates first of all that the kidney enzyme has similar properties at least as far as turnover is concerned, as does the liver enzyme; and also supports the view that the measurement of the kidney enzyme is not subject to artefact since it is unlikely that any other contaminating enzyme system has such characteristics.

It was obviously desirable to investigate the relationship of adrenocortical steroids to kidney ornithine decarboxylase further. Glucocorticoids are known to increase the RNA synthesis in liver in a manner reminiscent of the increase which follows partial hepatectomy (Feigelson et al., 1962). They might perhaps affect kidney RNA and therefore kidney ornithine decarboxylase similarly. On the other hand mineralocorticoids by definition affect kidney function: it would be hardly surprising if they affected kidney ornithine decarboxylase also. One of them (aldosterone) has already been shown to affect kidney RNA metabolism (Castles & Williamson, 1965).

Table 34 shows the effects on the ornithine decarboxylase of normal kidney of dexamethasone and deoxycorticosterone.

Dexamethasone is a synthetic glucocorticoid with no mineralocorticoid activity whereas deoxycorticosterone is a mineralocorticoid with no glucocorticoid activity (Goodman & Gilman, 1965). They were employed in preference to common adrenal steroids because the latter have both glucocorticoid and mineralocorticoid activity in varying degrees.

However as is shown in Table 34 dexamethasone (the glucocorticoid) was very potent in raising the kidney enzyme level, in fact to almost exactly the same level as unilateral nephrectomy. The mineralocorticoid was without effect on the kidney enzyme. These results show that the
Table 34.

Influence of adrenal hormones on kidney ornithine decarboxylase activity.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Test</th>
<th>Control (saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone phosphate 0.25mg/kg</td>
<td>235 ± 19 (3)</td>
<td>54.9 ± 13.5 (3)</td>
</tr>
<tr>
<td>Deoxycorticosterone acetate 2.5mg/kg</td>
<td>10.2</td>
<td>53.9</td>
</tr>
<tr>
<td></td>
<td>0.25mg/kg</td>
<td>55.1</td>
</tr>
<tr>
<td></td>
<td>0.025mg/kg</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.

Rats weighed 200g.

The steroids were administered by intraperitoneal injection in 0.5ml 0.9% (w/v) saline and the animals were sacrificed 4 hours later.
effect of stress on kidney ornithine decarboxylase is mediated by adrenal glucocorticoids and not mineralocorticoids.

In order to investigate further the relationship of stress and kidney growth to increased ornithine decarboxylase activity, adrenalectomised and control rats were unilaterally nephrectomised and the remaining kidneys assayed for enzyme activity after 4, 8 and 24 hours (Figure 21). The adrenalectomised rats showed no increase in activity at 4 hours or 8 hours but at 24 hours the activity was depressed below the preoperative level just as it was in the control animals. Similarly there was no increase in ornithine decarboxylase in the kidneys of sham-operated adrenalectomised rats. These results indicate that the increase in ornithine decarboxylase activity in the kidney is totally dependent on the presence of intact adrenal glands. In confirmation Figure 22 shows that in normal rats a variety of surgical procedures increased kidney ornithine decarboxylase though to a lesser degree than did unilateral nephrectomy. These increases also were prevented by prior adrenalectomy.

Figure 23 shows the results of a parallel series of experiments in which ornithine decarboxylase was measured in liver instead of kidney. Here again all the surgical procedures elicited increased activity when performed on normal intact rats, the greatest effect being produced by hepatectomy. Prior adrenalectomy greatly diminished the increase in each case but did not abolish it, as had been the case in kidney. Finally Table 35 shows that in the heart, unlike liver and kidney, the stress of surgery produces no increase in ornithine decarboxylase activity.

Whatever may be the role of the adrenals in controlling ornithine decarboxylase in liver and kidney the fact remains that the highest levels in liver and kidney are elicited by partial hepatectomy and
Figure 21.

Ornithine decarboxylase activity in the left kidneys of unilaterally nephrectomised and sham-operated rats, either normal or previously adrenalectomised.

Points and vertical bars represent means ± S.E.M.

The number of rats in each group is shown in parenthesis.

Rats weighed between 180 and 210g.
Ornithine decarboxylase
nmol/30min/g of kidney

Hours after unilateral nephrectomy
Effect of various operations in normal and adrenalectomised rats on kidney ornithine decarboxylase activity 4 hours later.

Histograms and vertical bars represent mean ± S.E.M. for number of animals in parenthesis.

Rats weighed between 185 and 205g.
Figure 23.

Effect of various operations in normal and adrenalectomised rats on liver ornithine decarboxylase 4 hours later.

Histograms and vertical bars represent mean ± S.E.M. for number of animals in parenthesis.

Rats weighed between 185 and 225g.
FIGURE 23

[Diagram showing the effects of different surgeries on liver function, comparing normal rats, adrenalectomised rats, sham nephrectomy, unilateral nephrectomy, laparotomy, and heptectomy. The y-axis represents percentage changes, and the x-axis represents time in minutes per gram of liver. The diagram includes shaded bars and error bars to indicate variability.]
Table 35.

Heart ornithine decarboxylase activity 4 hours after operation in normal rats weighing between 185 and 205g.

<table>
<thead>
<tr>
<th>Operation</th>
<th>Ornithine decarboxylase (nmol/30min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.744 ± 0.040 (2)</td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td>0.696 ± 0.295 (2)</td>
</tr>
<tr>
<td>Sham unilateral nephrectomy</td>
<td>0.849 ± 0.384 (2)</td>
</tr>
<tr>
<td>67% hepatectomy</td>
<td>1.22 (1)</td>
</tr>
<tr>
<td>Sham hepatectomy</td>
<td>0.774 (1)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.
unilateral nephrectomy respectively. Certainly in the liver and probably in the kidney there must be an increment in ornithine decarboxylase genuinely associated with compensatory growth and quite distinct from the increase produced by stress. To gain further information on this point it was desirable to attempt to measure the degree of stress imposed on our animals by the various surgical procedures. An approximation to this could be achieved by estimation of corticosterone which is the preponderant adrenal steroid found in rat plasma (Zenker & Bernstein, 1958).

The laboratory environment itself may be stressful and consequently "normal" levels for plasma corticosterone were measured both in rats in the animal house and in the laboratory. Animals were killed by sudden decapitation with a minimum of handling and blood was collected from the severed carotid arteries. Table 36 shows that by this criterion our rats were indeed stressed by mere removal from the animal house to the laboratory and that the stress appeared to increase after 1 hour in the laboratory. A very much greater stress was produced simply by ether anaesthesia. The effects of unilateral nephrectomy and sham operation, whether they were measured 30 minutes or 1 hour later, were indistinguishable and no greater than those produced by ether alone. There is thus no reason to suppose that removal of a kidney stresses the animal any more than sham operation. This would indicate that the higher ornithine decarboxylase levels found after unilateral nephrectomy as compared to those obtained after sham operation are attributable to the early stages of compensatory growth.

If this is the case the increase due to kidney growth is much smaller than, and very transient compared to, that occurring during liver regeneration. In fact the increase in ornithine decarboxylase
Table 36.

Changes in plasma corticosterone levels following operations in rats weighing between 180 and 200g.

<table>
<thead>
<tr>
<th>Description</th>
<th>Plasma corticosterone concentration (µg/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats killed immediately in animal house</td>
<td>0.036 ± 0.008 (3)</td>
</tr>
<tr>
<td>Rats killed immediately after arrival in laboratory</td>
<td>0.076 ± 0.023 (2)</td>
</tr>
<tr>
<td>Rats left in laboratory for 1 hour</td>
<td>0.175 ± 0.033 (3)</td>
</tr>
<tr>
<td>Blood withdrawn from jugular vein under ether anaesthesia</td>
<td>0.563 ± 0.045 (4)</td>
</tr>
<tr>
<td>60 min after unilateral nephrectomy</td>
<td>0.627 ± 0.063 (7)</td>
</tr>
<tr>
<td>60 min after sham operation</td>
<td>0.601 ± 0.035 (7)</td>
</tr>
<tr>
<td>30 min after unilateral nephrectomy</td>
<td>0.482 ± 0.017 (2)</td>
</tr>
<tr>
<td>30 min after sham operation</td>
<td>0.476 ± 0.116 (2)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis. Unless otherwise stated blood was collected from the carotid arteries after decapitation without anaesthesia.
is so small as to be useless as an early indicator of compensatory renal hypertrophy, especially when the tremendous variability of the kidney enzyme level is taken into account.

6. **Factors responsible for maintenance of high kidney ornithine decarboxylase activity.**

The relatively small increase in ornithine decarboxylase activity in the kidney is probably related to the very high normal level, 60-fold higher than that in the liver and nearly 70% of the peak activity found in regenerating liver. Since the enzyme has such a short half-life this high activity can only be maintained by a high rate of synthesis. It has been shown above that after the peak evoked by operation the enzyme activity falls to a level well below normal, as though the synthetic mechanism had temporarily failed and this might lead one to speculate whether the normal high level is sustained by the action of some hormone. Glucocorticoids are an obvious candidate for this role but are excluded by the fact that adrenalectomised rats have the same level of enzyme activity as normal rats (Figure 22). Another possibility is that sex hormones are responsible. The prostate is also very rich in the enzyme and kidney and prostate have a common embryological origin in the Wolffian duct (Tettenborn et al., 1971). Testosterone has been shown to be responsible for maintenance of the high ornithine decarboxylase activity in the prostate (Pegg et al., 1970) and injection of testosterone results in a slightly elevated activity in liver (Panko & Kenney, 1971). However kidney ornithine decarboxylase is as high in female rats as in males (Table 32). In Table 37 it can be seen that in an immature male and an immature female the kidney enzyme level appears to be lower than the average for normal adults but well within the normal range (which is, as we have seen, very wide). Only the proximal tubules are of the same
Table 37.

Ornithine decarboxylase in organs of immature rats and in cortex and medulla of adult rat kidneys.

<table>
<thead>
<tr>
<th></th>
<th>n mol/30min/g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ornithine decarboxylase</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Young female (30g)</strong></td>
<td></td>
</tr>
<tr>
<td>kidney</td>
<td>6.48</td>
</tr>
<tr>
<td>liver</td>
<td>5.46</td>
</tr>
<tr>
<td>uterus</td>
<td>6.57</td>
</tr>
<tr>
<td><strong>Young male (30g)</strong></td>
<td></td>
</tr>
<tr>
<td>kidney</td>
<td>9.44</td>
</tr>
<tr>
<td>liver</td>
<td>0.423</td>
</tr>
<tr>
<td><strong>Adult males (180-200g)</strong></td>
<td></td>
</tr>
<tr>
<td>kidney cortex</td>
<td>5.37</td>
</tr>
<tr>
<td>kidney medulla</td>
<td>2.38</td>
</tr>
</tbody>
</table>
Table 38.

Influence of testosterone and oestradiol on kidney ornithine decarboxylase activity in rats weighing between 190 and 200g.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ornithine decarboxylase (nmol/30min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone 25mg/kg</td>
<td>78.7 ± 26.1 (3)</td>
</tr>
<tr>
<td>Oestradiol 2.5mg/kg</td>
<td>52.1 ± 19.4 (3)</td>
</tr>
<tr>
<td>Sham injection saline : ethanol, 1:1</td>
<td>24.5 ± 2.2 (2)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.

Hormones were administered as intraperitoneal injections in 0.5ml physiological saline : ethanol, 1:1 and the rats were sacrificed 4 hours later.
Kidney ornithine decarboxylase activity in castrated and normal male and female rats weighing between 150 and 200g.

<table>
<thead>
<tr>
<th></th>
<th>Ornithine decarboxylase (nmol/30min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal males</td>
<td>6.38 ± 4.97 (5)</td>
</tr>
<tr>
<td>Orchidectomised males</td>
<td>0.927 ± 0.189 (7)</td>
</tr>
<tr>
<td>Normal females</td>
<td>9.73 ± 3.51 (3)</td>
</tr>
<tr>
<td>Ovariectomised females</td>
<td>4.30 ± 1.45 (5)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.

Albino rats (Wistar) were all obtained from Scientific Products Farm Ltd.

There was no significant difference between the values for normal and castrated males or normal and castrated females.
embryological origin as prostate and these are found predominantly in the cortex of the kidney. Ornithine decarboxylase activity was, however, found to be similar in both cortex and medulla of the same kidneys.

The experiment in Table 38 was intended to determine whether or not injection of testosterone or oestradiol could in fact stimulate the kidney ornithine decarboxylase level. Other glucocorticoid-inducible enzymes have been shown to be elevated in male and female rat liver after injection of oestradiol (Braidman & Rose, 1971) and ornithine decarboxylase is increased in rat uterus by oestradiol (Kaye et al., 1971; Russell & Taylor, 1971). The amounts of the steroids injected were the same as used by Panko & Kenney (1971) in an investigation of hormonal influences on liver ornithine decarboxylase activity. Table 38 shows that in fact, neither testosterone nor oestradiol significantly raised ornithine decarboxylase activity in the kidney and neither gave anywhere near the response as did dexamethasone. Nor was there any significant difference between the kidney enzyme levels in normal males and castrated males or between normal females and ovariectomised females although this may be due to the very wide variation in each group (Table 39). The relatively low enzyme activities found in this experiment, compared to the usual values obtained for male and female rats, may be due to the fact that the rats were used shortly after arrival from the supplier, instead of being taken from the animal house stock.

The outcome is that the high kidney ornithine decarboxylase activity does not appear to be maintained by any of the obvious possible hormonal influences, but appears to be an intrinsic property of the tissue. This raises the question whether ornithine decarboxylase is the rate limiting step in polyamine synthesis in kidney as it is reputed to be in liver (Raina et al., 1970). A priori this seems unlikely since the
polyamine content in normal kidney is no greater than in normal liver (Tabor & Tabor, 1964), in spite of the enormous difference in ornithine decarboxylase activity. It seemed however worth while to look at another enzyme involved in polyamine biosynthesis, S-adenosylmethionine. decarboxylase (SAMD).

7. **S-adenosylmethionine decarboxylase during compensatory renal hypertrophy.**

Figure 24 shows time courses for the SAMD assay using either HEPES or PO₄ buffers for both kidney and liver. The initial rates appear to be somewhat higher for the kidney preparation. This might simply be due to greater lability of the liver preparation since its time course is markedly curved. Although HEPES gave lower activities than phosphate buffer it was employed in subsequent experiments so that ornithine decarboxylase and SAMD could be assayed using the same supernatant preparation. Table 40 shows that 4, 8 and 24 hours after unilateral nephrectomy there were no significant changes in kidney SAMD activity compared to sham-operated controls. The SAMD in kidney (but not in liver) had much lower activities than the ornithine decarboxylase, indicating that ornithine decarboxylase is not the rate limiting step in polyamine biosynthesis in kidney. The lack of a marked response of SAMD even to stress, may indicate that the increase in kidney ornithine decarboxylase in response to stress has nothing to do with polyamine biosynthesis but is related to some quite different metabolic role normally fulfilled more actively in the kidney than in the liver. Such a metabolic role might be related to gluconoegenesis which is also more active in kidney than in liver (Krebs, 1963).

8. **Ornithine decarboxylase in BHK 21/Cl3 cells.**

Because the situation in kidney seems so complex it appeared well
Figure 24.

Time courses of SAMD from kidney and liver.

Supernatants were obtained from the organs of normal rats and assays were conducted as described in "Materials and Methods". Both 50mM-PO₄ buffer, 1mM-dithiothreitol, pH 7.2 and 100mM-HEPES buffer, 1mM-dithiothreitol, pH 7.2 were tested. Samples from the same organs were carried through from homogenisation to assay in both buffer systems.
<table>
<thead>
<tr>
<th></th>
<th>Normal rats</th>
<th>Unilateral nephrectomy</th>
<th>Sham operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.21 ± 0.14 (2)</td>
<td>1.56 ± 0.39 (3)</td>
<td>1.20 ± 0.07 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.01 ± 0.20 (4)</td>
<td>1.66 ± 0.08 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.57 ± 0.09 (4)</td>
<td>1.65 ± 0.07 (4)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for number of animals in parenthesis.

Rats weighed between 200 and 215g.
worth while looking at ornithine decarboxylase levels in the much simpler situation of cells in culture. The growth of several cell lines in culture can be varied by altering the serum concentration of the medium in which they are grown. Examples include 3T3 cells (Castor, 1971) and BHK 21/C13 cells (Fried & Pitts, 1968; Clarke et al., 1970). The latter was adopted for the present study and the conditions employed to obtain resting culture, and then to stimulate growth in them, were as described by Nicholas (1971). Normally C13 cells are grown attached to glass or plastic in Eagle's medium containing 10% bovine calf serum and 10% tryptose phosphate broth, commonly known as ETC.

If they are transferred to a medium containing only 1% serum and no tryptose phosphate (EC1) the rate of DNA synthesis declines steadily to zero over a period of 5 days (Nicholas, 1971; Howard D.K. unpublished results 1972). Such cells are normally described as "resting" cells. Addition of serum to such stationary phase cultures results in a wave of DNA synthesis at 14-18 hours followed by mitosis at about 20 to 24 hours. This is obviously an attractive model system of stimulated growth in which to study the relationship of the enzyme ornithine decarboxylase to growth.

Table 41 shows that C13 cells have much higher levels (160 to 200-fold) of ornithine decarboxylase activity when growing exponentially than when "resting". Not all enzyme activities decline in these circumstances: for example the deoxypurine nucleoside kinases are undiminished in activity in similarly "resting" cells (Durham, J.P., 1972, personal communication). This indicates that although the "resting" cells are viable, the ornithine decarboxylase activity has declined in parallel with cell growth.

Figure 25 shows the results of an experiment designed to establish whether the decline in DNA synthesis when C13 cells are deprived of
Table 41.

Ornithine decarboxylase activity in "resting" and logarithmically growing cultures of C13 cells.

<table>
<thead>
<tr>
<th>State</th>
<th>Activity (nmol/30 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Resting&quot;</td>
<td>0.0282</td>
</tr>
<tr>
<td></td>
<td>0.0262</td>
</tr>
<tr>
<td>Exponential</td>
<td>4.52</td>
</tr>
<tr>
<td></td>
<td>5.28</td>
</tr>
</tbody>
</table>

Exponentially growing cultures were 2 day old cultures in medium ETC at less than confluence.

"Resting" cultures had been transferred to medium ECI 5 days previously.

Each value represents the average of duplicate assays on a single culture. The assay procedure was as described in "Materials and Methods".
Figure 25a.

Effect of serum depletion of medium on DNA synthesis of

C13 cells. (Redrawn from Nicholas, 1971).
Figure 25A

Incorporation of [³H]thymidine c.p.m.

% LABELLED CELLS

DAYS

0 1 2 3 4 5

0 20 40 60

2500 5000 7500
Figure 25b.

Effect of serum depletion of medium on ornithine decarboxylase activity of Cl3 cells.

At time 0 cells were changed into EC1.
At time 49 hours serum was added back to 10% (v/v).
ORNITHINE DECARBOXYLASE in BHK 21/C13 CELLS

10% serum | 1% serum | 10% serum

3.0
2.0
1.0
0.0

MOL/0MIN/ MG PROTEIN

20 40 60
HOURS
serum is paralleled by a corresponding decline in ornithine decarboxylase.
Figure 25a redrawn from Nicholas (1971) illustrates the fall in DNA
synthesis over a 5 day period in EC1.  Figure 25b shows that ornithine
decarboxylase, astoundingly, declined precipitously within 4 hours.
It remained at this new low level (still somewhat higher than in cells
"resting" for 5 days) until serum was added back to 10%; 4 hours later
the activity had returned to the high value characteristic of
exponentially growing cells.  It appears that ornithine decarboxylase
responds almost instantaneously to alterations in the environment
which subsequently affect growth.  The response of DNA synthesis to
such changes is much slower.

The growth of C13 cells in culture can be varied by altering the
composition of the medium in which they are grown, especially with
respect to the serum concentration and the tryptose phosphate content.
In Figure 26 it can be seen that replacement of ETC with EC1 results
in a rapid fall in ornithine decarboxylase activity in C13 cells,
oticeable after only 1 hour and very marked by 4 hours when only
about 1.5% of the control activity remains.  4 hours after replacement
of ETC with EC10 (10% serum, no tryptose) however, the activity had
also fallen but only to 24% of the level in ETC.  C13a cells are
C13 cells which have been selected for their ability to grow well in
the absence of tryptose phosphate.  These also show a similar decline
in activity when EC10 is replaced by EC1, although the level of activity
in C13a cells (in EC10) is considerably less than that in C13 cells
(in ETC).  This correlates with the longer generation time of C13a cells
(about 20 hours) compared with that of C13 cells of about 12 to 14 hours
(Hay, J., personal communication, 1972).

It was considered valuable to perform analogous experiments with
polyoma-transformed C13 cells (PyY) whose growth is not inhibited to
the same extent by transfer to EC1 (Bürk, 1966).  4 hours after
Influence of composition of medium on ornithine decarboxylase activity of C13 and C13(a) cells.

C13 cells in ETC and C13(a) cells in EC10 were transferred to fresh medium at 0 time.

After the stated times, ornithine decarboxylase was assayed.

Tp refers to the addition of tryptose phosphate broth to 10%.
Figure 26

Ornithine decarboxylase
nmol/30 min/mg protein

Hours after medium change

EC10
EC1
Fresh ETC
EC10
EC1
Fresh EC10

C13 cells in ETC
C13(a) cells in ETC

C13 cells in ETC

H ours after medium change

EC10
EC1
Fresh ETC
EC10
EC1
Fresh EC10

C13 cells in ETC
C13(a) cells in ETC
Figure 27.

Influence of composition of medium on ornithine decarboxylase activity of PyY and PyA cells.

PyY cells in ETC and PyA cells in EC10 were transferred to fresh medium at 0 time.

4 hours later ornithine decarboxylase activity was assayed.
Figure 27

PyY cells in ETC

Transfer to EC1

Reinitine
ECarboxylase
nmol/30min/
mg protein

PyA cells in ETC

Fresh ETC

EC10

EC1

HOURS AFTER MEDIUM CHANGE
replacement of ETC with ECl 10% of the ornithine decarboxylase activity remained in the polyoma-transformed cells as against only 1.5% in the untransformed cells (Figure 27). Similar results were obtained with PyA cells (PyY cells selected for ability to grow well in the absence of tryptose phosphate). These experiments once more indicate a correlation between cell growth and ornithine decarboxylase activity.

The half-life of decay of ornithine decarboxylase in C13 cells after transfer from ETC to ECl (55 min) is almost exactly the same as that obtained after treatment with cycloheximide (60 min) (Figure 28). The half-life in C13 cells, while much longer than in rat liver and rat kidney, is nonetheless still very short compared with other enzymes (Schimke & Doyle, 1970). During the rapid fall in ornithine decarboxylase activity the rates of incorporation of labelled thymidine, and even labelled arginine, continue unabated suggesting that the ornithine decarboxylase activity decline may be one of the earliest responses to the new conditions and hence may be involved in controlling the more gradual run-down of other cellular functions such as DNA synthesis and even protein synthesis. In this connection it is interesting to speculate that ornithine decarboxylase is a part of, and possibly even a controlling factor in, the "pleiotypic response" proposed by Hershko et al. (1971). This model (Hershko et al., 1971) suggests that a pattern of biochemical events under a common control is related to growth.

Table 42 shows that the ornithine decarboxylase from C13 cells is inhibited by the presence of several amines in the assay mixture at a concentration of 1mM and in this respect is similar to the prostatic enzyme (Pegg & Williams-Ashman, 1968). The residual activity was 84% in the presence of putrescine, 51% with spermidine and 37% with spermine. The order of inhibition is the reverse of that observed
Figure 28.

Decay of ornithine decarboxylase activity following cycloheximide treatment or transfer to ECl.

Cl3 cells in ETC were treated with cycloheximide (5μg/ml medium) or were transferred to ECl. Ornithine decarboxylase was measured at various times thereafter.

Cl3 cells in plastic dishes in ETC were transferred to 5ml of ECl and pulsed for 1 hour periods with 10μCi/ml [Me-3H]thymidine and 1μCi/ml [U-14C]arginine. At the end of the 1 hour pulse the medium was removed and the cell sheet washed 3 times with 5ml ice-cold 5% (w/v) TCA. The precipitate was then scraped off and washed a further 3 times in 5ml ice-cold 5% (w/v) TCA by centrifugation at 600g. The precipitate was dissolved in 0.5ml Hyamine hydroxide and 10ml toluene based scintillation counting fluid was added. Double label scintillation counting was performed on a Philips liquid scintillation spectrometer.
**CYCLOHEXIMIDE**

- **Ornithine Decarboxylase**
  - $T_1 = 60$ min

**SERUM DEPLETION**

- $^{3}H$ Thymidine Incorporation
- $^{14}C$ Arginine Incorporation

- **Ornithine Decarboxylase**
  - $T_1 = 55$ min

**Figure 28**

- Log % of Control
- Hours

(Explanation of the graphs and data points not provided in the text.)
Table 42.

Inhibition of Cl3 cell ornithine decarboxylase by amines.

<table>
<thead>
<tr>
<th>Amine</th>
<th>dpm/30 min</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mM-putrescine</td>
<td>117</td>
<td>84.1</td>
</tr>
<tr>
<td>1mM-spermidine</td>
<td>71</td>
<td>51.1</td>
</tr>
<tr>
<td>1mM-spermine</td>
<td>51</td>
<td>36.7</td>
</tr>
<tr>
<td>1mM-3,3'diamino dipropylamine</td>
<td>109</td>
<td>78.5</td>
</tr>
<tr>
<td>Control</td>
<td>139</td>
<td></td>
</tr>
</tbody>
</table>

Each assay contained 100μM D,L-ornithine in the standard assay described in "Materials and Methods". Duplicate assays were conducted with the addition of the above amines to a concentration of 1mM.
for the prostatic enzyme. An analogue of spermidine, bis 3,3'-diaminodipropylamine, leaves 79% of the activity as against spermidine, 51%. This suggests that the inhibition may be relatively specific for the naturally occurring polyamines and may indicate that ornithine decarboxylase in C13 cells is under feedback control.

Although the high ornithine decarboxylase level in C13 cells appears to be to some extent dependent on the presence of tryptose phosphate broth (which enhances cell growth) the most marked influence on enzyme activity is that due to serum (Figure 26). Attempts have been made to identify the nature of this stimulatory factor in serum. Table 43 shows that it is not diffusable during dialysis. This need not rule out the possibility that glucocorticoids are responsible, since glucocorticoids may be tightly bound to serum proteins (Keller et al., 1969). In analogy to the effects of stress in raising the liver and kidney ornithine decarboxylase levels it was considered that the stimulation in C13 cells may be a consequence of the glucocorticoids and other hormones present in serum. Consequently both growth hormone and dexamethasone phosphate at high concentrations were tested for their effect on C13 cell ornithine decarboxylase activity. As can be seen in Table 43 both were ineffective. This suggests that the C13 cell ornithine decarboxylase increase is in no way related to the stress response.

There is the possibility that the tryptose effect may simply be due to induction of the enzyme by its substrate, ornithine, and not growth itself. Table 43 also shows that neither arginine nor ornithine stimulated the enzyme activity, although tryptose phosphate was efficacious in this respect. Ornithine in fact depressed the C13 cell enzyme concentration.

The results shown in Figure 29 indicate that the serum factor
Table 43.

Composite table of two experiments; the first testing possible causes of the serum effect; the other testing possible causes of the tryptose effect.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Cl3 cells in medium ETC transferred to medium EC1 6 hours before additions</th>
<th>Ornitline decarboxylase activity nmol/30min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control (ETC)</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>Control (EC1)</td>
<td>0.200</td>
</tr>
<tr>
<td></td>
<td>+ serum to 10%</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>+ dialysed serum</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>+ dexamethasone phosphate to 10μM</td>
<td>0.113</td>
</tr>
<tr>
<td></td>
<td>+ growth hormone to 100μg/ml</td>
<td>0.236</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Cl3a cells in medium EC10</th>
<th>Ornitline decarboxylase activity nmol/30min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control (EC10)</td>
<td>0.160</td>
</tr>
<tr>
<td></td>
<td>+ L-ornithine to 1mg/ml</td>
<td>0.0564</td>
</tr>
<tr>
<td></td>
<td>+ L-ornithine to 0.1mg/ml</td>
<td>0.0303</td>
</tr>
<tr>
<td></td>
<td>+ L-arginine to 1mg/ml</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>+ L-arginine to 0.1mg/ml</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td>+ tryptose phosphate broth to 10% (v/v)</td>
<td>0.721</td>
</tr>
</tbody>
</table>

Cells were harvested and ornithine decarboxylase activity was measured 4 hours after additions were made.
Serum stimulation of ornithine decarboxylase of C13 cells.

Rough fractionation of serum factor on a Sephadex G-25 column.

C13 cells in ECl had the following additions made:

(a) Serum to 10% (v/v)

(b) Void volume fraction of Sephadex G-25 serum separation
   (Fraction I); equivalent to 10% serum.

(c) Small molecular weight (phenol red containing) fraction
   of Sephadex G-25 serum separation (Fraction II);
   equivalent to 10% serum.

(d) Fraction I + Fraction II, equivalent to 10% serum.

(e) Tryptose phosphate broth to 10% (v/v).

(f) Serum to 10% (v/v) + Tryptose phosphate broth to 10% (v/v).

4 hours later ornithine decarboxylase activity was measured
as described in "Materials and Methods".
Figure 29

NMOL/30MIN/MG PROTEIN

EC1 + serum + fraction I + fraction II + fractions I+II + Tp + serum + tryptose
which is capable of stimulating ornithine decarboxylase activity in C13 cells passes through in the excluded volume of a Sephadex G-25 column (Fraction I). The small molecules of serum (Fraction II) lack this stimulative influence. Tryptose phosphate can be seen to have a small effect in the presence of 1% serum but a much larger effect in the presence of 10% serum. This is further evidence for a role for ornithine decarboxylase in the growth process, since factors in serum which cause cell growth have the same characteristics (Bürk, 1966).

When C13 cells which had been maintained in ECI for 6 days, and had consequently virtually stopped synthesising DNA, had serum added back to 10% a marked but transient increase in ornithine decarboxylase resulted after 5 hours (Figure 30). The enzyme activity declined precipitously between 6 and 8 hours. Other smaller peaks of ornithine decarboxylase activity occurred at 14 to 19 hours but may simply represent a "hunting about a mean". The results in Figure 30 are a part of a much larger experiment in which the activities of DNA polymerase, deoxycytidine, thymidine, deoxyadenosine, deoxyguanosine and uridine kinases and nucleoside phosphotransferases with deoxyadenosine, deoxyguanosine and thymidine as nucleoside acceptor were also determined on the same preparations as the ornithine decarboxylase (Figure 31). In addition RNA and DNA synthesis were measured by incorporation of $[^3H]$uridine and $[^3H]$thymidine, and the percentage of cells incorporating thymidine was followed by autoradiography. Ribosomal RNA synthesis was determined by isolating 28S and 18S RNA on polyacrylamide gels and determining the specific activity of the UTP pool (Howard, D.K., Durham, J.P., Melvin, W.T., Abrahams, J.C. and Hay, J., unpublished results, 1972). Uridine, deoxyadenosine and deoxyguanosine kinase activities did not decline
Elevation of ornithine decarboxylase activity in C13 cells after stimulation to growth with serum.

C13 cells ("resting" in EC1 for 5 days) were stimulated to grow by addition of serum to 10%. Ornithine decarboxylase was measured at various times thereafter.

Ornithine decarboxylase activity was expressed relative to the protein concentration of the homogenate rather than to the protein concentration of the 60,000g supernatant as previously.
Figure 30

C13 CELLS after SERUM ADDITION

Ornithine Decarboxylase

NMOL/30MIN/MG PROTEIN

Hours after SERUM ADDITION
Biochemical changes after serum stimulation of C13 cells.


C13 cells "resting" in ECl for 5 days had serum added to 10% (v/v) at 0 time. All enzymes were measured on the same homogenate prepared from cells from 2 Winchester bottles. Measurement of rates of labelling of RNA and DNA were performed in dishes.
in serum-depleted cells and did not show marked changes upon addition of serum. All other enzyme activities showed markedly decreased levels after serum depletion and a further decrease within 4 hours of addition of serum. DNA polymerase and thymidine and deoxycytidine kinases showed increases in activity at about the time of the peak of DNA synthesis (15-16 hours) but there was no change as early or as marked as the increase in ornithine decarboxylase activity at 5 hours.

All the above evidence obtained from experiments on C13 cells in culture supports the view that ornithine decarboxylase is an important part of the growth regulating system. The enzyme level appears to respond immediately to changes in the medium which subsequently will affect cell growth. This may be evidence for the operation of a sensitive and rapidly acting growth regulating mechanism and it appears that ornithine decarboxylase may be one of the earliest responders in a programmed sequence of biochemical changes.

9. Ornithine decarboxylase during pseudorabies infection of C13 cells.

Pseudorabies virus grows in C13 cells and the sequence of biochemical events during infection, leading to viral DNA synthesis within 6 hours, can be regarded as a simple model of organisation and growth and therefore can be compared with the sequence of events during the growth of C13 cells themselves. For this reason, and in order to investigate polyamine metabolism during viral infection, it was decided to measure ornithine decarboxylase activity in C13 cells after pseudorabies virus infection.

Table 44 shows that infection at a multiplicity of infection of 20p.f.u./cell results, 4 hours later, in an elevation of the "resting" cell ornithine decarboxylase activity about 7-fold and of the exponential level about 2-fold. By 8 hours these effects are less marked. Infection does not cause the same net increase in ornithine decarboxylase
Table 44.

Effect of pseudorabies virus infection of C13 cells on ornithine decarboxylase activity of "resting" and logarithmically growing cultures.

<table>
<thead>
<tr>
<th></th>
<th>Mock-infected control</th>
<th>Pseudorabies infected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ornithine decarboxylase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>nmol/30min/mg protein</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4 hours after infection

- "Resting"  
  - Mock-infected control: 0.0282  
  - Pseudorabies infected: 0.205
- Exponential  
  - Mock-infected control: 4.52  
  - Pseudorabies infected: 8.05

8 hours after infection

- "Resting"  
  - Mock-infected control: 0.0262  
  - Pseudorabies infected: 0.0222
- Exponential  
  - Mock-infected control: 5.28  
  - Pseudorabies infected: 7.60

Exponentially growing cultures were 2 day old cultures in medium ETC which were not yet confluent.

"Resting" cultures had been transferred to medium ECl 5 days previously.

Virus infection and assays were conducted as described in "Materials and Methods".
Figure 32.

Ornithine decarboxylase activity in C13 cells following infection with pseudorabies virus.
Figure 32

Ornithine Decarboxylase
nmol/30min/mg protein

Hours after infection with pseudorabies virus
activity in each case, since the overall activity of infected "resting" cells is much less than that of infected exponentially growing cells. Figure 32 shows the results of several experiments performed on cells either growing exponentially or "resting" in low serum medium for various periods of time. In each case a transient increase in ornithine decarboxylase activity is observed, but again the peak level reached is characteristic of the normal level of the cells used.

The increased activity may be to meet a requirement for increased synthesis of polyamines for use by the virus. Spermidine and spermine have recently been shown to be an integral part of the structure of the Herpes simplex virion (a virus closely related to pseudorabies) (Gibson & Roizman, 1971). In fact the changes in ornithine decarboxylase activity found here are mirrored by changes in ornithine decarboxylase activity when E. coli is infected with T2 bacteriophage (Bachrach & Ben-Joseph, 1971). Bacteriophage T2 has also been shown to contain the polyamines (Tabor & Tabor, 1964).

10. Discussion.

It would certainly appear both from the work on rats and the work on C13 cells in culture that an increase in ornithine decarboxylase, sometimes transient, sometimes sustained, is an early event in the biochemical prelude to growth. The transient increase in activity in C13 cells in culture further supports the view that the transient increase found after unilateral nephrectomy could be related to the growth process and is not necessarily simply a response to stress. This view is further supported by the published work of Hogan (1971) which appeared during the course of our work on C13 cells. Hogan has found high ornithine decarboxylase activity in exponentially growing HTC cells (rat hepatoma cells) in suspension culture which declines progressively (over 100-fold) with increasing cell density.
During this time the specific activity of the enzyme tyrosine transaminase remained virtually unchanged. Dilution of high-density cultures with fresh medium resulted in a transient increase in ornithine decarboxylase activity with a 60-fold peak after 4 hours.

Putrescine, the product of ornithine decarboxylase, has been shown to be a growth factor for human fibroblasts in culture (Pohjanpelto & Raina, 1972). This might indicate that ornithine decarboxylase, acting through its product putrescine may have a controlling role in the commitment to growth of BHK cells. Alternatively the control of ornithine decarboxylase may be linked to the control of ribosomal RNA synthesis or accumulation. It has been shown that the rate of rRNA synthesis is reduced in contact inhibited cells (Emerson, 1971; Weber, 1972) and the turnover of ribosomal RNA is increased (Weber, 1972), thus preventing the accumulation of ribosomal RNA. There is a parallel with the changes found in ornithine decarboxylase in growing and non-growing cells, but the enzyme level would appear to be more related to the rate of rRNA accumulation than to rRNA synthesis, in keeping with the possible role of polyamines in ribosomal function.

This relationship of ornithine decarboxylase to RNA synthesis, function or accumulation, may be the common denominator for the enzyme response both to stress and to growth. The striking correlation between RNA and polyamine levels in growing tissues has already been pointed out and in the kidney the accumulation of RNA is an early event in compensatory renal hypertrophy. In the liver stress causes increased RNA synthesis mimicking the increase during liver regeneration (Bucher & Swaffield, 1969), but the effect is only transient as is the rise in ornithine decarboxylase. In fact imposition of surgical stress 4.5 to 8 hours prior to partial hepatectomy accelerates the DNA synthetic peak, suggesting that many early events may be common to both
the stress response and the growth process (Moolten et al., 1970). If such a situation obtains in the kidney, as well as in the liver, the transient increase in ornithine decarboxylase after unilateral nephrectomy may be related to both stress and growth at one and the same time.

There is however at least one system in which the proposed relationship of ornithine decarboxylase activity to ribosomal RNA synthesis does not hold true. It has been shown that synthesis of ribosomal RNA in fact decreases after pseudorabies infection (Rakusanova et al., 1971), whereas, as we have seen above, the ornithine decarboxylase level increases, albeit transiently.

Again, in the kidney, ornithine decarboxylase activity seems to be inexplicably high in relation to RNA synthesis. Although enzyme activity increases early during compensatory renal hypertrophy the response is not sufficiently marked to justify its use as an early indicator of growth. In addition it is difficult to differentiate between the increase due to stress and the increase due to growth. The enzyme S-adenosylmethionine decarboxylase is similarly of no use as an indicator of growth. One might speculate that in the kidney the rate of polyamine synthesis is not regulated by the activities of these enzymes alone. Ornithine-keto acid aminotransferase for example, could compete with ornithine decarboxylase for available ornithine. It is known that ornithine-keto acid aminotransferase is higher in kidney than in liver (Raiha, 1971) and perhaps this may necessitate the high levels of kidney ornithine decarboxylase. This might also explain the glucocorticoid induction of ornithine decarboxylase. Ornithine-keto acid aminotransferase is involved in gluconeogenesis and increased activity might well be induced by glucocorticoids. A corresponding increase in ornithine decarboxylase
might then be required to maintain normal rates of polyamine biosynthesis.
CONCLUSION
CONCLUSION.

Several important conclusions emerge from the work described in the preceding sections. First, and perhaps most important, the changes in plasma urea and creatinine levels after unilateral nephrectomy show that the remaining kidney cannot to any significant degree compensate for the absence of its partner by increasing its own rate of function. The same point is demonstrated more precisely and even more convincingly by the measurements of glomerular filtration rate. The fact that the glomerular filtration rate does not increase significantly in the first 24 hours after unilateral nephrectomy suggests that increased work, in the thermodynamic sense, is not a primary stimulus to growth since the thermodynamic work of the kidney is coupled to the glomerular filtration rate (Pitts, 1968).

The renal insufficiency consequent on unilateral nephrectomy is tolerated remarkably well. Once the immediate effects of the anaesthetic and surgery have worn off the animals show no obvious signs of impaired vitality and, except in respect of urea and creatinine, there is little change in their blood chemistry. However, despite the fact that unilateral nephrectomy seems to make little difference to the animal's well-being, the surviving kidney is within a matter of hours stimulated to an active growth which does not cease until the deficit has been almost, if not quite, made good. It seems a fair presumption that some sort of homeostatic mechanism determines the amount of kidney tissue which an animal is to possess.

There is here an interesting parallel with the liver. Just as unilateral nephrectomy halves kidney function as measured by glomerular filtration rate, so partial hepatectomy diminishes liver function, as measured by bromsulphthalein clearance, in proportion to the amount
of tissue removed (R.W. Jubb, unpublished results, 1968). This hepatic insufficiency seems to be as well tolerated as is renal insufficiency after unilateral nephrectomy. Even so, it provokes almost immediately a compensatory growth even more rapid than that in the kidney. Here again a homeostatic control of tissue mass seems a fair presumption. However neither in the case of the liver nor the kidney is there any evidence relating to the nature of this homeostatic regulator.

There is evidence (summarised in the "Introduction") that both compensatory renal hypertrophy and liver regeneration are initiated within a few hours of the operation, and possibly even within the first few minutes. Consequently any system of homeostatic regulation would have to respond very rapidly to the decrease in functional capacity or tissue mass. The only immediate change known to occur after unilateral nephrectomy which could conceivably be a stimulus to growth is the 20 to 25% increase in renal blood flow in the remaining kidney. However in view of the fact that glomerular filtration rate does not show a concomitant increase this may be regarded as an unlikely possibility. A parallel exists here with liver regeneration. The liver remnant also has an increased blood flow but the available evidence (as summarised in the "Introduction") indicates that this is unnecessary for liver regeneration to occur.

The first unequivocal manifestation of kidney growth is the accumulation of RNA detectable twelve hours after the operation. These twelve hours are presumably occupied, first, by the progressive and perhaps fairly rapid changes in blood chemistry consequent on the 50% diminution in renal function. It is presumably the extent of one (or perhaps several) of these which triggers the sequence of events which leads ultimately to overt growth. In spite of the experiments
detailed in the section on "Nucleic Acid Metabolism" the nature of this sequence remains puzzling. The contrast between the rate of RNA synthesis in normal kidney and in compensatory renal hypertrophy is so dramatic that it should be easy to demonstrate by incorporation of radioactively labelled precursors or by the assay of the corresponding enzymes. The same might be expected to hold true, though to a lesser extent, of DNA synthesis. However the numerous attempts, described in "Chapter 3", to demonstrate such changes all gave results which were either negative or equivocal.

The one dramatic biochemical change early in the process of compensatory renal hypertrophy, as in liver regeneration, is the sudden increase in ornithine decarboxylase activity, though even the significance of this is difficult to assess. The tissue culture experiments described in "Chapter 4" leave no doubt that there is a real relationship between ornithine decarboxylase and growth. However even in C13 cells this relationship may not be exclusive. Ornithine decarboxylase activity may be related directly to some other cellular function, such as RNA synthesis, and hence only indirectly to growth.

In the whole animal ornithine decarboxylase appears to be related to many factors, only one of which is growth. There is, first of all, the inexplicably high activity in kidney compared to almost all other tissues. It seems impossible to relate this to any known aspect of kidney metabolism. One consequence is that ornithine decarboxylase would appear not to be the rate limiting step in polyamine biosynthesis in the kidney, as it has been suggested to be in the liver (Raina et al., 1970). More serious, the experiments described in "Chapter 4" show that ornithine decarboxylase in liver, and more especially in kidney, can increase markedly in response not only to quite trivial local
insults, but also to the stress of any sort of surgery under general anaesthesia.

The meaning of all this is obscure. The most likely interpretation, though admittedly very tentative, is that ornithine decarboxylase activity is linked to, and an extraordinarily sensitive indicator of, increased RNA biosynthesis and that such increased biosynthesis can be evoked not only (in sustained form) by compensatory growth but also (in transient form) by stress and quite trivial local damage.
SUMMARY
SUMMARY OF EXPERIMENTAL RESULTS.

1. The control of compensatory growth in liver and kidney has been studied with particular reference to the early events of the process.
2. Unilateral nephrectomy resulted in elevated plasma creatinine and urea levels soon after operation. However the operation appeared to cause no serious metabolic upset since plasma amino acid levels were remarkably unaffected.
3. A method has been devised for the determination of PAH and inulin clearances in anaesthetised rats by continuous infusion and periodic measurements of the levels of these compounds in the blood plasma. By these methods it was found that although renal blood flow in the remaining kidney increased by about 25% within an hour of unilateral nephrectomy the glomerular filtration rate showed no corresponding increase. After 24 hours it appeared that glomerular filtration rate was increasing but to a lesser extent than the kidney weight. Renal growth on a high gelatin diet similarly preceded functional adaptation and there was no increase in renal blood flow as in the solitary kidney after unilateral nephrectomy.
4. A preliminary investigation was made of the biochemical events in the early stages of compensatory renal hypertrophy. Measurements of RNA synthesis in vivo, RNA polymerase, DNA polymerase and changes in HnRNA metabolism showed no marked changes.
5. The accumulation of RNA by 16 hours after unilateral nephrectomy, normally amounting to a 17% increase, was inhibited by anaesthesia and anaesthetic agents.
6. The possibility of the use of the enzyme ornithine decarboxylase as an early indicator of growth was investigated. It was found to increase 90-fold in activity early during liver regeneration and was
maintained at an elevated level for at least 48 hours. 10% hepatectomy and sham operation also provoked similar but transient increases.

7. After unilateral nephrectomy ornithine decarboxylase activity showed a transient peak at 4 hours in the remaining kidney. Sham operation caused a similar but somewhat smaller response indicating that in the kidney also, stress, probably mediated by glucocorticoids, is partly responsible for increased ornithine decarboxylase activity. However in addition to the response to stress there was an added increase in enzyme activity at 4 hours, attributable to the process of compensatory growth, both in liver and kidney. Analysis of plasma corticosterone showed that unilateral nephrectomy and sham operation were equally stressing.

8. Ornithine decarboxylase was found to be much higher (about 60-fold) in the kidney than in heart, spleen, uterus, pancreas, parotid or liver. The only other normal tissue with a comparably high level was prostate.

9. The enzyme S-adensoylmethionine decarboxylase which is thought to be coupled to polyamine biosynthesis showed no marked changes during the first 24 hours of compensatory renal hypertrophy.

10. The relationship of ornithine decarboxylase activity to growth was investigated in C13 cells in culture. The enzyme was high in exponentially growing cells but low in "resting" cells. Stimulation of "resting" cells to growth resulted in a sharp peak in ornithine decarboxylase activity.
APPENDIX 1

MATERIALS AND METHODS
MATERIALS AND METHODS.

1a. Chemicals.

Corticosterone (4-pregnen-11β,21-diol-3,20-dione), sodium dodecyl sulphate, Hyflo Super-Cel, acrylamide, vitamins, amino acids and 2,5-diphenyloxazole (PPO) were obtained from Koch-Light Laboratories, Colnbroock, Bucks., England.

Cytidine 5'-triphosphate, uridine 5'-triphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, testosterone, β-oestradiol, deoxycorticosterone acetate, pyridoxal-5'-phosphate, ribonucleic acid from yeast, deoxyribonucleic acid from calf thymus, spermidine free base, spermine free base, putrescine dihydrochloride, D,L-ornithine HCl, L-ornithine HCl, spermidine phosphate, spermine diphosphate, tris and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) were obtained from Sigma, London.

Diethylpyrocarbonate, thioacetamide, atropine, p-aminohippuric acid, creatinine, inulin, N-1-naphthylethylenediamine dihydrochloride, orcinol and ethanolamine were obtained from British Drug Houses, Ltd.

Cleland's reagent (dithiothreitol) and cycloheximide (actidione) were from Calbiochem, London.

Deoxyribonuclease I, RNase free and salmon testes deoxyribonucleic acid were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

Polyvinylsulphuric acid and 3,3' diaminodipropylamine were obtained from Eastman Kodak Ltd., Kirkby, Liverpool.

Penicillin, streptomycin and Adexolin were obtained from Glaxo Laboratories Ltd., Greenford, Middlesex, England.
The following compounds were obtained from the sources listed:

α-amanitin; Boehringer Corporation, Germany:
bovine serum albumin; Pentex Incorporated:
calf serum; Biocult Laboratories and Flow Laboratories:
dexamethasone phosphate (decadron phosphate); Merck, Sharp & Dohme International
ethylene diacrylate; Borden Incorporated, Philadelphia:
halothane (Fluothane); Imperial Chemical Industries Ltd.:
heparin (Pularin); Evans Medical Ltd.:

Hyamine hydroxide (1M-solution in methanol); Nuclear Enterprises Ltd.,
Edinburgh:
metyrapone (Metopirone); Ciba Laboratories Ltd., Horsham, Sussex:
Nembutal; Abbott Laboratories Ltd.:
Sephadex; Pharmacia AB, Uppsala, Sweden:
Tryptose phosphate broth; Difco Laboratories, East Molesey, Surrey:
vinblastine (Velbe); Eli Lilly & Co. Ltd., Basingstoke, England.

Phenol (AnalaR grade) was obtained from British Drug Houses Ltd.,
and redistilled.

Human growth hormone preparation was a gift from Dr Webster of
the Pathology Department, Royal Infirmary, Glasgow.

Other chemicals were of AnalaR grade where possible.

lb. Radiochemicals.

[Me-$^3$H]dTTP (50mCi/mmol) was obtained from Schwarz Bioreserach
Incorporated, Orangeburg, N.Y., U.S.A.

D,L-[1-$^{14}$C]ornithine monohydrochloride (4.63mCi/mmol) and
D,L-[5-$^{14}$C]ornithine monohydrochloride (4.25mCi/mmol) were obtained
from New England Nuclear.

Other radiochemicals, obtained from the Radiochemical Centre,
Amersham, Bucks., England, were:-
[5-3H]uridine, 31Ci/mmol;
[8-3H]adenosine 5'-triphosphate ammonium salt, 11Ci/mmol;
[2-3H]p-aminohippuric acid, 150mCi/mmol;
D,L-[1-14C]ornithine monohydrochloride, 37mCi/mmol; 17mCi/mmol;
D,L-[5-3H]ornithine monohydrochloride, 2.7Ci/mmol;
[1,4-14C2]putrescine dihydrochloride, 50mCi/mmol;
S-adenosyl-L-[carboxy-14C]methionine, 61mCi/mmol;
Na214CO3, >50mCi/mmol;
[3H]inulin, 100-300mCi/mmol;
Inulin ([14C]carboxylic acid), 5-15mCi/mmol;
[U-14C]arginine, >270mCi/mmol;
[Me-3H]thymidine, 18.9Ci/mmol.

1c. Solutions.

Polyvinylpyrrolidone solution (Periston) had the composition shown in Table 45.

Tryptose phosphate broth contained 2.95% (w/v) dehydrated tryptose phosphate broth in water.
PBS(a) contained 8g NaCl, 0.2g KCl, 1.15g Na2HPO4 and 0.2g KH2PO4 in 800ml distilled water.

Toluene based scintillation fluid contained 0.5% (w/v)PPO in toluene.
Dioxan based scintillation fluid contained 0.7% (w/v)PPO and 10% (w/v) naphthalene in dioxan.

RSB buffer contained 0.1M-Tris-HCl, pH7.4, 0.1M-NaCl, 0.0015M-MgCl2.
### Composition of polyvinylpyrrolidone (PVP) solution (Periston)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyvinylpyrrolidone</td>
<td>35g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>8g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.42g</td>
</tr>
<tr>
<td>Calcium chloride hexahydrate</td>
<td>0.50g</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>0.005g</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>1.68g</td>
</tr>
<tr>
<td>1M-hydrochloric acid</td>
<td>17.1ml</td>
</tr>
</tbody>
</table>

Distilled water to 1 litre.
2. **Animals.**

Adult male albino rats and mice from the departmental colony were used in all experiments unless otherwise stated. Rats weighed between 150 and 250g and mice between 25 and 35g. During actual experiments rats were caged individually under thermostatic conditions (26°C) and were normally fed on a diet of "Rat Cubes".

Adrenalectomised, castrated and ovariectomised rats were bought from Scientific Products Farm Ltd. along with control males and females. All weighed between 150 and 250g and were kept in quarantine until used. Adrenalectomised rats were maintained on drinking water containing 0.9% sodium chloride and 10% sucrose. Animals were killed by cervical dislocation except where otherwise stated.

3. **Diets.**

The composition of the isocaloric high-gelatin and control diets was as shown in Tables 46-49 (Halliburton, 1966). Animals weighing about 200g were maintained on these diets for 3 days and were offered 7g at 10.00 hours and 8g at 16.00 hours.

4. **Surgery.**

All operations were performed between 9.00 hours and 12.00 hours unless otherwise stated.

   a. **Unilateral nephrectomy.**

Right unilateral nephrectomy was performed under ether anaesthesia through a midline abdominal incision. The kidney was decapsulated and the renal pedicle ligated with linen thread at a point about 3mm from the kidney which was then excised. The wound was closed in 2 layers, first with cotton thread and then with Michel Suture Clips (Thackray Ltd.). When required kidneys were washed in ice cold isotonic saline, dried and weighed on a torsion balance. When necessary they were frozen in a mixture of alcohol and solid carbon dioxide and stored.
**Table 46.**

Composition of semi-synthetic diets.

<table>
<thead>
<tr>
<th></th>
<th>15% Casein</th>
<th>15% Casein 30% Gelatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (g)</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Margarine (g)</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Glucose (g)</td>
<td>429</td>
<td>309</td>
</tr>
<tr>
<td>Potato starch (g)</td>
<td>129</td>
<td>9</td>
</tr>
<tr>
<td>V.M.R. * (g)</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Gelatin (g)</td>
<td>--</td>
<td>240</td>
</tr>
</tbody>
</table>

* See Table 47.

A 200g animal was offered 15g per day of one of these diets.

The physiological calorie equivalents are:

- **Protein**: 4 calories per gram.
- **Carbohydrate**: 4 calories per gram.
- **Fat**: 9 calories per gram.
- **V.M.R.**: 3.5 calories per gram.

The diets as made up therefore had a calorie equivalent of 4.18 calories per gram of diet.
Table 47.

Composition of vitamin-mineral-roughage (V.M.R.) mixture.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>32.5g</td>
</tr>
<tr>
<td>* Salt mixture &quot;446&quot;</td>
<td>130.0g</td>
</tr>
<tr>
<td>† &quot;Vitamins in starch&quot;</td>
<td>250.0g</td>
</tr>
<tr>
<td>Agar powder</td>
<td>62.5g</td>
</tr>
<tr>
<td>Margarine</td>
<td>77.5g</td>
</tr>
</tbody>
</table>

* See Table 48.
† See Table 49.

1g α-tocopherol acetate was mixed with 17.64ml Adexolin. 1ml of this was mixed with the above mixture.
### Table 48.

Composition of salt mixture "446".

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>243.2g</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>533.0g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>174.0g</td>
</tr>
<tr>
<td>CaH₂PO₄</td>
<td>800.0g</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>368.0g</td>
</tr>
<tr>
<td>Ferric citrate.3H₂O</td>
<td>36.0g</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.4g</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.2g</td>
</tr>
<tr>
<td>K₂Al₂(SO₄)₄.24H₂O</td>
<td>0.2g</td>
</tr>
<tr>
<td>NaF</td>
<td>0.002g</td>
</tr>
<tr>
<td>MgCO₃</td>
<td>92.0g</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>2.8g</td>
</tr>
<tr>
<td>KI</td>
<td>0.1g</td>
</tr>
<tr>
<td>ZnCO₃</td>
<td>0.1g</td>
</tr>
</tbody>
</table>
Table 49.
Composition of vitamins in starch.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>25mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>25mg</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>25mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>100mg</td>
</tr>
<tr>
<td>Menaphthone</td>
<td>5mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>5mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>200mg</td>
</tr>
<tr>
<td>Para-amo acid benzoic acid</td>
<td>500mg</td>
</tr>
<tr>
<td>Inositol</td>
<td>1.0g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>10.0g</td>
</tr>
<tr>
<td>Folic acid</td>
<td>trace</td>
</tr>
<tr>
<td>Potato starch</td>
<td>to 500g</td>
</tr>
</tbody>
</table>
at -70°C. Sham unilateral nephrectomy was performed by making a similar incision, exposing the right kidney, and closing the wound as described above.

b. Partial hepatectomy.

Under ether anaesthesia a midline abdominal incision was made from about 1cm above the xiphoid process to about 2cm below it. Using gentle pressure on the lower part of the thorax and upper abdomen, the median and left lateral lobes of the liver were delivered through the incision. 67% hepatectomy was performed by ligating these lobes with linen thread followed by excision. In a 10% hepatectomy a loop of linen thread was placed over the left radicle of the median lobe and tightened. This radicle was then excised. The abdominal wound was closed in a single layer with interrupted sutures.

Two types of sham operation were performed. In sham hepatectomy the median and left lateral lobes were delivered through the incision and returned to the peritoneum with manipulation. In laparotomy the incision was made but the liver was not disturbed. In both cases the wound was closed as described previously.

c. Cross-circulation experiments.

Cross-circulation was established between litter-mates of rats of the same sex and body weight by the method of Alston & Thomson, (1963). Both animals were anaesthetised with an intraperitoneal injection of pentobarbital (Nembutal) (45mg/kg). A midline skin incision was then made from the chin to the anterior end of the sternum. Through this incision the right external jugular vein was located and gently dissected free from connective tissue for 5-10mm, distal to its junction with the axillary vein. It was then cannulated with a 30cm nylon cannula (bore, 0.75mm; external diameter 0.94mm) previously
filled with physiological saline containing 5mg/ml heparin (Pularin; Evans Medical Ltd.) and clipped about halfway down its length.

Through the same incision the right common carotid artery was located by blunt dissection deep between the sterno-mastoid and sterno-cleoid muscles and similarly cannulated. By the same procedure the free ends of these cannulae were introduced into the corresponding blood vessels of the second animal, so that the carotid artery of each animal was connected to the jugular vein of its partner. The clips were then removed to allow exchange of blood and the animals were transferred to a baby incubator (30°C, 90% humidity)(Oxygenaire Ltd.). Animals were maintained under anaesthesia for the duration of the experiment by intraperitoneal injections of 3% phenobarbital in physiological saline. Anaesthesia was monitored using leg and conjunctival reflexes. Cross-circulation was established between 09.00 hours and 12.00 hours and an injection of heparin (5mg/kg) was given at 17.00 hours to each animal. Both animals were killed at about 03.00 hours next day after cross-circulation for 16 hours. At this time both cannulae were found to be patent.

d. Decerebration.

Male rats were decerebrated by the method of Gillespie et al. (1970). Under ether anaesthesia a midline skin incision was made as described previously and the trachea was located and cannulated. Through this cannula the rats were adequately ventilated by a Palmer small animal pump at 96 strokes/minute. Decerebration was performed by inserting a steel rod (13 s.w.g.) through the orbit and the foramen magnum and the rats were pinned down on a cork board in the incubator at 30°C to prevent hypothermia.

It was found that if ether anaesthesia was used initially, the tracheal cannula became blocked. Subsequently, therefore, halothane-
oxygen anaesthesia was employed. A simple halothane vapouriser was made as described by Parbrook (1966) in which a jet of 100% oxygen is directed onto the surface of halothane. No blockage of the tracheal cannula occurred in animals thus anaesthetised. The heart rate of decerebrate rats was satisfactory for at least 8 hours.

5. Clearance measurements.


Rats were maintained under anaesthesia by intramuscular and subcutaneous injections of a 1 in 5 dilution (v/v) of Nembutal in physiological saline. An injection of 5ml/100g body weight of polyvinylpyrrolidone (PVP) solution (Table 45) containing 6g inulin/100ml was given intraperitoneally and at the same time a priming injection of 0.75ml/100g body weight of physiological saline containing 4g inulin/100ml was given via the exposed right jugular vein. At suitable time intervals samples of peritoneal fluid and of blood (from the left jugular vein) were withdrawn for inulin determinations. Blood samples (0.2ml) were added to 3.8ml 0.9% saline and the blood cells removed by centrifugation at 700g for 10 minutes. Peritoneal samples were diluted 1 in 2000 with glass distilled water. Inulin was determined on these blood and peritoneal samples by the method of Heyrovsky (1956) against inulin standards and blood and reagent blanks. Estimations were performed in duplicate. The total inulin clearance was calculated by dividing the loss of inulin from the peritoneum by the concentration of inulin in the blood. The result was expressed as ml of blood cleared per minute per 100g body weight.

b. Single injection clearance.

Rats were kept under anaesthesia by intraperitoneal injection of a 1 in 2 dilution (v/v) of Nembutal in 0.9% saline. The jugular veins were exposed, 14μCi [3H]PAH was injected into the right jugular
vein and at various times thereafter blood samples (0.2ml) were withdrawn from the left jugular vein. Samples were centrifuged at 600g for 15 minutes and 0.05ml aliquots of separated plasma were removed to scintillation vials. Hyamine hydroxide (0.3ml) was added and the vials heated in a 60°C incubator for 30 minutes. Toluene scintillator (10ml) was added and the samples counted as described in section 9d. The results were plotted semilogarithmically and analysed according to the method of Blaufox & Cohen (1970) based on the mathematical treatment of Sapirstein et al. (1955). Clearance was expressed as ml of plasma/minute/100g body weight.

c. Clearance determinations by continuous infusion.

Rats were kept anaesthetised throughout the experiment by injection of a 1 in 5 dilution (v/v) of Nembutal in physiological saline (8mg pentobarbital over 80 minutes) via a cannula (25g x ½" needle) inserted into a tail vein, after induction with ether. The solution to be infused was prepared as follows. 0.9g of NaCl and 0.7g of PAH were dissolved in a small volume of distilled water and brought to pH 7.0-7.4 using 1.0M-NaOH. Inulin (7g) was then dissolved in the solution and the volume made up to 100ml with distilled water. The above solution was infused into normal and sham-operated animals. Unilaterally nephrectomised animals were infused with a dilution (generally 1 in 2) of this solution in physiological saline so that the concentration of inulin and PAH presented to a solitary kidney was similar to that presented to two kidneys in the intact animal. The concentration of the infusion solution (after dilution 1 in 4000) was checked in the standard assays for inulin and PAH. Infusion was carried out in an incubator at approximately 30°C and 90% humidity.

Immediately after an initial priming dose (35mg inulin, 3.5mg PAH in a volume of 0.5-1.0ml delivered intravenously) infusion of the
prepared solution into the exposed left external jugular vein was started at a constant rate of 0.01ml/minute, using a Sage model 234-3 infusion pump. At 60, 70 and 80 minutes after the start of the infusion 0.5ml blood samples were withdrawn from the right external jugular vein using heparinised syringes. These samples were centrifuged at 600g for 15 minutes and two 0.1ml aliquots of plasma were obtained from each (one for inulin assay and one for PAH assay). These plasma samples were stored at -55°C until assayed. Since the clearance of PAH above a threshold plasma concentration (when $T_m$ is exceeded) is decreased (Pitts, 1968), experiments in which the plasma PAH concentration exceeded 3.0mg/100ml were excluded from the calculations. Experiments where the three estimations of plasma inulin or PAH varied by more than 10% of their mean were also excluded. This limit is reasonably narrow, since total renal failure occurring at the time of the first blood sample leads to an increase over the three samples of about 100% for inulin and 200% for PAH. Respiratory failure or cardiac arrest during infusion usually resulted in very low clearance values. Such experiments were excluded from the Tables.

6. Chemical estimations.

a. Inulin.

A modification of the method of Heyrovsky (1956) was employed. The assay was carried out (in duplicate) scaled down x 2 on a 1 in 25 dilution of plasma with distilled water. Colour was developed by incubating the tubes in a water bath at 50°C for 1 hour and extinction was measured spectrophotometrically at 530nm. Standards and reagent blanks were run with each assay. Several plasma samples containing no inulin were assayed and found to give an extinction at 530nm similar to that of reagent blanks. Heparin has no effect on the reaction.
b. **PAH estimation.**

Acid cadmium sulphate was prepared by dissolving 17.34g CdSO$_4$.8H$_2$O in 84.55ml 0.5M-H$_2$SO$_4$ and making the total volume up to 500ml with distilled water. To 0.1ml plasma, 1.0ml distilled water, 0.3ml of the above acid cadmium sulphate and 0.1ml 1.1M-NaOH were added. After shaking and standing for a few minutes another 1.0ml distilled water was added and deproteinization completed by centrifuging at 4000g for 15 minutes. 1.0ml aliquots of supernatant were assayed (in duplicate) as described by Varley (1954), the assay being scaled down x 10. Standards and reagent blanks were run with each assay. Several plasma samples containing no PAH were assayed and found to give an extinction at 520nm corresponding to that of reagent blanks. Heparin has no effect on the reaction.

c. **Creatinine (Table 1)**

0.4ml samples of serum from blood (allowed to clot and centrifuged at 600g for 15 minutes) from the inferior vena cava were deproteinised and assayed for creatinine as described by Henry (1964). The method was scaled down x 5.

**Creatinine Table 2**

1ml blood samples from the exposed right jugular vein, before operation, and 5ml blood samples from the inferior vena cava, at death, were taken using syringes containing a trace of heparin. Plasma samples (obtained after centrifugation at 600g for 15 minutes) were stored at -55°C until assayed (autoanalyser) using the Jaffé reaction (Henry, 1964).

d. **Plasma amino acid analysis.**

**Method used for results in Table 3.**

Blood was collected in a heparinised syringe from the right jugular vein under ether anaesthesia. Plasma (obtained after centrifugation
at 600g for 10 minutes) was deproteinised by mixing with an equal volume of 6% (w/v) sulphosalicylic acid and immediately cooled. After centrifugation (600g for 10 min) at 4°C the supernatant was stored overnight at -20°C. The sample was thawed and centrifuged at 60,000g (Rav.) for 20 minutes. The resulting supernatant was diluted 1 in 2 with distilled water and 0.8ml was applied to both columns of a Jeolco amino acid analyser (JLC-5AH). Acidic and neutral amino acids were eluted from the long column (70cm, 0.8cm internal diameter) of resin LCR1 initially at 45°C with 0.2M-sodium citrate buffer pH 3.28 and later at 58°C with 0.2M-sodium citrate buffer pH 4.25. Basic amino acids were eluted from the short column (15cm, 0.8cm internal diameter) of resin LCR1 with 0.35M-sodium citrate buffer pH 5.25 at 58°C. Amino acids were estimated by the ninhydrin reaction using a cell with 2mm light path. Standard amino acid samples were also run.

Method used for results in Table 4.

Deproteinised plasma was prepared as described above. 0.25ml was applied to the 25cm column (0.9cm internal diameter) of a Locarte amino acid analyser. Amino acids were eluted with lithium citrate buffers 0.3M pH2.81; 0.3M pH4.15; 1.2M pH4.15 using temperatures of 37°C and 64°C. Amino acids were estimated by the ninhydrin reaction using a cell with a 10cm light path. Standard amino acid samples were also run.

e. Estimation of RNA, DNA and protein content of kidneys.

The method was essentially that of Schneider (1945). Kidneys were removed at operation or at death and stored as described in section 4a until assayed. Each kidney was homogenised in 50ml ice-cold glass-distilled water in a Nelco Blender at full speed for 3 minutes. Aliquots of 5ml were pipetted into ice-cold centrifuge
tubes and 2.5ml ice-cold 15% (w/v) TCA (trichloroacetic acid) was added to each. Estimations were normally performed in quadruplicate or quintuplicate from this stage onwards. After thorough mixing, the mixture was allowed to stand at 0°C for 10 minutes and then centrifuged at 900g for 10 minutes. The precipitate was washed with ice-cold 5% (w/v) TCA twice, the supernatant and washings being discarded. In experiments in which protein was estimated the precipitate was dissolved in 5ml 0.3M-NaOH at 37°C for 1 hour and 20μl was removed for protein assay. 0.85ml 5M-PCA (perchloric acid) was then added to the remainder and the mixture heated at 70°C for 30 minutes. After centrifugation at 900g for 10 minutes the supernatant was used for RNA and DNA estimation.

In experiments where protein was not measured the washed TCA precipitate was extracted with 5ml 0.5M-PCA at 70°C for 30 minutes and the supernatant used for RNA and DNA assay. In one experiment (Table 24) the TCA precipitate was dissolved in 5ml 0.3M-NaOH and 1ml 4.5N PCA added. The solution was allowed to stand for 10 minutes at 0°C and centrifuged at 900g for 10 minutes. The resulting supernatant was used for estimation of RNA. 5ml 0.5M-PCA was added to the precipitate and the mixture was heated at 70°C for 30 minutes. After centrifugation at 900g for 10 minutes the supernatant was used for DNA assay.

f. **RNA assay.**

The method was a modification of that of Kerr & Seraidarian (1945). 0.5ml of the supernatant obtained above was diluted with 1.5ml distilled water and 2ml 0.03% (w/v)FeCl₃ in concentrated hydrochloric acid (AnalaR, S.G. 1.18) was added. After addition of 0.1ml 26.7% (w/v) orcinol in ethanol, and mixing, the tubes were placed in a vigorously boiling water bath for 30 minutes. After cooling (ice-water) RNA
was estimated by the extinction at 665nm. Standard curves and reagent blanks were run with each set of assays.

g. DNA assay.

The method of Burton (1956) was employed. 1ml of the supernatant, prepared as in e. above, was taken for analysis. 1ml 0.5M-PCA was added followed by 4ml mixed Burton reagent (500ml glacial acetic acid + 7.5ml concentrated sulphuric acid + 7.5g diphenylamine + 2.5ml 1.6% (w/v) acetaldehyde). The mixture was left overnight and DNA estimated by measuring the extinction at 600nm. A standard curve and reagent blanks were run with each set of assays.

h. Protein assay (analysis of kidneys)

The 0.2ml sample for protein estimation (section 6e. above) was added to 9.8ml distilled water and mixed. 1.5ml aliquots were taken for estimation of protein by the method of Lowry et al. (1951) against a standard of bovine serum albumin. Blanks were also run. The extinction was measured at 625nm.

i. Protein assay (ornithine decarboxylase experiments)

Since HEPES interferes with the Lowry protein estimation, measurement of protein content of supernatants for ornithine decarboxylase assay was made by the Biuret method (Bailey, 1962) against a standard of bovine serum albumin. Appropriate blanks of the same buffer were also run.

j. Corticosterone assay.

Rats were killed by decapitation and the blood flowing from the severed carotid arteries was collected in heparinised containers. Plasma was obtained by centrifugation at 600g for 10 minutes. Extraction and measurement of corticosterone were as described by Zenker & Bernstein (1958). Fluorescence was measured using an Aminco-Bowman spectrophotofluorometer (4-8202 SPF) with an excitation
wavelength of 470nm and measuring emission at 530nm. Blanks and standard curves were also run.

7. Enzyme assays.

a. DNA polymerase.

The left kidneys from freshly killed rats were washed in ice-cold homogenisation medium (0.25M-sucrose, 5mM-MgSO₄, 0.02M-tris-HCl buffer pH 7.5) and were then homogenised in 5 vol. of the same medium using a Potter-Elvehjem homogeniser (Sireica). The homogenate was centrifuged at 600g for 10 minutes and the supernatant re-centrifuged at 105,000g for 60 minutes (Spinco model L ultracentrifuge, Type 50 Titanium Rotor). The supernatant after ultracentrifugation was used as source of enzyme in the DNA polymerase assays. The 600g pellet was washed twice more in homogenisation medium before final suspension (in 5ml) to be used as a source of nuclear DNA polymerase activity. The integrity and purity of nuclei were checked by fluorescent microscopy after staining with 1% (w/v) Acridine Orange.

DNA polymerase activity was assayed according to the method described by Lindsay et al. (1970). After incubation of the nuclear and supernatant fractions (20μl) at 37°C for 60 minutes in a total volume of 0.25ml with 5μmol of tris-HCl buffer, pH 7.5, 2μmol of MgCl₂, 15μmol of KCl, 0.1μmol of EDTA, 1.5μmol of 2-mercaptoethanol, 100μg of DNA and 50nmol each of dATP, dCTP, dGTP and [Me⁻³H]dTTP (50nCi/μmol), the reaction was terminated by addition of 0.05ml 2M-NaOH and the samples incubated overnight at 37°C before precipitation of DNA with 2ml of ice-cold 5% (w/v) trichloracetic acid (TCA) containing Hyflo Super-Cel (20g/l). 5ml of ice-cold 5% TCA containing 0.05M-Na₂P₂O₇ was also added and the mixture centrifuged at 800g for 10 minutes. The precipitate was collected on a pad of Hyflo Super-Cel on a disk of Whatmans No. 1 filter paper (2.5cm diameter) and washed
with 3 x 10ml 5% (w/v) TCA, 0.05M-\(\text{Na}_2\text{P}_2\text{O}_7\), 10ml ethanol and 2 x 5ml ether (all ice-cold). The DNA was dissolved by heating with 0.5ml 1M-Hyamine hydroxide for 10 minutes at 60°C in a counting vial. Radioactivity was measured as described in section 9d.

DNA used in the assay was from salmon testis. Denatured DNA was prepared by heating a solution of native DNA in 50mM-KCl (2mg/ml) at 100°C for 10 minutes and then rapidly cooling in ice water.

Results were expressed as specific activity. Protein was measured by the Lowry method (section 6h above).

b. RNA polymerase.

Preparation of nuclei: Each kidney was chilled in ice and washed with ice-cold RSB (0.1M-tris-HCl buffer, pH 7.4, 0.1M-NaCl, 0.0015M-MgCl₂). It was then minced with scissors and homogenised in 8ml RSB. A loose fitting teflon pestle was used and homogenisation was complete after 3 slow up and down movements. The homogenates of 2 kidneys were mixed and centrifuged at 600g for 5 minutes at 0°C. This preparation was passed through 2 layers of muslin to filter off clumps of connective tissue and unbroken cells. The filtrate was centrifuged at 150g for 15 minutes at 0°C. The supernatant was discarded and the pellet resuspended in 5ml ice-cold RSB. This preparation was centrifuged again at 150g for 15 minutes at 0°C. The supernatant was discarded and the nuclear pellet was resuspended to a final volume of 3ml in 0.25M-sucrose. Each stage in the nuclear preparation was checked by phase contrast microscopy for purity of nuclei. The procedure resulted in a 44% recovery of nuclei as estimated by analysis of DNA content.

Enzyme assay: The procedure adopted for the assay of RNA polymerase was essentially that of Widnell & Tata (1966) except that the assay time adopted was 5 minutes. In addition the
preincubation was omitted from the assay of the ammonium sulphate stimulated reaction since it was found that maximum incorporation was reached by 5 minutes and remained constant thereafter.

The incubation mixture for the low salt, Mg\textsuperscript{2+} activated RNA polymerase contained in a final volume of 0.5ml: 50\mu mol tris-HCl buffer (pH 8.5), 2.5\mu mol of MgCl\textsubscript{2}, 10\mu mol of cysteine, 3\mu mol of NaF; 0.3\mu mol each of GTP, CTP and UTP, 0.015\mu mol of non-radioactive ATP, 5\mu Ci [8-\textsuperscript{3}H]ATP and 0.1ml of nuclear suspension.

The incubation mixture for the RNA polymerase reaction in the presence of ammonium sulphate was as follows: the incubation medium contained in 0.5ml: 50\mu mol of tris-HCl buffer (pH 7.5), 2\mu mol of MnCl\textsubscript{2}, 0.05ml of a solution of ammonium sulphate saturated at room temperature and adjusted to pH 7.5 with NH\textsubscript{4}OH, 0.3\mu mol each of GTP, CTP and UTP, 0.015\mu mol of non-radioactive ATP, 5\mu Ci [8-\textsuperscript{3}H]ATP and 0.1ml of nuclear suspension.

After incubation for 5 minutes each assay was terminated by the addition of 5ml of ice-cold 0.5M-perchloric acid containing 0.05M-sodium pyrophosphate and the precipitate was collected, washed and prepared for counting as described for DNA polymerase.

Results were expressed relative to the amount of DNA in the incubation. DNA was estimated as described in sections 6e and 6g.

c. Ornithine decarboxylase.

Tissues (0.5 to 2g) were homogenised in 10ml 50mM-sodium-potassium phosphate buffer, pH 7.2 (later with the addition of 2.5mM-mercaptoethanol and later still in 100mM-HEPES, pH 7.2, 1mM-dithiothreitol) with 10 up and down strokes of the teflon pestle of a Sireica homogeniser. The homogenate was centrifuged at 60,000g for 20 minutes (Spinco model L ultracentrifuge, Type 50 Titanium Rotor) and the supernatant used immediately for enzyme assay.
All steps in this preparation were conducted at 0°C and without any delay.

Ornithine decarboxylase activity was measured by one of the following methods.

cl. By collection of $^{14}$CO$_2$

The method was essentially that of Russell & Snyder (1968). Enzyme activity was determined by measuring the liberation of $^{14}$CO$_2$ from carboxyl-labelled substrate. Incubations were carried out initially in Warburg flasks and subsequently in 25ml conical flasks specially fitted with glass centre wells. The neck was stoppered with a tightly fitting rubber bung pierced with a 21g x 1⅜" hypodermic syringe needle curved at the tip. The centre well contained 0.25ml of a CO$_2$ trapping solution (ethanolamine: ethylene glycol, 1 : 2). Some experiments were performed with 25ml flasks fitted with rubber stoppers supporting polyethylene centre wells (all supplied by Kontes Glass Co., Vineland, New Jersey).

Incubation mixtures consisted of 0.1μmol of pyridoxal phosphate, 0.5 to 1ml of supernatant (from roughly 50 to 100mg of tissue), 0.5μCi of D,L-[$^{14}$C]ornithine (200nmol) all in 0.05M-sodium-potassium phosphate buffer, pH 7.2, to make a final volume of 2ml. All components except for the substrate were agitated for 10 minutes at 37°C prior to the addition of substrate, and the incubation was continued for 30 minutes at 37°C. The reaction was stopped by injecting 1ml of 2M-citric acid into the reaction mixture via the in situ needle. The syringe was left in place to close the system to the atmosphere during absorption of evolved CO$_2$ (30 minutes shaking at 37°C). From the centre well 0.2ml of trapping solution was removed to a counting vial and 2ml ethanol and 10ml toluene scintillator were added. Scintillation counting was performed as described in section 9d.
All values were corrected for a zero-time blank which was found to be the same as, but more reproducible than, incubated heated enzyme blanks.

c2. Modifications.

As described in the Chapter entitled "Polyamine Metabolism" modifications were introduced into the above method. It was found that the addition of 2.5 mM-mercaptoethanol to all solutions gave higher activities and this was employed routinely until the assay was modified further. Dithiothreitol (1 mM) was found at a later stage to give higher activities as was HEPES buffer (100 mM, pH 7.2). Consequently the mercaptoethanol was discarded and all solutions (from homogenising buffer to assay) contained 1 mM-dithiothreitol in 100 mM-HEPES (pH 7.2) rather than 2.5 mM-mercaptoethanol in 50 mM-sodium-potassium phosphate (pH 7.2). A linear time course resulted from this modification and higher activities were obtained.

It was found that the tissue culture supernatants (lower protein content) had less stable ornithine decarboxylase activity, and therefore in these assays 2 mg bovine serum albumin was added. This led to higher activities.

These modified methods were used routinely for measurement of ornithine decarboxylase activity. Activity was expressed per g of tissue or (in the cell culture experiments) per mg protein in supernatant. Protein was measured by the Biuret assay (section 6i).

c3. By extraction of labelled putrescine.

This method is a modification of that of Russell & Snyder (1968) which was found to be unsatisfactory in its original form. After incubation as described in (c1) and (c2) (but with [5-3H]ornithine rather than [1-14C]ornithine) the reaction was terminated by addition of 1 ml of 1 M-NaOH saturated with sodium chloride. Solid sodium
chloride was added and the tube was Whirlimixed periodically for several minutes. The amine product was extracted into 7 ml of 1-butanol by vigorous agitation on a mechanical shaker for 30 minutes. The organic phase was washed once by Whirlimixing for 30 seconds with 1 ml of 1M NaOH saturated with NaCl, after which 1 ml of the organic phase was transferred to a counting vial along with 2 ml ethanol and 10 ml toluene scintillator. Radioactivity was measured as described in section 9d. In some experiments the butanol phase was evaporated to dryness and the residue chromatographed on Whatmans No. 1 paper by descending chromatography in 1-propanol : NH₄OH, 3 : 1. The area analogous to authentic putrescine standard was cut out, eluted with ethanol and counted.

d. **S-adenosylmethionine decarboxylase.**

Homogenates and supernatants were prepared in 100 mM-HEPES, pH 7.2 containing 1 mM-dithiothreitol as described for ornithine decarboxylase. The incubation mixture contained in a total volume of 0.2 ml: 0.1 ml supernatant, 0.01 μmol pyridoxal phosphate, 0.1 μCi S-adenosyl-L-[carboxyl-¹⁴C] methionine and 0.5 μmol putrescine, all in 100 mM-HEPES, pH 7.2, 1 mM-dithiothreitol. The assay was conducted in 4" x 20 mm test tubes fitted with rubber stoppers and polyethylene centre wells containing 0.2 ml of CO₂ trapping solution (ethanolamine : ethylene glycol, 1 : 2). The tubes were shaken at 30°C during the assay (30 min) and gas absorption (30 min). The reaction was terminated by injection of 0.5 ml 2M-citric acid and, after CO₂ absorption, the centre well was transferred to a scintillation vial containing 2 ml ethanol and 10 ml toluene scintillator and counted as described in section (9d).

Activity was expressed per g of tissue.

8. **Cell culture.**

Cells were cultured in Eagles medium (Glasgow modification; twice
with added serum and tryptose phosphate broth.

Medium EC1 was supplemented with 1% (v/v) calf serum.
Medium EC10 was supplemented with 10% (v/v) calf serum.
Medium ETC was supplemented with 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth.
Media also contained 100U/ml of penicillin/streptomycin.

Experiments were normally conducted in rotating 80oz winchester bottles except for those on incorporation of labelled arginine or thymidine which were performed in 50mm Nunc vented tissue culture dishes. Cell density was less than a monolayer.

Cells were supplied by the Wellcome Cell Culture Unit of the Department. The BHK21/C13 cell line and line PyY derived from it were propagated continuously in ETC. Cell lines C13a and PyA, selected for ability to grow well in the absence of tryptose phosphate broth, were propagated continuously in EC10. Sterility and mycoplasma checks were routinely carried out.

Pseudorabies virus was a plaque purified strain originally obtained from Dr A.S. Kaplan, Philadelphia.

Cells were normally grown in 180ml ETC or EC10. Transfer to EC1 was accomplished by pouring off old medium, washing the cell sheet with 50ml of Eagle’s medium and addition of 120ml of EC1.

Virus infection at 20 p.f.u. per cell was performed by allowing adsorption in only 25ml of medium for 1 hour, before replacing with the remainder of the medium. Mock infection of control cultures was carried out.

For ornithine decarboxylase assay the medium was poured off and the bottle cooled in a bath of ice-cold water. The cell sheet was washed with 20ml ice-cold PBS(a) and then scraped off in a further
10ml. Cells were collected by centrifugation at 300g for 10 minutes and homogenised in 3ml 100mM-HEPES, pH 7.2, 1mM-dithiothreitol as described in section 7c. Ornithine decarboxylase was assayed as described in section 72. 0.5ml of supernatant preparation was used in each of two duplicate ornithine decarboxylase assays and 0.5ml in each of two duplicate protein assays (section 6i).


a. Preparation of nucleoplasmic RNA.

The method was that of Willems et al. (1969). Mice (adult, male) were injected with [5-³H]uridine (250μCi) intraperitoneally for 1 hour before killing. Four kidneys from two mice were homogenised and the method carried out as described by Willems et al. (1969). Extracted RNA was subjected to electrophoresis on 2.8% acrylamide gels with ethylene diacrylate as cross-linker. Gels were scanned at 260nm (Gilford Spectrophotometer 240) and sliced, and the slices were hydrolysed for 30 minutes with 0.5ml Hyamine hydroxide at 60°C. Toluene based scintillator (10ml) was added and radioactivity was counted as described in section 9d.

All glassware and solutions were autoclaved before use. In some experiments ribonuclease inhibitors makaloid (up to 0.1% w/v), diethylpyrocarbonate (0.1% to 1%) and polyvinylsulphate (up to 0.1% w/v) were employed alone or in conjunction.

Homogenisation in RSB buffer (0.01M-tris, pH 7.4 at 20°C, 0.01M-NaCl, 0.0015M-MgCl₂) was found to give greater yields of nuclei, and nuclear RNA was extracted directly. Even when metal screens were substituted for ultracentrifugation (AB & Malt, 1970) and the entire RNA extraction procedure, from killing to precipitation of RNA, was accomplished in 30 minutes at 0°C, no reproducible pattern corresponding to the results reported by Willems et al. (1969) could be obtained.
b. **Rate of RNA synthesis in vivo.**

Rats were injected with radioisotopically labelled uridine or orthophosphate. At death the kidneys were removed immediately and homogenised as described in section 6e. Aliquots of 5ml were pipetted immediately into ice-cold centrifuge tubes containing 2.5ml ice-cold 15% (w/v) TCA. After centrifugation a 0.2ml aliquot of each supernatant was taken for scintillation counting in 10ml dioxan based scintillator. The precipitate was washed 3 times more with 5ml of ice-cold 5% TCA and in some experiments was then dissolved in 1ml Hyamine hydroxide for 10 minutes at 60°C. The sample was then transferred to a scintillation counting vial along with 10ml dioxan based scintillator. In other experiments the TCA precipitate was washed 3 times with 5ml of ice-cold 5% TCA and once with 5ml ice-cold ethanol before preparation for scintillation counting as above.

Radioactivity measurement was as described in section 9d.

c. **Sephadex G-25 chromatography of inulin.**

The method was essentially that of Cohen (1969) except that the Sephadex G-25 (fine) column was 50cm long and 1cm in diameter capped with 1cm of Sephadex G-25 (course). The column was run in phosphate buffer (0.03M- Na2HPO4 + 0.02M-KH2PO4) under gravity and timed fractions were collected with an Isco fraction collector. Aliquots (0.05ml) of each fraction were taken for scintillation counting along with 2ml ethanol and 10ml toluene based scintillator.

Peak samples were pooled and lyophilised before re-application to the column.

d. **Assay of radioactivity.**

[3H] or [14C] labelled samples were dissolved in toluene or dioxan based scintillator fluids and counted in a Phillips Liquid Scintillation Spectrometer with automatic quench correction.
(external standard). In some experiments where results are expressed as c.p.m. rather than d.p.m., a Nuclear Chicago Liquid Scintillation Spectrometer was used. $[^{32}\text{P}]$ labelled samples in dioxan based scintillator were counted in a Nuclear Chicago Scintillation Spectrometer.

e. Statistical Analysis.

Tests of statistical significance were performed where appropriate by analysis of variance (Snedecor, 1946) and Student's t test. The expressions $P<0.05$ and $P<0.01$ are used in the conventional sense to indicate significance at the 5 per cent and 1 per cent levels respectively.
APPENDIX 2

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