Regeneration of Renal Proximal Tubules after Mercuric Chloride Injury is Accompanied by Increased Binding of Aminoacyl-Transfer Ribonucleic Acid

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Homogenates of rat kidney cortex obtained 1, 3 or 14 days after a single injection of HgCl_2 were used to prepare the post-microsomal pH5 supernatant fraction. The activity of this fraction for peptide synthesis from [^{14}C]phenylalanyl-tRNA was significantly increased at 1 and 3 days, at which time the proximal tubules are regenerating [Cuppige & Tate (1967) *Am. J. Pathol.* 51, 405–429]. This increased activity could not be attributed to a decreased inhibitory activity, but was due to an increased aminoacyl-tRNA binding, i.e. elongation-factor-1 activity, in the supernatant fraction.

A number of experimental techniques have been used to induce general growth in the mammalian kidney (Goss & Dittmer, 1969), and this growth has been correlated with increases in DNA, RNA and protein content [reviewed by Halliburton (1969) and by Bucher & Malt (1971)]. The increases in RNA and protein after contralateral nephrectomy appear to result from a decreased degradation (Hill *et al.*, 1974; Hill & Malamud, 1974; Hill, 1975). In addition, evidence has been obtained for an increased synthesis of proteins in this situation (Coe & Korte, 1967; Sendeck *et al.*, 1973; Nicholls *et al.*, 1975). Moreover, this growth is accompanied by an increased activity of the machinery of protein synthesis, since there is an increase in the activity of elongation factor 1, which promotes the factor-dependent binding of aminoacyl-tRNA to ribosomes (Sendeck *et al.*, 1973; Nicholls *et al.*, 1975). Similar changes were found in kidney responding to growth-hormone administration (Girgis & Nicholls, 1973) or undergoing hypertrophy in response to folic acid (Nicholls *et al.*, 1975). An increase in aminoacyl-tRNA binding also was observed during the increased synthesis of specific kidney proteins, such as the soluble ribonuclease inhibitor during aminonucleoside nephrosis and the microsomal proteins during the induction of kidney enzymes by DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] and polychlorinated biphenyls (Girgis & Nicholls, 1971; Bishay & Nicholls, 1973; Nicholls & Markle, 1974; Cappon & Nicholls, 1974b; Cappon *et al.*, 1976).

Although numerous different cells are believed to be involved in the general growth response of the kidney, the glomerular cells are especially affected in aminonucleoside nephrosis. Hence it was decided to examine protein synthesis in a situation where only the tubular cells were involved in the growth response. Such a condition has been described by Cuppage &

Tate (1967), who observed the appearance of early regenerating cells and many mitotic figures in the middle and terminal portions of the proximal tubules, but not elsewhere. This condition occurred in the kidney of rats on the third day after tubular injury by a single dose of HgCl_2 . Accordingly, the incorporation of aminoacyl-tRNA into peptide was studied during the injury and repair of rat kidney after a single injection of HgCl_2 .

Experimental

Animals

Male rats (180–220g) of the Wistar strain were used and had free access to food (Master Fox Cubes; Maple Leaf Milling Co., Georgetown, Ont., Canada) and water throughout the experiments. HgCl_2 (BDH, Toronto, Ont., Canada), dissolved in water as a 0.05% solution, was injected once intraperitoneally (1.5mg/kg body wt.), either 24 or 72h or 14 days before removal of the kidneys. Control rats received the same volume of water. Metabolic cages were used for experiments involving the measurement of urine volume.

Chemicals

L-[U- ^{14}C]Phenylalanyl-tRNA (specific radioactivity 385mCi/mmol, 0.156 $\mu\text{Ci}/\text{mg}$ of tRNA) containing 19 other unlabelled aminoacyl-tRNA species from *Escherichia coli* strain B was obtained from New England Nuclear Corp., Lachine, Que., Canada. Sodium fusidate was a gift from the Squibb Institute for Medical Research, New Brunswick, NJ, U.S.A., and from Leo Pharmaceutical Products, Copenhagen, Denmark. Other chemicals were obtained as previously described (Girgis & Nicholls, 1972).

Liver ribosomes

A 20% (w/v) homogenate of liver obtained from control rats was prepared in 0.25M-sucrose dissolved in Buffer 1 [composed of 50mM-Tris/HCl (pH 7.8 at 25°C)/80mM-KCl/6mM-MgCl₂/10mM-2-mercapto-ethanol]. The ribosomes were preincubated, treated with 1% (w/v) sodium deoxycholate, and salt-washed as described by Cappon & Nicholls (1974a) to remove the endogenous mRNA and bound elongation factors 1 and 2. Salt-washed ribosomes were centrifuged for 2h at 2°C in a Spinco type 40 rotor at 105000g (r_{av} , 5.9cm) through a layer of 0.5M-sucrose in Buffer 1. The liver-ribosome pellets were resuspended in 0.25M-sucrose in Buffer 1 containing 10% (v/v) glycerol and stored at -20°C.

Kidney homogenate and pH 5 supernatant fraction

Both kidneys were placed on ice and all subsequent preparation was carried out at 2-4°C. The region of the hilum was removed by dissection and the kidneys were weighed and homogenized in a glass homogenizer with eight to ten passes of a Teflon pestle in 4vol. of 0.25M-sucrose in Buffer 1. The homogenate was centrifuged for 15min at 2°C in a Spinco type 40 rotor at 15000g (r_{av} , 5.9cm). The postmitochondrial supernatant fraction was centrifuged for 2h at 105000g (r_{av} , 5.9cm) to remove the microsomal fraction, and the resultant postmicrosomal supernatant fraction (S105) was used for preparation of the pH5 supernatant fraction, which contained elongation factors 1 and 2 (Girgis & Nicholls, 1972).

The S105 fraction was freed of synthetases and RNA, including tRNA, by adjusting the pH to 5.2 with 1M-acetic acid. After standing for 30min, the precipitate was removed by centrifugation at 15000g for 10min. The supernatant fluid was adjusted to pH7.8 by the addition of 1M-KOH. This resultant pH5 supernatant fraction, containing a final concentration of 15% (v/v) glycerol, was stored at -20°C.

Preparation of liver elongation factors 1 and 2

The pH5 supernatant fraction of control rat liver was prepared as described above for kidney, and elongation factor 1 was prepared as described previously by using Sephadex G-200 gel-filtration chromatography (Girgis & Nicholls, 1971; Goodchild & Nicholls, 1976). Elongation factor 2 was prepared by hydroxyapatite chromatography followed by DEAE-Sephadex chromatography (Petryshyn & Nicholls, 1976).

Measurement of protein and RNA

Hot-5%-(w/v)-trichloroacetic acid-insoluble protein was prepared for radioactivity counting as described by Nicholls *et al.* (1970) and the counting

efficiency was 85%. Incorporation and binding are expressed as d.p.m./mg of rRNA. These values were corrected by subtraction of values for tubes with no added elongation factors or pH5 supernatant, which were approx. 70 d.p.m./tube. Protein concentration was determined by the methods of Lowry *et al.* (1951) and of Tombs *et al.* (1959). The protein concentration of the urine was determined by the method of McAllister (1967) after sedimentation of cellular debris by centrifugation at 500g for 10min. RNA was estimated from the E_{260} ($E_{1cm}^{1\%} = 230$). Ribonuclease was determined as described previously (Nicholls & Bishay, 1971). The results given in the Figures are the mean values of duplicate tubes, representative of those obtained in three or more separate experiments in which one or more pairs of rats were studied. The means $\pm$ S.E.M. are shown in the Tables.

Results

The wet weight of the kidney undergoing repair after injection of HgCl₂ was increased significantly at 24 and 72h, but not at 14 days. The percentage dry weight was decreased comparably, so that the weight increase depended chiefly on water retention (Table 1). The content of protein was decreased slightly both in the postmicrosomal fraction (S105) and in the pH5 supernatant fraction at 24 and 72h (Table 1). This probably reflects the smaller size of the early regenerating cells (Cuppige & Tate, 1967).

The control animals gained weight, but the treated animals failed to gain weight at 24 and at 72h. After 14 days the treated rats remained slightly lighter than their paired controls (results not shown). After the injection of HgCl₂, the (daily) excretion of urine was markedly increased at 48h and 72h but returned to normal by 9 days. The (daily) excretion of protein was markedly increased during the periods of increased urine volume (results not shown). Cuppige & Tate (1967) did not observe a marked polyuria, but they observed a marked proteinuria, together with decreased urine osmolarities and a failure to concentrate the urine after water deprivation, in the early reparative phase.

Table 2 shows that the incorporation of [¹⁴C]-phenylalanyl-tRNA into peptide is significantly increased from 1 to 3 days but not at 14 days after the injury caused by HgCl₂ injection. The increase, however, is much greater at 3 days than at 1 day, and thus occurs at the time of increased cell proliferation as measured by mitotic figures and [³H]thymidine incorporation. When the pH5 supernatant fraction containing the elongation factors 1 and 2 was added at increasing concentrations to incubation mixtures containing liver ribosomes and excess of exogenous mRNA [i.e. poly(U)], the incorporation of [¹⁴C]-phenylalanyl-tRNA into peptide increased with increasing amounts of pH5 supernatant and was

Table 1. *Weight of the kidney and the soluble protein concentration in homogenate fractions during regeneration of the proximal-tubule cells*

The wet weight was determined for the kidneys immediately after dissection. The percentage dry weight was determined for several portions of these kidneys. For protein concentrations, the fractions were prepared and the protein determined as described in the Experimental section. Abbreviations: C, control; H, HgCl₂-treated. Results are means $\pm$ s.e.m. for the numbers of animals in parentheses. *P* values were obtained by Student's *t* test. **P* < 0.05; †*P* 0.10–0.05.

Period of regeneration (days)	Kidney weight				Protein (mg/100mg dry wt.)			
	Wet wt. (g)		Dry wt. (% of wet wt.)		S105 fraction		pH5 supernatant fraction	
	C	H	C	H	C	H	C	H
1	2.10 $\pm$ 0.02 (4)	2.40* $\pm$ 0.08 (5)	22.7 $\pm$ 0.19 (4)	17.5* $\pm$ 0.26 (4)	19.2 $\pm$ 0.8 (4)	16.3* $\pm$ 0.9 (5)	14.9 $\pm$ 1.0 (4)	13.0† $\pm$ 0.4 (5)
3	2.08 $\pm$ 0.02 (7)	2.62* $\pm$ 0.06 (7)	22.4 $\pm$ 0.09 (12)	18.4* $\pm$ 0.16 (12)	18.7 $\pm$ 0.7 (7)	16.3* $\pm$ 0.8 (7)	13.9 $\pm$ 0.3 (7)	11.7* $\pm$ 0.5 (7)
14	2.32 $\pm$ 0.01 (5)	2.45 $\pm$ 0.08 (5)	22.4 $\pm$ 0.03 (8)	21.1 $\pm$ 0.48 (8)	19.4 $\pm$ 0.8 (4)	19.6 $\pm$ 0.9 (4)	14.9 $\pm$ 0.2 (4)	14.3 $\pm$ 0.4 (4)

 Table 2. *Incorporation of [¹⁴C]phenylalanyl-tRNA into peptide by using pH 5 supernatant fraction obtained from kidney during regeneration of the proximal-tubule cells*

An excess of salt-washed liver ribosomes (94 μ g of RNA) was incubated with 50 μ g of pH5-supernatant protein from control or treated kidney for 10 min at 37°C in 0.40 ml of medium containing 50 mM-Tris/HCl (pH 7.6), 80 mM-KCl, 6 mM-MgCl₂, 0.2 mM-GTP, 10 mM-mercaptoethanol, [¹⁴C]phenylalanyl-tRNA (13700 d.p.m., 40 μ g of tRNA) and 100 μ g of poly(U). **P* < 0.05.

Period of regeneration (days)	10 ⁻³ $\times$ Incorporation (d.p.m./mg of RNA)		Increase (%)
	C	H	
1	7.93 $\pm$ 0.45 (4)	11.2* $\pm$ 0.28 (4)	41
3	7.91 $\pm$ 0.41 (6)	17.4* $\pm$ 0.90 (6)	120
14	8.11 $\pm$ 0.77 (5)	9.86 $\pm$ 1.10 (5)	22

consistently higher in the regenerating-kidney preparation (Fig. 1). Prolonging the incubation time from 10 to 30 min resulted in greater peptide synthesis in both preparations, and the incorporation in the treated-kidney preparation was increased twofold above that in the control preparation (Fig. 1).

The addition of high concentrations of GTP did not remove the difference between the pH5-supernatant-fraction activity of the regenerating kidney and the control preparation (Fig. 2), as might be expected if a GTP-sensitive inhibitor were present in the control kidney, as it is in control liver (Scornik *et al.*, 1967; Nolan & Hoagland, 1971; Rupniak & Quincey, 1973).

Further, the addition of increasing amounts of poly(U) (Fig. 2) did not remove the increased activity, as might be expected if differential amounts

of ribonuclease activity were responsible for the effects observed at 72 h.

As with poly(U) addition, in other experiments the increased activity was still present when amounts of either ribosomes or phenylalanyl-tRNA were increased in the incubation mixture. Thus the decrease in free and total ribonuclease activity that was detected in the regenerating kidney (results not shown), and which would conserve mRNA and other RNA species, cannot account for the increase in the activity of the pH5 supernatant fraction. Further evidence in support of this conclusion is the observation that the addition of increasing amounts of exogenous pancreatic alkaline ribonuclease to the kidney supernatant fractions inhibited both the regenerating kidney and the control preparation to a similar extent (Fig. 3).

Because of the possibility that initiation factors might be solubilized during the preparation of the homogenate and might have a greater activity in the pH5 supernatant fraction from the regenerating kidney, experiments were carried out with aurintricarboxylic acid, which is believed to inhibit the binding of mRNA to ribosomes, as well as possibly other steps in initiation (Stewart *et al.*, 1971). Fig. 3 shows that no difference in the degree of inhibition produced by aurintricarboxylic acid could be detected in experiments using the supernatant from control rats compared with that in experiments using the supernatant from treated rats.

Since elongation factors 1 and 2 were present in the kidney pH5 supernatant fraction and not on the salt-washed control liver ribosomes used in these experiments, it was desirable to see whether either of these factors was increased in the kidney pH5 supernatant of the HgCl₂-treated rats. The addition of various amounts of sodium fusidate, which inhibits

Fig. 1. Effect of concentration of pH 5 supernatant and incubation time on the incorporation of [^{14}C]phenylalanyl-tRNA into peptide

Incubation was as described for Table 2. (a) Incubation was for 10 min, and the amount of pH 5 supernatant per tube was as indicated. (b) The amount of pH 5 supernatant per tube was $50 \mu\text{g}$ (of protein), and the incubation time was as indicated. $\circ$, Control kidney; Δ , 3-day-regenerating kidney.

the translocation step (Malkin & Lipmann, 1969), did not introduce a differential inhibition of the incorporation of [^{14}C]phenylalanyl-tRNA into peptide (Fig. 4). Since fusidate has additional effects (at least in prokaryotes) when an excess of EF-G, relative to ribosomes, is used (Burns *et al.*, 1974),

Fig. 2. Effect of GTP (a) and poly(U) (b) concentration

Incubation was as described for Table 2. $\circ$, Control kidney; Δ , 3-day-regenerating kidney.

further experiments were carried out to examine the role of elongation factor 2 (Girgis & Nicholls, 1971). When the pH 5 supernatant fraction of kidney from treated and control rats was supplemented with increasing amounts of control-liver elongation factor 2 (Fig. 5), there was no change in the amount of labelled peptide formed. These results suggest that excess of elongation-factor-2 activity is present in

Fig. 3. Inhibition of peptide synthesis by pancreatic ribonuclease (a) and aurintricarboxylic acid (b)

Incubation was as described for Table 2. $\circ$, Control kidney; Δ , 3-day-regenerating kidney.

both preparations and that it is unlikely that elongation-factor-2 activity is altered in kidney undergoing repair.

When the reaction mixture was supplemented with liver elongation factor 1, the addition of 100 μ g of protein to the tubes containing control-kidney pH 5 supernatant fraction increased the incorporation of [14 C]phenylalanyl-tRNA, to a value equal to that seen with unsupplemented pH 5 supernatant from the treated rats, showing that elongation-factor activity was limiting in the control preparation. An increased incorporation was also observed when liver elongation factor 1 was added to the pH 5 supernatant from treated rats, showing that elongation-factor activity was also limiting in this preparation (Fig. 5).

Fig. 4. Inhibition of peptide synthesis by fusidate

Incubation was as described for Table 2 and fusidic acid was added as indicated. $\circ$, Control kidney; Δ , 3-day-regenerating kidney.

Since elongation-factor activity causes the poly(U)-directed binding of [14 C]phenylalanyl-tRNA to ribosomes at low Mg^{2+} concentrations, the binding activity of control and treated kidney preparations was measured by the Millipore-filter assay described by Malkin & Lipmann (1969), by using 4 mM-sodium fusidate to inhibit translocation. The stimulation of binding by the pH 5 supernatant fraction from the treated rats was significantly greater than that by the pH 5 supernatant from control rats (Fig. 6). Further, the addition of 70 μ g of control-liver elongation factor 1 to the supernatant obtained from control rats resulted in increasing the binding of [14 C]-phenylalanyl-tRNA to that reached with the supernatant obtained from treated rats.

Discussion

The results show that there is a marked increase in the capacity for protein synthesis in the soluble post-microsomal fraction of kidney when the proximal-tubule cells are regenerating after injury by a single injection of $HgCl_2$. This increased capacity is due to an increased activity for binding aminoacyl-tRNA to ribosomes, which depends on elongation-factor-1

Fig. 5. Effect of supplementing the incubation mixture with purified liver elongation factor 2 or elongation factor 1

The incubation was as described for Table 2 except that either purified liver elongation factor 1 (a) or 2 (b) was added as indicated. ○, Control kidney; Δ, 3-day-regenerating kidney.

activity. The increase was highest at a time when the mitotic index and incorporation of [^3H]thymidine into the regenerating cells of the proximal tubules were greatest and when no such changes could be

Fig. 6. Binding of [^{14}C]phenylalanyl-tRNA to ribosomes by using pH 5 supernatant fraction obtained from kidney on the third day of regeneration

An excess of preincubated salt-washed liver ribosomes (100 μg of RNA) was incubated with pH5-supernatant protein from control or treated kidney for 5 min at 37°C in 0.40 ml of medium containing 50 mM-Tris/HCl (pH 7.6), 80 mM-KCl, 6 mM-MgCl₂, 0.2 mM-GTP, [^{14}C]phenylalanyl-tRNA (13700 d.p.m., 40 μg of tRNA), 100 μg of poly(U) and 4 mM-sodium fusidate. The reaction was terminated by adding ice-cold Buffer 1 (see the Experimental section), and the samples were washed with three portions of buffer on Millipore filters and counted for radioactivity as described in the Experimental section. Blanks containing all the reactants except supernatant were subtracted. (a) Effect of pH5-supernatant concentration. (b) The pH5 supernatant (150 μg of protein) was supplemented with elongation factor 1 purified from control rat liver. ○, Control kidney; Δ, 3-day-regenerating kidney.

detected in the glomeruli or distal and collecting tubules (Cuppige & Tate, 1967). The increased activity of the regenerating-kidney preparation could not be attributed to the decreased alkaline ribonuclease activity that was detected and which might preserve the activity of the mRNA, tRNA or ribosomes during the incubation procedure. Moreover, no evidence was found to support the view that a GTP-sensitive inhibitor of peptide synthesis was involved, such as that reported in control and regenerating liver preparations (Scornik *et al.*, 1967; Rupniak & Quincey, 1973).

For regenerating liver and liver during microsomal-enzyme induction, evidence has been obtained for a decrease in the activity of an inhibitor of aminoacyl-tRNA binding (Goodchild & Nicholls, 1976; Tominaga *et al.*, 1975), as well as an increase in the activity of elongation factor 1 (Cappon & Nicholls, 1974a; D. M. Nicholls & J. Carey, unpublished work). Since this liver inhibitor is obtained from particulate rather than soluble fractions and is assayed in the presence of excess of pH 5 supernatant fraction, a decrease in its activity would not be detected in the present experiments if it were present in regenerating kidney. In experiments with increasing amounts of pH 5 supernatant, there was no evidence for changes in the activity of a soluble inhibitor of peptide synthesis, such as that found in muscle of genetic dystrophic mice (Petryshyn & Nicholls, 1976). Further, the absence of a differential effect on initiation suggests that changes in an inhibitor affecting initiation, such as reported for tissues other than kidney, cannot account for the results reported here (Gross & Rabinowitz, 1972; Lodish & Desalu, 1973; Levin *et al.*, 1975; Bester *et al.*, 1975).

The increased incorporation with the supernatant fraction of regenerating kidneys could not be attributed to the amount of mRNA in the preparation, since the increase was present when excess of poly(U) directed the peptide formation as well as when endogenous mRNA in the control-liver ribosome preparation directed incorporation. Moreover, the pH 5 precipitation step removes mRNA and also removes free and charged tRNA, and synthetases. Thus differences in the amount of endogenous aminoacyl-tRNA species in the supernatant fraction from treated rats compared with control supernatant fraction were not involved in the effects reported here. It is of course possible that differences in the amounts and/or activity of synthetases, aminoacyl-tRNA and mRNA may occur between control kidney and the kidney of HgCl₂-treated rats. If so, these changes cannot account for the results obtained in the present experiments.

The accumulation of mercury, especially in kidney (Haber & Jennings, 1964) and in contrast with liver (Clarkson, 1972), may involve its transport as a mercury-amino acid complex (Richardson *et al.*,

1975). The deleterious effect of mercury on the proximal tubular cells is presumed to be due to the involvement of these cells in transport (Vostál & Heller, 1968) and may be mediated by its effects on the mitochondria and lysosomes, which are histologically and functionally altered (Southard & Nitisewojo, 1973; Ware *et al.*, 1975; Verity & Brown, 1970). Membrane structure and function in muscle as well as in renal proximal tubules also appear to be affected (Cuppige & Tate, 1967; Shamoo *et al.*, 1976). After the disruption of the architecture of the proximal tubular cells, Cuppage & Tate (1967) described the process of repair to the tubular epithelium by the production of new cells. It is not known whether these new cells are the ones which are induced to produce the metal-binding protein, metallothionein (Cherian & Clarkson, 1976). This protein appears to be doubled in amount as early as 3 days after two injections, each of 0.5 mg/kg, of HgCl₂ (Piotrowski *et al.*, 1974), and thus its appearance is correlated with the peak of mitotic activity (Cuppige & Tate, 1967) and the increase in aminoacyl-tRNA binding observed in the experiments reported here.

The present observations of an increased aminoacyl-tRNA-binding activity in preparations obtained from kidney where the proximal tubule cells are regenerating resemble those in preparations obtained from kidney during generalized growth (Girgis & Nicholls, 1973; Nicholls *et al.*, 1975) and during the induction of specific enzymes (Girgis & Nicholls, 1971; Nicholls & Markle, 1974; Cappon & Nicholls, 1974b; Cappon *et al.*, 1976). Similar results have been found in rat liver during growth (Nicholls, 1973; Nicholls *et al.*, 1974) and during enzyme induction (Cappon & Nicholls, 1974a; Cappon *et al.*, 1976), in chicken liver after oestrogen treatment (Smith *et al.*, 1976) and in rat spleen after immunization (Willis & Starr, 1971).

These observations, taken together with those of changed activity of other translational proteins [Hopkinson *et al.* (1974); Pain & Henshaw (1975); for reviews see Pain & Clemens (1973); Manchester (1975); Hogan (1975)], suggest that there is usually an increased activity of the machinery of protein biosynthesis during increased protein production in mammals. Evidence for such a view has appeared for prokaryotic cells as well (Krauss & Leder, 1975; Furano, 1975; Furano & Wittel, 1976). Although variations in mRNA concentration and initiation-factor activity appear to be important for the regulation of translation [for a review see Lodish (1976)], there is a growing body of evidence to support the view that elongation factors may also be involved in regulation (see, for example, Nicholls *et al.*, 1974, 1975; Smith *et al.*, 1976; Haschemeyer & Persell, 1973).

Because of the difficulties in purifying elongation

factor 1, which is a multimeric protein that has different activity in different states of aggregation and that contains lipid (Collins *et al.*, 1972; Moon *et al.*, 1973; Drews *et al.*, 1974; Legocki *et al.*, 1974; Slobin & Möller, 1975), it is not yet possible to decide whether the increased activity of aminoacyl-tRNA binding is due to an increase in amount and/or reactivity of elongation factor 1. In either case, changes in growth of a given cell type in kidney seem to induce a change in aminoacyl-tRNA binding that is similar to that found after the induction of kidney enzymes or to that found after an increase in growth of a number of cell types such as in compensatory renal hypertrophy.

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