Localization of D-Myoinositol 1:2-Cyclic Phosphate 2-Phosphohydrolase in Rat Kidney

By N. CLARKE and R. M. C. DAWSON

*Department of Biochemistry, Agricultural Research Council Institute of Animal Physiology,
Babraham, Cambridge CB2 4AT, U.K.*

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1. On subcellular fractionation of rat kidney homogenates by differential and density-gradient centrifugation, the bulk of the inositol 1:2-cyclic phosphate 2-phosphohydrolase activity remains with the alkaline phosphatase activity, suggesting localization in the brush borders of the proximal tubules. 2. Histochemical studies with a medium containing inositol 1:2-cyclic phosphate and *Escherichia coli* phosphomonoesterase show Gomori staining around the brush borders of the proximal tubules in the outer cortex only. 3. Serial sections across the kidney from cortex perimeter to papilla suggest that the inositol 1:2-cyclic phosphate 2-phosphohydrolase has a limited distribution along the proximal tubule of the nephron, probably being limited to the pars convoluta, whereas the alkaline phosphatase extends along the pars recta.

We reported that animal tissues contain a specific phosphodiesterase that hydrolyses D-inositol 1:2-cyclic phosphate, producing D-inositol 1-phosphate (Dawson & Clarke, 1972). The cyclic phosphate itself is a product of the catabolism of phosphatidylinositol (Dawson *et al.*, 1971). In the rat the phosphodiesterase was present in very high concentration in the kidney cortex, its specific activity being some 18 times higher than the next richest tissue source, brain, and well over 100 times higher than its activity in the liver. Because of this unusual tissue distribution, experiments were made to try to determine its precise location in kidney-cortex cells. Subcellular fractionations and histochemistry demonstrated that the enzyme is located in the brush borders of the proximal tubules but that its distribution along each tubule is more limited than that of alkaline phosphatase.

Experimental

Subcellular fractionation

The kidneys removed from two rats (male, albino adults) were dissected to remove cortical material (approx. 2.5 g), which was homogenized in ice-cold 0.25 M-sucrose at pH 7.4. The conditions of homogenization and the separations of the primary fractions were as used by McMurray & Dawson (1969) for studies on rat liver, the operations being carried out in a cold-room at 3°C.

Density-gradient centrifugation at 0°C of the light mitochondrial and microsomal fractions was based on the procedure of Whittaker *et al.* (1964) for preparing cerebral-cortex subfractions. Although the discontinuous density gradients were initially set up

by employing the sucrose solution described by these authors, it was found that 5 ml samples of 1.3 M-, 1.1 M-, 0.85 M-, 0.6 M- and 0.4 M-sucrose gave somewhat improved separation for the kidney fractions. The light mitochondrial or microsomal fractions from kidney cortex (0.5–10 mg of protein/tube) were loaded on top of the gradient in 0.25 M-sucrose. The lower protein loading improved the separation but the protein determinations became more inaccurate because of the interference of sucrose in the protein determinations. The Lustroid tubes were centrifuged in a Spinco model L, SW 25 rotor, for 2 h at 23 000 rev./min (53 500 $g_{av.}$). The gradients were removed with a syringe, and the various interfacial and pellet fractions combined and stored at 0°C for later examination.

Assay of D-inositol 1:2-cyclic phosphate 2-phosphohydrolase activity

The assay relies on the addition of *Escherichia coli* phosphomonoesterase, which liberates P_i from any inositol 1-phosphate formed by hydrolysis of the cyclic compound. The phosphomonoesterase preparation was without action on inositol 1:2-cyclic phosphate, whereas inositol 1-phosphate could be quantitatively hydrolysed provided Ca^{2+} was added. The action of the latter might be to decrease any inhibition of the phosphomonoesterase activity by P_i released in the reaction (Kay, 1928) by precipitating this as calcium phosphate.

The tissue homogenate or membrane fraction was incubated at 37°C in a medium containing 0.9 mM-D-inositol 1:2-cyclic phosphate; 12.5 mM-2-amino-2-methylpropane-1,3-diol-HCl buffer, pH 8.3, 10 mM-MgCl₂, 4 mM-CaCl₂ and 0.04 mg of *E. coli* alkaline

phosphatase (type III; Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.) in a total volume of 1 ml. Sufficient tissue protein was added to cause a 15–30% break-down of the substrate in 20 min, e.g. with kidney-cortex homogenate approx. 0.05 mg of protein. At the end of the incubation the mixture was treated with 9.3 ml of ice-cold 7.8% HClO_4 , centrifuged at $3000g_{av}$ for 10 min and P_i was measured in the supernatant by the procedure of Bartlett (1959). Appropriate controls were run either in the absence of substrate or of tissue enzyme in the incubation medium. Under such conditions kidney-cortex homogenate gave a P_i release that was linear with respect to protein concentration (up to 0.05 mg of protein/ml) and practically linear with respect to time (up to 20 min).

Histochemical location of alkaline phosphatase and D-inositol 1:2-cyclic phosphate 2-phosphohydrolase

Frozen sections ($8\mu\text{m}$ thick) of rat kidney were cut on a microtome and air-dried for 1–2 h on a glass slide. A section was covered with a drop of incubation medium and incubated for 15–60 min at 37°C . The incubation medium contained 0.014 M-2-amino-2-methylpropane-1,3-diol-HCl buffer, pH 8.9, 9 mM- MgCl_2 , 36 mM- CaCl_2 , 27 mM- NaCl and one of the following substrates: (1) 1.7 mM-sodium glycerol-2-phosphate; (2) 1.7 mM-ammonium D-inositol 1-phosphate; (3) 1.7 mM-ammonium D-inositol 1:2-cyclic phosphate; (4) 1.7 mM-ammonium D-inositol 1:2-cyclic phosphate + 0.11 mg of *E. coli* alkaline phosphatase (type III Sigma, dialysed for 2 h against water)/ml. The deposited calcium phosphate was then fixed by a Gomori-type technique (Pearse, 1960); the section was counterstained with Haematoxylin, dehydrated and mounted.

Preparation of D-inositol 1:2-cyclic phosphate

The anterior portion of ox small intestine was washed with 0.15 M- NaCl , and the mucosa scraped with a glass plate and frozen in liquid N_2 . The frozen tissue was crushed to a powder and stored at -20°C . A sample (500 g) of the powder was pasted and then mixed with 2.8 litres of water at 2°C . The suspension was centrifuged at $15000g_{av}$ for 30 min and the supernatant collected by decanting through glass-wool to remove floating fatty particulate matter.

Phosphatidylinositol (0.4 g of P as the calcium salt) prepared from soya-bean lipids (Dawson & Clarke, 1972) was recovered from chloroform and the last traces of solvent were removed. Sodium acetate-acetic acid buffer (0.1 M, pH 5.5, 800 ml) at 40°C was added and the phospholipid completely dispersed by swirling for 1 h with glass beads. To the turbid phospholipid suspension was added 600 ml of the intestinal mucosal extract, and the mixture was incubated for

75 min at 37°C with gentle shaking and then centrifuged at $1000g$ for 15 min to remove most of the lipid and some protein. The clear supernatant was shaken with 3 vol. of chloroform-methanol (2:1, v/v) and the emulsion centrifuged. The upper phase containing the water-soluble phosphorus products from phosphatidylinositol degradation was collected. After adjustment to pH 8 with aq. NH_3 it was evaporated to dryness in batches below 50°C and the residue dissolved in water (0.1 vol. of the starting vol.). The solution was shaken with 4 vol. of chloroform-methanol (2:1, v/v), centrifuged and the upper phase collected, leaving the lower phase and proteinaceous material at the interface. The upper phase was evaporated to dryness below 50°C , the residue treated with 100 ml of water and any insoluble material removed by centrifuging. The solution was passed down a column (21 cm long $\times$ 2 cm diam.) of Dowex 1 (X8 ; SO_4^{2-} form; 100–200 mesh), which had been previously treated with 200 ml of 1 M- NaOH and then washed with 1 litre of water. The eluate was discarded and the column then eluted with 125 ml of 1.8 M- NH_3 and then 500 ml of 0.05 M- $(\text{NH}_4)_2\text{SO}_4$. Fractions (25 ml) were collected and monitored for phosphate esters by paper ionophoresis (Dawson *et al.*, 1971; Dawson & Clarke, 1972). Inositol 1:2-cyclic phosphate emerged soon after commencing the elution with the $(\text{NH}_4)_2\text{SO}_4$, followed immediately by inositol 1-phosphate and then by P_i , which overlaps the latter. The appropriate fractions were collected as a solution (pH 10) containing about 0.2 mg of P/ml and stored at 0°C . No SO_4^{2-} could be detected with BaCl_2 in either the inositol 1:2-cyclic phosphate or the inositol 1-phosphate fractions. About 40% of the original starting lipid P was recovered as inositol 1:2-cyclic phosphate.

Marker enzyme activity assays

NADPH-cytochrome *c* reductase (EC 1.6.2.1) and 5'-nucleotidase activities (EC 3.1.3.5) were determined as described by Jungalwala *et al.* (1971), and glucose 6-phosphatase (EC 3.1.3.9), alkaline phosphatase (EC 3.1.3.1) and acid phosphatase activities (EC 3.1.3.2) by the procedure of Hübscher & West (1965). The alkaline phosphatase values were, when necessary, corrected for a small inhibition produced by assaying fractions in a medium containing high sucrose concentrations.

Protein and RNA determinations

Protein was determined by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. The effect of sucrose on the colour development was allowed for as described by Gerhardt & Beevers (1968). RNA was measured by the procedure of Fleck & Munro (1962).

Staining of kidney sections

Periodic acid-Schiff staining was done as described by Pearse (1960). This gave a very-clear differentiation of the glomeruli, and moreover the brush borders of the pars recta stained a deeper red than those of the pars convoluta (Schmidt & Dubach, 1968).

Results

Location in subcellular fractions of kidney cortex

The distributions reported are typical of two or more fractionations and were completely reproducible in the salient features. In the primary fractionation of the kidney cortex it is clear that the inositol 1:2-cyclic phosphate 2-phosphohydrolase is membrane-bound: very little activity was present in the 100000g supernatant (Figs. 1 and 2). Maximal specific activity was found in the 'light mitochondrial' fraction, although the microsomal fraction also showed high activity. The initial debris fraction contained a large amount of lower specific activity material, but this contained many whole cells and unbroken tubular material. Distribution in the primary fractions was somewhat similar to that of NADPH-cytochrome *c* reductase (endoplasmic-reticulum marker), 5'-nucleotidase (plasma-membrane marker) and acid phosphatase (lysosomal marker high in glomerulus in kidney; Mattenheimer, 1968), except these consistently showed higher specific activity in the microsomal fraction than the 'light mitochondrial' fraction (Fig. 1). The distribution of activity in the primary fractions showed a more close similarity to alkaline phosphatase (Fig. 2), an enzyme reported to be very selectively localized in the brush borders of rat kidney tubules (Wilfong & Neville, 1970).

When either the 'light mitochondrial' fraction or the microsomal fraction was centrifuged on a discontinuous sucrose density gradient the bulk of the inositol 1:2-cyclic phosphate 2-phosphohydrolase sedimented with the more-heavy membrane fractions, maximal specific activity occurring at the interface between 1.1M- and 1.3M-sucrose (density 1.161–1.174) (Figs. 3 and 4). The major activity could be clearly distinguished from those of NADPH-cytochrome *c* reductase and 5'-nucleotidase, whose highest specific activity occurred in less-dense membrane fragments (Figs. 3 and 4). In addition, the activity did not correlate with the ribonucleic acid/protein ratio as determined along the gradient. The activity was similar in distribution throughout 'light mitochondrial' fraction and microsomal fraction gradients to that of alkaline phosphatase (Fig. 4).

The specific activity of the small amount of membrane protein occurring at the 0.4–0.6M-sucrose interface (density 1.055–1.083) of 'light-mitochondrial'-fraction sucrose gradients consistently tended to

Fig. 1. Distribution of inositol 1:2-cyclic phosphate 2-phosphohydrolase and marker enzyme activities in primary subcellular fractions obtained by differential centrifuging of kidney-cortex homogenates

(a) Inositol 1:2-cyclic phosphate 2-phosphohydrolase; (b) NADPH-cytochrome *c* reductase; (c) 5'-nucleotidase; (d) acid phosphatase. The specific activities of the enzymes are represented by the height of the histograms and the width represents the % of total protein recovered in the same fraction. The area therefore gives an indication of the % of total enzyme activity recovered in each particular fraction. Recoveries as % of activity or protein in whole homogenate are: inositol cyclic phosphate 2-phosphohydrolase, 108%; 5'-nucleotidase, 86%; acid phosphatase, 112%; protein, 114%.

Fig. 2. Distribution of inositol 1:2-cyclic phosphate 2-phosphohydrolase and alkaline phosphatase activities in primary subcellular fractions obtained by differential centrifuging of kidney-cortex homogenates

(a) Inositol 1:2-cyclic phosphate 2-phosphohydrolase; (b) alkaline phosphatase. The specific activities of the enzymes are represented by the height of the histograms and the width represents the % of total protein recovered in the same fraction. The area therefore gives an indication of the % of total enzyme activity recovered in each particular fraction. Recoveries as % of activity or protein in whole homogenate are: inositol cyclic phosphate 2-phosphohydrolase, 136%; alkaline phosphatase, 138%; protein, 139%.

have a higher specific activity of inositol 1:2-cyclic phosphate 2-phosphohydrolase than expected from the sequence of the gradient, suggesting a component other than that containing the bulk of the activity with maximum specific activity at density 1.161–1.174 (Figs. 3 and 4). The bimodal distribution of inositol 1:2-cyclic phosphate 2-phosphohydrolase activity along the gradient could be accentuated by a

preliminary 'washing' of the 'light mitochondrial' and microsomal fractions to remove very light components and soluble protein before centrifuging on a sucrose gradient. With the 'light mitochondrial' fraction the specific activity of the membrane protein of density 1.055–1.083 became very much higher than that of membrane fractions of density 1.161–1.174 (Fig. 5). However, the activity in the lighter component only amounted to a few per cent of the total activity. The bimodal distribution of activity was also seen in washed microsomal fractions (Fig. 5). The activity of the alkaline phosphatase also showed a similar bimodal distribution along the gradient from both fractions (Fig. 5). However, the ratio of inositol 1:2-cyclic phosphate 2-phosphohydrolase to alkaline phosphatase activities was always double in the membrane component of density 1.055–1.083 from the 'light mitochondrial' fraction compared with the membrane component of density 1.161–1.174 (Table 1). The ratio did not change in the same way with the microsomal fraction. With these washed 'light mitochondrial' and microsomal fractions there was no correlation of the inositol 1:2-cyclic phosphate 2-phosphohydrolase activity along the gradient with acid phosphatase, glucose 6-phosphatase or 5'-nucleotidase activities (Fig. 5).

Histochemical location of enzyme activity

Since the subcellular distribution studies had indicated that inositol 1:2-cyclic phosphate 2-phosphohydrolase activity was closely associated with that of alkaline phosphatase (a brush-border marker), experiments were performed to attempt to localize the enzyme by histochemical means. By using kidney slices with either glycerol-2-phosphate or D-inositol 1-phosphate as substrate the deposited calcium phosphate formed by alkaline phosphatase action could be clearly seen in the lumen of the tubules (Plate 1a, b). This is consistent with a brush-border localization for this enzyme. By using D-inositol 1:2-cyclic phosphate as substrate and relying on intrinsic alkaline phosphatase to hydrolyse any liberated D-inositol 1-phosphate little reaction occurred. However, in localized regions, usually towards the periphery of the kidney slice, some of the lumen of the tubules showed calcium phosphate deposition (Plate 1c, d). If *E. coli* alkaline phosphatase was added to the incubation medium, the Gomori staining was more intense, and, in spite of some structural disruption probably caused by the enzyme preparation, deposition of calcium phosphate had again occurred inside the lumen of the tubules (Plate 1e). It was also observed that, whereas the staining with glycerol-phosphate or inositol 1-phosphate as substrate extended throughout the cortical regions, that obtained with D-inositol 1:2-cyclic phosphate + *E. coli* alkaline phosphatase was largely localized in the

EXPLANATION OF PLATE I

Histochemical experiments were done with different substrates and released P_i was located by the Gomori technique

Conditions are as described in the Experimental section. The substrates were: (a) glycerol-2-phosphate, 15 min incubation; (b) D-inositol 1-phosphate, 15 min incubation; (c) D-inositol 1:2-cyclic phosphate, 15 min incubation; (d) D-inositol 1:2-cyclic phosphate, 30 min incubation; (e) D-inositol 1:2-cyclic phosphate + 0.11 mg of *E. coli* alkaline phosphatase/ml, 15 min incubation. Magnification $\times 98$.

EXPLANATION OF PLATE 2

Macroscopic appearance of rat kidney slices after Gomori staining

Incubation was for 30 min under conditions described in the Experimental section. (a) D-Inositol 1-phosphate as substrate; (b) D-inositol 1:2-cyclic phosphate as substrate + 0.11 mg of *E. coli* alkaline phosphatase/ml. Magnification $\times 6.6$.

Fig. 3. Distribution of inositol 1:2-cyclic phosphate 2-phosphohydrolase activity, marker enzyme activities and ribonucleic acid after centrifuging 'light mitochondrial' and microsomal fractions from kidney cortex on a sucrose density gradient

(a) Inositol 1:2-cyclic phosphate 2-phosphohydrolase; (b) NADPH-cytochrome *c* reductase; (c) 5'-nucleotidase; (d) ribonucleic acid. The specific activities of the enzymes are represented by the height of the histograms and the width represents the % of total protein recovered in the same fraction. The area therefore gives an indication of the % of total enzyme activity recovered in each particular fraction. Recoveries as % of activity or protein in whole homogenate are: inositol cyclic phosphate 2-phosphohydrolase, 91% ('light mitochondrial' fraction), 106% (microsomal fraction); protein, 110% ('light mitochondrial' fraction), 101% (microsomal fraction).

Fig. 4. Distribution of inositol 1:2-cyclic phosphate 2-phosphohydrolase and marker enzyme activities after centrifuging the 'light mitochondrial' and microsomal fractions from kidney cortex on a sucrose density gradient

(a) Inositol 1:2-cyclic phosphate 2-phosphohydrolase; (b) alkaline phosphatase; (c) 5'-nucleotidase. The values for 'light mitochondrial' and microsomal fractions represent two different experiments. The specific activities of the enzymes are represented by the height of the histograms and the width represents the % of total protein recovered in the same fraction. The area therefore gives an indication of the % of total enzyme activity recovered in each particular fraction. Recoveries as % of activity or protein in whole homogenate are: inositol cyclic phosphate 2-phosphohydrolase, 109% ('light mitochondrial' fraction), 99% (microsomal fraction); protein, 108% (light mitochondrial' fraction), 96% (microsomal fraction).

outer cortex (Plate 2). This suggested that the inositol 1:2-cyclic phosphate 2-phosphohydrolase may not be so widely distributed throughout the tubules of the nephron as alkaline phosphatase. Moreover, as far

as could be judged from the number of stained nuclei surrounding each region of calcium phosphate deposition, the activity was more confined to the proximal tubules rather than to the distal ones (Ham, 1953).

Fig. 5. Distribution of inositol 1:2-cyclic phosphate 2-phosphohydrolase and various marker enzyme activities after centrifuging 'washed' 'light mitochondrial' (a) and microsomal (b) fractions from kidney cortex on a sucrose density gradient

The values for 'light mitochondrial' and microsomal fractions represent two different experiments. The 'light mitochondrial' fraction was resuspended in 0.25M-sucrose, centrifuged at 5000g for 10min and a very small sediment rejected, then the washed 'light mitochondrial' fraction was recovered by centrifuging the supernatant at 10000g for 15min. The microsomal fraction was suspended in 0.25M-sucrose, centrifuged at 10000g for 10min and a very small sediment rejected, then the washed microsomal fraction was recovered by centrifuging the supernatant at 36000g for 90min. ○, Inositol 1:2-cyclic phosphate 2-phosphohydrolase; ●, alkaline phosphatase; □, acid phosphatase; △, glucose 6-phosphatase; ▲, 5'-nucleotidase. Recoveries as % of activity or protein in whole homogenate are: inositol cyclic phosphate 2-phosphohydrolase, 109% ('light mitochondrial' fraction), 123% (microsomal fraction); alkaline phosphatase, 122% ('light mitochondrial' fraction), 85% (microsomal fraction); acid phosphatase, 85% (microsomal fraction); protein, 104% ('light mitochondrial' fraction), 88% (microsomal fraction).

Fig. 6. Typical distribution of D-inositol 1:2-cyclic phosphate 2-phosphohydrolase and alkaline phosphatase activities in rat kidney

Specific activities of D-inositol 1:2-cyclic phosphate 2-phosphohydrolase (○) and alkaline phosphatase (●) were measured in slices cut from a frozen segment of kidney. The values are plotted against the distance of the centre of the slice from the outer cortex perimeter. The divisions judged from the gross macroscopic appearance are indicated approximately by the arrows.

Table 1. Ratio of inositol 1:2-cyclic 2-phosphohydrolase/alkaline phosphatase activities on a density-gradient centrifugation of kidney-cortex primary fractions

Washing procedures are described in the legend to Fig. 5. Activities are in mg of P_i released/h per mg of protein.

Fraction subjected to centrifugation	1.1–1.3M-sucrose interface	0.4–0.6M-sucrose interface
Washed microsomal	1.22	0.77
Washed 'light mitochondrial' (Prep. 1)	1.09	2.21
(Prep. 2)	1.50	3.00
(Prep. 4)	0.94	2.35

Comparison of enzymic activities in serial slices of kidney from cortex perimeter to papilla

To confirm the results of the histochemical experiments, serial sections of a segment of rat kidney were cut with a freezing microtome to obtain a sequence of samples from the outer cortex through the inner cortex and the medulla to the papilla. Inner and outer cortical regions in a rat can often be distinguished because the outer is a deep chocolate brown whereas the inner is a lighter brown. Enzymic determinations showed that the inositol 1:2-cyclic phosphate 2-phosphohydrolase activity was largely localized in the outer cortex whereas the alkaline phosphatase activity was present throughout the cortex, even extending a little into the medulla (Fig. 6). Such observations were completely reproducible,

and thus the histochemical observation on the enzyme's gross distribution within the kidney tissue was confirmed.

Discussion

Subcellular fractionation of rat kidney suggests that the inositol 1:2-cyclic phosphate 2-phosphohydrolase follows a distribution pattern that is more similar to that of alkaline phosphatase than to that of any other marker enzyme studied, i.e. 5'-nucleotidase, NADPH-cytochrome *c* reductase, glucose 6-phosphatase, acid phosphatase. In the hamster and rat kidney alkaline phosphatase is greatly enriched in isolated brush-border preparations (Miller & Crane, 1961; Wilfong & Neville, 1970), its specific activity in the rat brush border being

15 times greater than the starting homogenate. Moreover, during the fractionation procedure used to prepare a brush-border preparation from the rat kidney the specific activity at each purification step correlated with the increasing concentration of brush borders when assayed by phase microscopy (Wilfong & Neville, 1970). In addition, histochemical studies (Maengwyn-Davies & Friedenwald, 1950; Molbert *et al.*, 1960), amply confirmed in the present studies, indicate that alkaline phosphatase is highly concentrated in the brush borders of the proximal tubule.

In the rather vigorous homogenization procedure used in the present investigation, it is likely that the large fragments of brush border initially produced would be further disrupted and the microvilli pinched off, yielding vesicles (Thuneberg & Rostgaard, 1968). This would account for the highest specific activity of both inositol 1:2-cyclic phosphate 2-phosphohydrolase and alkaline phosphatase being in the smaller membrane fragments ('light mitochondrial' and microsomal fractions). On examination by electron microscopy of the heavy fragments obtained by density-gradient centrifugation of these fractions there was no clear indication of intact brush borders or microvilli being present even though the vesicles and membrane fragments revealed showed a high specific activity of both alkaline phosphatase and inositol 1:2-cyclic phosphate 2-phosphohydrolase.

The partial separation of 5'-nucleotidase and alkaline phosphatase activities (especially by density-gradient centrifuging of the microsomal fraction; Figs. 3, 4 and 5) was surprising in view of the failure of Taylor *et al.* (1971) to separate these enzymes on zonal centrifugation of a collagenase-treated rat kidney-cortex suspension. The brush border containing alkaline phosphatase is only part of the total plasma membrane of the rat tubule cell, and the fragments of the latter occurring in the microsomal fraction could possibly separate on the basis of different densities. Alternatively, some dissociation of brush-border membrane may have occurred during the fractionation with separation of the enzymes. Aminopeptidase and alkaline phosphatase are located in different parts of the brush-border membrane, but were only separated after treatment with papain and detergents (Thomas & Kinne, 1972).

Although the histochemical experiments were far from perfect technically owing to a tendency for some tissue disruption to occur in the presence of the inositol 1:2-cyclic phosphate substrate and *E. coli* alkaline phosphatase, they were nevertheless indicative of a brush-border localization for inositol 1:2-cyclic phosphate 2-phosphohydrolase. Since the Gomori staining was much heavier in the presence of the added bacterial alkaline phosphatase, it is reasonable to assume that the inositol 1-phosphate liberated by the phosphodiesterase was largely

broken down by the bacterial enzyme rather than by the intrinsic alkaline phosphatase in the kidney tissue itself. If this is so the obvious association of the liberated P_i with the lumen of the proximal tubules is consistent with the primary enzyme in the sequence being in the brush borders. If the deposition of calcium phosphate were merely due to a diffusion of liberated inositol 1-phosphate from elsewhere to the brush border where it is hydrolysed by kidney alkaline phosphatase, one would expect the *E. coli* alkaline phosphatase to make little difference to the degree of staining. In addition it is clear from the macrodeposition of Gomori staining that the inositol 1:2-cyclic phosphate 2-phosphohydrolase has a more limited distribution than the alkaline phosphatase: whereas the former is localized in the outer cortex the latter appeared to be distributed throughout the cortex. This result is fully confirmed by the enzymic assays done on serial sections cut from segments of kidney, which even suggests that alkaline phosphatase activity may extend into the outer medullary regions. By using microdissection techniques Bonting *et al.* (1960) found that in the rat nephron alkaline phosphatase was mainly in the proximal convolutions, although considerable activity was found in the outer medullary zone when this was isolated by macrodissection.

Our observations on the rat kidney stained by the periodic acid-Schiff procedure showed that the glomeruli were confined to the outer cortex extending in from the perimeter to a maximum distance of 1.7 mm. The inner cortex contains both proximal and distal tubules of the nephron, the brush borders of the proximal tubules staining a deeper red than those in the outer cortex, suggesting they are limited to the straight portion or pars recta (Schmidt & Dubach, 1968). Thus, in confirmation of the results of Davison & Conning (1968), it would appear that the rat kidney cortex can be differentiated into two zones comprising an outer zone containing glomeruli and the convoluted portions of the proximal tubules (pars convoluta) and an inner zone consisting of the straight parts. The enzymic results could therefore be explained by assuming that the inositol 1:2-cyclic phosphate 2-phosphohydrolase is confined to the pars convoluta whereas the alkaline phosphatase, although also present in the convoluted portion, extends much further along the tubule into the pars recta and maybe even into the loop of Henle. A number of histochemical studies have indicated that in the rat alkaline phosphatase extends throughout the proximal tubule (Longley & Fisher, 1954; Baumann *et al.*, 1964; Deimling & Noltenius, 1964), which is in contrast with that of the mouse nephron (Hardonk & Koudstaal, 1968). Schmidt & Dubach (1968) by using a microdissection technique have measured the distribution of alkaline phosphatase along the proximal convolution of the rat kidney and have found that even with

this enzyme the distribution and total activity can vary between a short (superficial) and a long (juxta-medullary) nephron. Alkaline phosphatase was found usually to increase along the proximal tubule of both types, although the reverse was true for the superficial nephron of the male rat.

The limited distribution of inositol 1:2-cyclic phosphate 2-phosphohydrolase in the brush borders of the initial part of the proximal tubule may be connected with the distinct cytological segments of the proximal tubule. Maunsbach (1966) has differentiated the rat proximal tubule into at least three such segments by using phase-contrast, fluorescence and electron microscopy. Pronounced differences were apparent in cell size, the frequency of lysosomes, the length of brush-border projections, the mitochondria and the extent to which the proximal cells interdigitated. Further, certain enzymic differences have been reported between the cells in the convoluted and straight portions of the proximal tubule of the rat kidney: glucose 6-phosphatase activity is restricted to the cells of the pars convoluta (Jacobsen *et al.*, 1967) whereas nucleoside diphosphatase activity in the endoplasmic reticulum occurs principally in the terminal part of the proximal tubule (Novikoff *et al.*, 1962). In the mouse kidney D- β -hydroxybutyrate dehydrogenase activity occurs exclusively in the mitochondria associated with the cells lining the pars recta (Walker, 1963).

In the rat the pars recta is probably the site of alkaline phosphodiesterase I, which can in this way be clearly differentiated from inositol 1:2-cyclic phosphate 2-phosphohydrolase (N. Clarke & R. M. C. Dawson, unpublished work). This differing distribution of enzymic activities along the brush borders of the tubule could possibly explain why some limited fractionation of alkaline phosphatase and inositol 1:2-cyclic phosphate 2-phosphohydrolase activities was achieved during density-gradient centrifuging.

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