Bioluminescence Procedures for the Measurement of NAD(P) Dependent Enzyme Catalytic Activities in Submicrogram Quantities of Rabbit and Human Nephron Structures

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Summary: Reduced flavin mononucleotide dependent luciferase (EC 1.14.14.3) from *Photobacterium fischeri* has been used to measure NAD(P) dependent enzymes in submicrogram quantities of tissue homogenates and isolated structures of rabbit and human kidney. The procedure for measuring NAD(P)H was optimized, with internal standardization, to give a linear constant signal between 1 and 100 pmol.

This method was applied to the measurement of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) along the various structures of the rabbit nephron, microdissected from fresh tissue slices. Blank and recovery measurements were performed on each structure, and enzyme catalytic activities were calculated on the basis of tubular length and protein.

Glucose-6-phosphate dehydrogenase was found to be present in all nephron structures with highest catalytic activities in glomeruli, thin limbs of *Henle*'s loop and medullary collecting tubules. Lowest catalytic activities were detected in the pars recta of proximal tubules, the distal convoluted and the connecting tubule.

The mitochondrial 3-hydroxybutyrate dehydrogenase exhibited a different distribution pattern: Highest catalytic activities were found in the cortical ascending limbs of *Henle*'s loop, the proximal and distal convoluted tubule. An unusual internephron heterogeneity for this enzyme was found in the distal convoluted tubule. Catalytic activities in thin limbs of *Henle*'s loop, the medullary ascending limb, and the cortical and medullary collecting tubule were not significantly different from blank activities. The results obtained in isolated nephron segments agreed with those calculated from cortex and medullary homogenates.

In a prelininary experiment on human kidney it could be demonstrated that the procedures can be applied to fresh human biopsy samples.

The bioluminescence method offers several advantages (simplicity, rapidity) in comparison with the classical techniques used for ultramicro analysis of tissue enzymes (enzymatic cycling, radiochemical tests). The results confirm the biochemical heterogeneity of nephron structures and allow conclusions about species dependent functional differences of the pentose-phosphate cycle and renal ketone body metabolism along the nephron.

Biolumineszenzverfahren zur Messung NAD(P)abhängiger Enzymaktivitäten in einzelnen Nephronstrukturen der Kaninchen und Menschen

Zusammenfassung: Eine FMNH abhängige Luciferase aus *Photobacterium fischeri* wurde zur Messung NAD(P)H bildender Enzyme in Gewebshomogenaten und mikrodissezierten Nephronabschnitten eingesetzt. Die benötigte Gewebemenge aus Kaninchen- und menschlicher Niere lag unter einem Mikrogramm Frischgewicht. Die Methode wurde so adaptiert, daß 1–100 pmol NAD(P)H ein lineares und zeitkonstantes Lichtsignal ergaben. Die Kalibrierung erfolgte durch interne Standardisierung.

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Dieses Verfahren diente der Messung der Glucose-6-phosphatdehydrogenase (EC 1.1.1.49) und 3-Hydroxy-butyratdehydrogenase (EC 1.1.1.30) in verschiedenen Abschnitten des Kaninchennephrons. Die Segmente wurden aus frischen Gewebeschnitten disseziert und für jede untersuchte Struktur Leerwerte und Wiederfindungsraten gemessen. Die Enzymaktivitäten wurden auf Tubuluslänge und den Proteingehalt bezogen.

Die Glucose-6-phosphatdehydrogenaseaktivität war in allen untersuchten Nephronabschnitten nachweisbar. Die höchste spezifische Aktivität fand sich in Glomeruli, dünnen absteigenden Abschnitten der Henleschen Schleife und medullären Sammelrohren. Die niedrigsten katalytischen Aktivitäten wurden in der pars recta des proximalen Tubulus sowie im distalen Konvolut und Verbindungsstück gemessen.

Die mitochondriale 3-Hydroxybutyratdehydrogenase wies ein anderes Verteilungsmuster auf: Dieses Enzym zeigte seine höchsten katalytischen Aktivitäten im corticalen aufsteigenden Schenkel der Henleschen Schleife sowie im proximalen und distalen gewundenen Tubulus. Eine ungewöhnlich hohe Streuung zeichnete das distale Konvolut aus. Die katalytischen Aktivitäten in den dünnen Schleifenabschnitten, dem medullären dicken aufsteigenden Schenkel und dem corticalen und medullären Sammelrohr waren nicht signifikant von endogenen Aktivitäten verschieden.

Die in dissezierten Nephronabschnitten erhaltenen Ergebnisse stimmten mit denen aus Homogenaten berechneten überein. In einem vorläufigen Versuch mit menschlicher Niere konnte nachgewiesen werden, daß die beschriebene Methode auch für frische menschliche Biopsieproben anwendbar ist.

Die Biolumineszenzmethodik bietet einige Vorteile (Einfachheit, Schnelligkeit) gegenüber klassischen Verfahren der Ultramikroanalyse von Enzymaktivitäten in Gewebe (enzymatisches Cycling, radiochemische Methoden).

Die Ergebnisse bestätigen die biochemische Heterogenität der verschiedenen Nephronstrukturen und erlauben Rückschlüsse über speziesspezifische funktionelle Unterschiede der Rolle des Pentose-Phosphat-Zyklus und des renalen Ketonkörperstoffwechsels entlang des Nephrons.

Introduction

Studies on organ enzyme catalytic activities in health and disease have been performed to obtain a deeper insight into the pathomechanisms leading to enzyme activity changes in extracellular fluids (1, 2). In earlier studies, homogenates were used as samples and enzyme activities were determined by spectrophotometric techniques. Introduction of microdissection by O. Lowry about 30 years ago together with the use of enzymatic cycling techniques (3) markedly increased our knowledge about enzyme distribution in heterogenous organs like kidney and liver (4, 5). These techniques, however, need special training, air-conditioned laboratories and some special equipment, which limit their general use. The introduction of radiochemical enzyme tests (6, 7) together with microdissection techniques from fresh tissues after collagenase treatment (7, 8) made possible the introduction of ultramicromethods into standard laboratories; these tests could be performed in microliter volumes and detection procedure needed only standard equipment.

Recently bioluminescence techniques have been applied to the measurement of pmol quantities of ATP and NAD(P)H (9). The availability of pure luciferase and cofactors made it possible to measure these coenzymes with nearly unlimited sensitivity. We applied this technique to the measurement of enzymes in single microdissected structures of mammalian nephron. The present report summarizes our experience in standardizing this technique and gives some examples of its application to rabbit and human nephron structures.

Materials and Methods

Microdissection procedure

Tissue preparation and microdissection of tubular segments were performed by procedures similar to those described by Imbert et al. (7) and Vandewalle et al. (8): Rabbit left kidney was perfused in situ through the renal artery with 10 ml of a cold modified Hank's medium containing (in mmol/l): NaCl, 137; KCl, 5; $MgSO_4$, 0.8; Na_2HPO_4, 0.33; KH_2PO_4, 0.44; $MgCl_2$, 1; $CaCl_2$, 1; Tris HCl, 10 (all from Merck, Darmstadt), including 1 g/l each of collagenase (CLS II, Worthington, Freehold, New Jersey) and bovine serum albumin (pure defatted, Serva Heidelberg). After ligation of the renal vein and the ureter another 10 ml were injected under pressure until the renal capsule disrupted. The kidney was cut into pyramids containing cortex and medulla and incubated at 35 °C for 30 minutes in the same collagenase medium bubbled with a stream of oxygen. After incubation the pyramids were rinsed thoroughly in ice-cold microdissection medium which was identical to the perfusion medium, except that collagenase was omitted and $CaCl_2$ reduced to 0.25 mmol/l.

A sample of human kidney obtained after nephrectomy performed for kidney malformation was cut into pyramids and preincubated in collagenase-containing medium as described for rabbit kidney. Nephrectomy was performed 15 minutes after ligature of the renal artery, and the sample analysed proved histologically normal.

Microdissection was performed by hand using fine steel needles in a petri dish containing ice-cold microdissection medium under a stereomicroscope (8). The following segments (abbreviations according to l.c. (7)) were isolated (fig. 1):

glomerulus (GL),

proximal convoluted (PCT) and

straight tubule (PR),

thin descending limb of *Henle*'s loop (TDL),

medullary (MAL) and

cortical portion of the thick ascending limb (CAL),

the distal convoluted tubule (DCT),

the connecting tubule (CNT) and

the cortical (CCT) and

medullary collecting tubule (MCT).

After isolation each tubule segment was transferred with a glass capillary pipette to an inverse microscope to be photographed for measurement of tubular length (8) and then transferred in 5 µl incubation medium to the reaction tube, which was frozen in dry ice and lyophilized overnight at −20 °C.

Protein and tubular length measurement and homogenates

Protein content was measured by a modified *Bradford* procedure described previously (8). Albumin was omitted from the microdissection medium when protein was to be determined and 2−10 structures corresponding to 5−10 mm were pooled in one sample. Protein values were calculated on the basis of bovine serum albumin as standard and related to the number of glomeruli or mm tubule. Tubular length was measured with a curvimeter from projections of film negatives of each tubular structure (8).

For comparison cortical and medullary homogenates were prepared by *Potter* homogenization (Mini-potter, Braun Melsungen, Melsungen) in a 10-fold volume of dissection medium and diluted to give the same protein concentration as the single structure in dissection medium.

Measurement of enzyme catalytic activities

Glucose-6-phosphate dehydrogenase

To the test tube containing the tubule or diluted homogenate in 10 µl dissection medium, 40 µl reaction mixture was added containing (in mmol/l) NADP, 1.875; glucose-6-phosphate, 1.25 in triethanolamine HCl buffer, 50, containing EDTA, 4, pH 8.0. All substrates and coenzymes were obtained from Boehringer, Mannheim. Blanks were incubated under the same conditions omitting glucose-6-phosphate. Incubations were performed for 30 minutes at 25 °C in a thermostat (Eppendorf 3401, Hamburg). Incubation was stopped by 3 min heating at 95 °C, cooling on crushed ice and addition of 50 µl potassium phosphate buffer pH 6.8, 100 mmol/l. Measurement of NADPH was performed on the same day after equilibration of samples at 25 °C.

The spectrophotometric procedure was performed in a total volume of 700 µl, using the same solutions and 10−100 µl of supernatant from a 1:10 homogenate (prepared by 2 min centrifugation at 1000 g).

3-Hydroxybutyrate dehydrogenase

Samples (10 µl) were mixed with 40 µl reaction or blank mixture containing (in mmol/l) D,L-3-hydroxybutyrate, 12.5; NAD, 6.2; (Boehringer, Mannheim) and dithioerythritol, 1.25 (Serva, Heidelberg) in Tris buffer pH 8.2, 50. 3-Hydroxybutyrate was omitted from blank incubations. After 15 min at 25 °C the reaction was stopped by 3 min heating to 95 °C. After cooling the samples on ice 50 µl of potassium phosphate buffer pH 6.8 was added and NADH measured as described below.

Measurement of NADH (NADPH)

Reduced pyridine nucleotides were measured by a modification of the procedure described by *Hastings* (in l.c. (9), pp. 125−134) on a SAI 3000 integrating photometer (Zinsser, Frankfurt) using luciferase (EC 1.14.14.3) and NAD(P)H: FMN oxidoreductase (EC 1.6.8.1) from *Photobacterium fisheri* (Boehringer, Mannheim). The chamber was supplemented with an additional conic mirror which brings the standard reaction tubes into the focus, thereby increasing sensitivity and reducing assay volume (fig. 2). The assay mixture (kept on crushed ice) was prepared freshly in dark glass vials by mixing 7 volumes of 100 mmol/l potassium phosphate pH 6.8 with one volume of each of the following solutions:

1. dithioerythritol 2 mmol/l in H_2O,

2. FMN, 0.05 mmol/l in 100 mmol/l potassium phosphate, pH 6.8 prepared daily, kept dark and diluted 1:10 in phosphate buffer before use.

3. myristinaldehyde, 0.46 mmol/l in H_2O.

Solution 2 and 3 were stored in the dark at 4−6 °C over one week. The stock solution 3 is diluted with two volumes 1 g/l triton X-100 p.a. (Serva, Heidelberg) in 5 g/l albumin in H_2O shortly before use.

4. NAD(P)H: FMN oxidoreductase 2 kU/l (1 g/l) in 400 ml/l glycerol containing 1 mmol/l EDTA, 0.1 mmol/l dithioerythritol and 50 mmol/l phosphate buffer pH 6.8. This dilution was kept at 4 °C−6 °C for up to two weeks.

5. FMNH dependent luciferase: 20 mg lyophilized powder (Boehringer, Mannheim, 476480) was dissolved in 2 ml distilled water and stored in 100 µl aliquots at −35 °C. One cup (for 80 assays) was diluted with 300 µl distilled water shortly before use. From a sample equilibrated to 25 °C in two minutes 40 µl were added to 10 µl distilled water in an Eppendorf cup and reaction started by addition of 60 µl reaction mixture. Integrated luminescence was measured for 30 seconds after 30 seconds delay. The photomultiplier was adapted to the appropriate sensitivity (between 3.8 and 5.5) giving rise to 1000−2500 counts per pmol NAD(P)H in 30 s.

Standardization

To correct for possible effects of buffers and assayed structures on the luminescence yield, each sample was remeasured containing 10 µl of a standard NAD(P)H solution (1 µmol/l) prepared in distilled water and checked spectrophotometrically. The difference between sample and sample + standard was taken as counts per 10 pmol and used for calculation of NAD(P)H content of the sample. Recovery of added NAD(P)H was calculated by analysis of a NAD(P)H solution in microdissection buffer in the absence of tissue.

Calculations and statistical evaluation

All results are given as pmol/min (µU) related to mm tubule length or µg tissue protein. Because normal distribution of results could not be verified with several nephron segments results are presented as median + 25 percentiles. Statistical significance was tested with the signed rank test of *Wilcoxon, Mann & Whitney* (10).
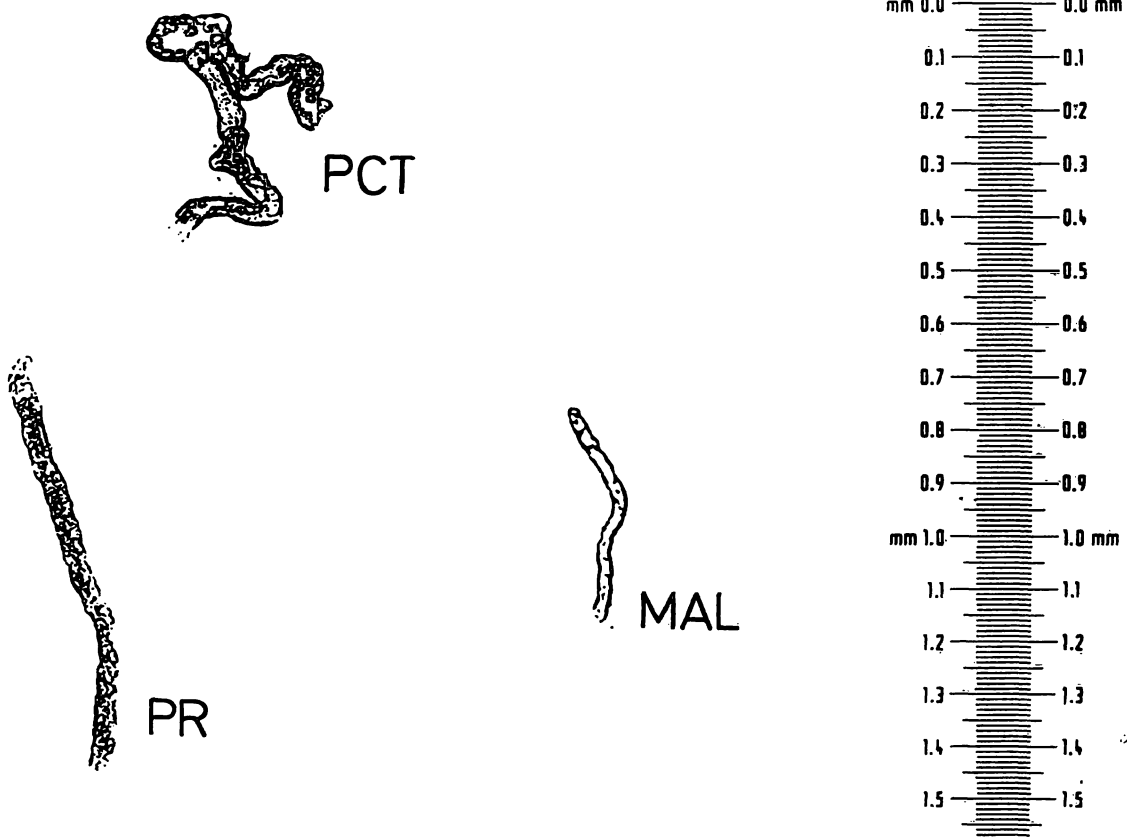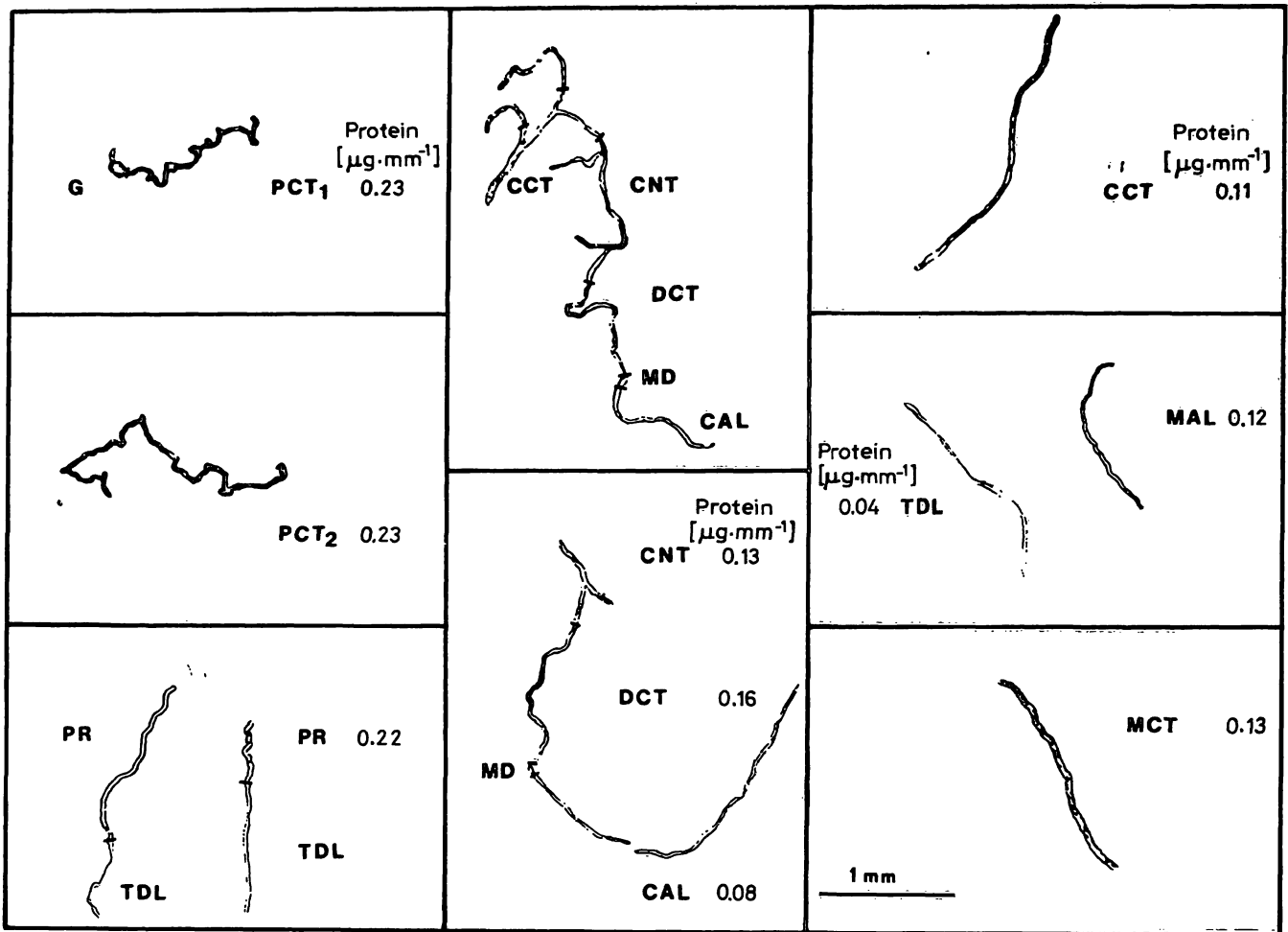
Fig. 1. Isolated nephron structures of rabbit (a) and human (b) nephron. For abbreviations see Methods. Protein content of dissected structures was calculated from the length measurement, using the data of *Vandewalle* et al. (8).

Results

Measurement of NAD(P)H

The analytical sensitivity needed to measure NAD(P) dependent enzyme catalytic activities in single segments of the nephron was obtained by the use of FMNH dependent luciferase from *Photobacterium fisheri*, using myristinaldehyde as cosubstrate. In order to get the sensitivity needed with the lowest amount of enzymes, the test was standardized for this purpose.

Temperature

With the photometer used no temperature stabilization was possible. Therefore samples were equilibrated to 25 °C outside the photometer, transferred, luciferase mixture (at 6 °C—8 °C) added and the reaction terminated after 1 min. This procedure gives a temperature of 20 °C during the reaction period.

Volume

The light signal is markedly dependent on the concentration of various assay components (see below). In order to get high sensitivity with low amounts of enzymes the volume was reduced and an additional mirror positioned in the chamber (fig. 2).
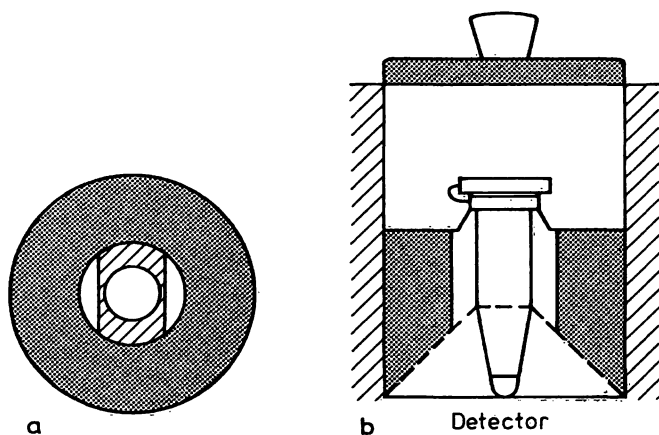

Fig. 2. Sketch of additional mirror for the bioluminescence chamber of the SAI 3000 integrating photometer, kindly made by the mechanical workshops of the Max-Planck-Institute of Biochemistry, Munich.
a. Top view with the center hole for the reaction vessel.
b. Cross section through measuring chamber with the mirror inside.

Figure 3 demonstrates the volume dependence of the light signal when either the amounts or concentrations of reaction components were kept constant. Standard assays were performed with 110 µl of test volume containing 60 µl of the luciferase mixture (see Methods).
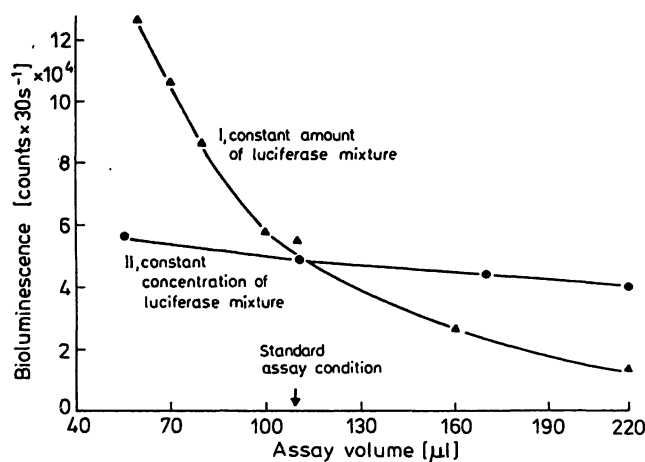

Fig. 3. Dependence of light emission upon assay volume containing 20 pmol NADPH.
Curve I: volume changed by addition of buffer (50 µl of luciferase mixture present) (see Methods).
Curve II: volume changed by addition of different amounts of standard luciferase mixture (see methods).

Concentrations of enzymes and cofactors

As can be seen from figure 4 the light signal emitted was largely dependent on the concentration of assay constituents. It increased linearly with myristinaldehyde, luciferase and FMN-reductase concentration over the whole range tested and reached a plateau with regard to dithioerythritol. FMN exhibited a striking maximum at 2—3 µmol/l with higher concentrations interfering with optimal light emission. In the final assay mixture the concentrations indicated by arrows (fig. 5) were used (see Methods).

pH and salt concentration

In accordance with the experience of others (9) a pH of 6.8 was found to be optimal for measuring light emission under the present conditions. Buffers and neutral salts led to an additional quenching of bioluminescence. Therefore salt and buffer concentrations in the enzyme tests and assay constituents were kept as low as possible and pH was brought to 6.8—7.0 before measurement of NAD(P)H by addition of phosphate buffer pH 6.8 (see Methods).

Internal standardisation and recovery

With the procedure thus standardized a linear signal was obtained with standard solutions of NADH and NAD(P)H from 1—100 pmol/sample. Intraassay variation was below 10%. In spite of this acceptable
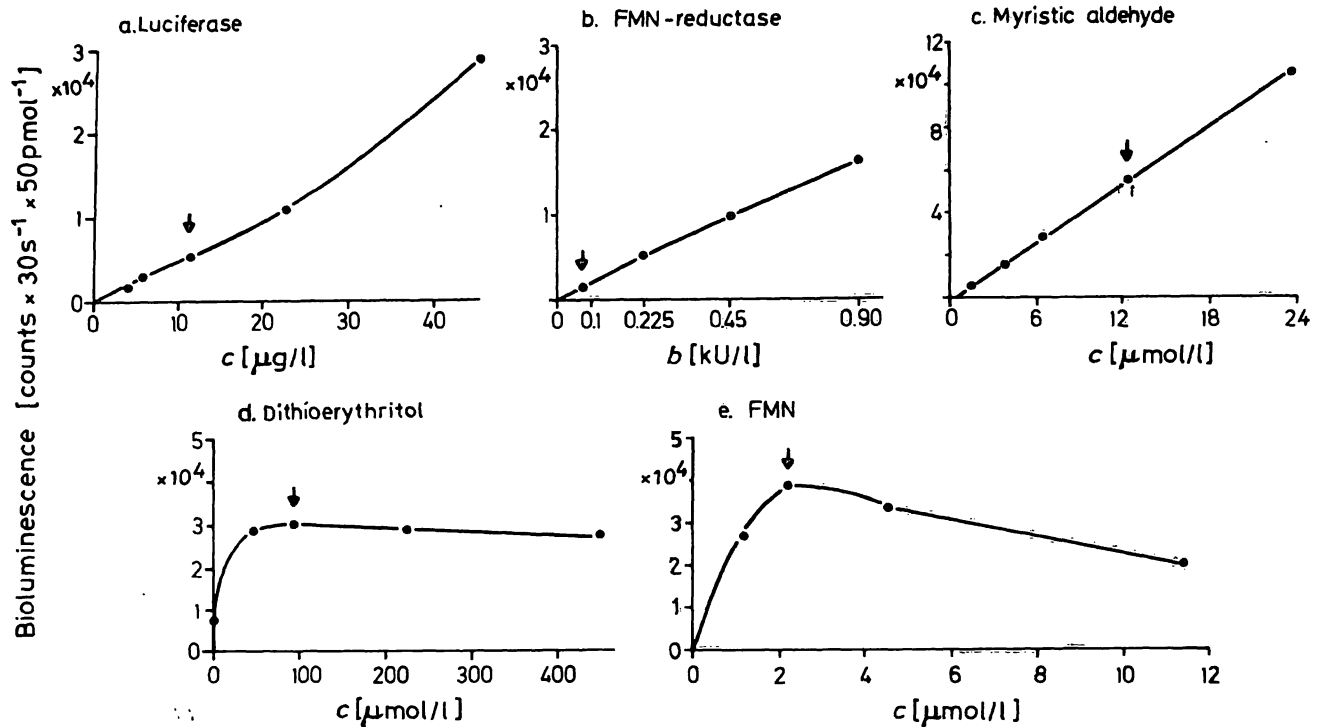
Fig. 4. Dependence of light emission on the catalytic concentration (b) of enzymes and on the concentration (c) of cofactors. 50 pmol NADH in 50 μl of the 3-hydroxybutyrate dehydrogenase assay mixture were mixed with luciferase mixture, but the substance added in 5 μl was varied to result in the final concentrations indicated. Concentration of all other constituents was as indicated by arrows, which represent the final assay condition. Final volume was 110 μl.
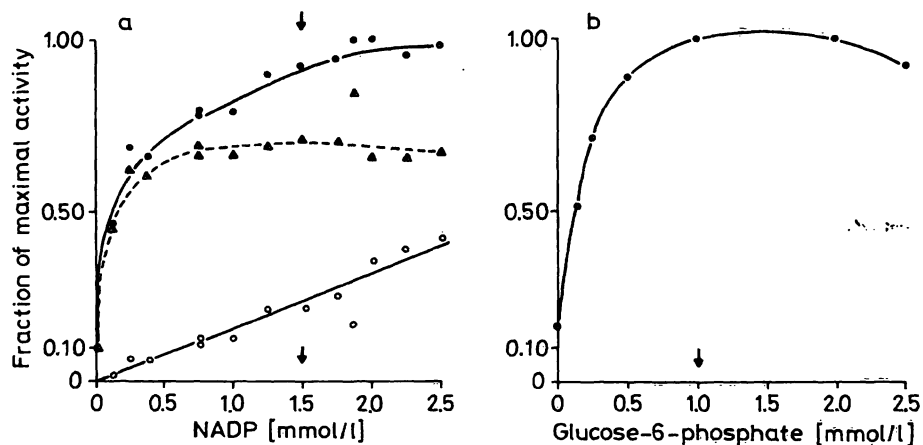

Fig. 5. Dependence of glucose-6-phosphate dehydrogenase upon NADP and glucose-6-phosphate concentration. 1:10 w/v homogenate of rabbit kidney cortex was diluted 1:200 in assay buffer (see Methods), and glucose-6-phosphate dehydrogenase was assayed by incubation with the indicated NADP (a) and glucose-6-phosphate (b) concentrations over 15 minutes at 37 °C. The NADPH signal was measured in the absence (O) and presence (⊙) of 1 mmol/l glucose-6-phosphate (a). Arrows indicate the concentrations used in the standard procedure (see methods). The dotted line in a was obtained by subtraction of endogenous activity (O) from glucose-6-phosphate dependent activity (⊙).

reproducibility the standard curve was not used for calculation of NADH, because interfering factors derived from the enzyme assay mixture and the tissue samples could not be excluded. Therefore all calculations were performed on the basis of an internal standard added to one half of the split sample (see Methods). In addition standards were added to

samples before and after incubation of the enzyme assays to exclude loss of NAD(P)H during incubation. Recovery of added standards was measured by comparison with tissue free samples and was between 79 and 106% with NADPH (see tab. 1) and between 72 and 104% with NADH.(see tab. 2). No correction was done for recovery.'

Tab. 1. Catalytic activities of glucose-6-phosphate dehydrogenase in microdissected rabbit nephron structures and homogenates from renal cortex and medulla.

A. Dissected structures	Glucose-6-phosphate dehydrogenase		n	Recovery of added NADPH	n
	(pmol/min × mm)	(μmol/min × g protein		(%)	
Glomeruli	1.05*	12.65	23	105	17
PCT	1.23	5.39	13	100	13
PR	0.64	2.93	15	111.5	15
TDL	0.48	12.97	20	98.6	12
MAL	0.60	4.8	20	106	11
CAL	0.34	4.3	20	89.2	10
DCT	0.47	2.95	22	104.9	14
CNT	0.36	2.85	20	98.5	10
CCT	0.51	4.67	19	93.1	8
MCT	1.15	9.05	20	94.9	14

B. Homogenates	Luciferase method	Spectrophotometric method
	μmol/min × g protein (n)	
Cortex	2.5 (12)	3.1 (8)
Medulla	3.77 (12)	7.1 (8)

* per glomerulus

Tab. 2. Catalytic activities of 3-hydroxybutyrate dehydrogenase in microdissected segments of rabbit nephron and homogenates from rabbit renal cortex and medulla.

A. Dissected structures	3-Hydroxybutyrate dehydrogenase		n	Recovery of added NADPH	n
	(pmol/min × mm)	(μmol/min × g protein		(%)	
PCT	2.37	10.4	42	96.1	14
PR	1.9	8.72	17	105	10
TDL	n.s.	n.s.	6	110	3
MAL	n.s.	n.s.	9	101	7
CAL	1.51	19.1	19	104	9
DCT	(1.88)*	(11.8)*	13	71.6	8
CNT	1.45	11.5	19	77.1	12
CCT	n.s.	n.s.	19	95.8	7
MCT	n.s.	n.s.	14	99.4	10

B. Homogenates

Cortex	8.3	(5)
Medulla	6.4	(5)

n.s.: activity not significantly different from blank.
* : large internephron heterogeneity.

Measurement of glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase catalytic activity measured with the luciferase procedure in diluted rabbit kidney homogenates showed the substrate and coenzyme dependence given in figure 5. In presence of homogenate there was a NADP-dependent rise in luminescence when no glucose-6-phoshate was present. In contrast to the substrate dependent activity this increase exhibited no saturation kinetics (fig. 5a). Therefore all subsequent enzyme assays were performed in the absence and presence of glucose-6-phosphate. Saturation was reached with 1 mmol/l of glucose-6-phosphate and NADP (fig. 2b). Addition of 6-phospho-gluconate dehydrogenase to increase sensitivity did not increase the glucose-6-phosphate dependent signal of tissue activities, but by itself increased the blank, probably due to its contamination with traces of glucose-6-phosphate dehydrogenase (not shown).

Using the final assay procedure enzyme catalytic activity could be measured in 0.1—1.0 μg tissue protein. As can be seen from figure 6, catalytic activity was dependent on tubule length and time of incuba-
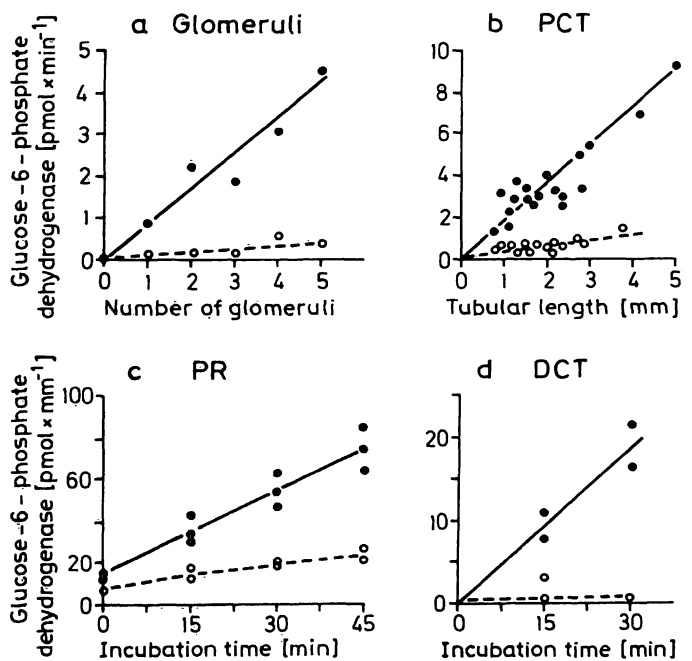
Fig. 6. Tissue and time dependence of glucose-6-phosphate dehydrogenase activity in microdissected structures of rabbit nephron. Glomeruli (a), PCT (b), PR (c) and DCT (d) structures were dissected, pooled and incubated as described in the methods section. (O) Activity in the absence (●) activity in the presence of glucose-6-phosphate.

tion for various nephron structures tested. Figure 7 summarizes catalytic activities found in various rabbit nephron structures, related to tubular length. Relatively high activities were found in glomeruli, proximal convoluted and medullary collecting tubules. When activities in the absence of glucose-6-phosphate were subtracted and the difference related to protein, the results summarized in table 1 were obtained. Due to its low protein content, thin descending limbs of *Henle*'s loop exhibited the highest activities. The higher activities obtained from medullary structures are also reflected in the enzyme activities obtained from cortical and medullary homogenates, performed on the same kidneys (tab. 1). The difference between cortex and medulla was highly significant ($2\alpha < 0.01$) and even more prominent when the enzyme was measured spectrophotometrically with the same assay mixture (tab. 1). The spectrophotometric results were in the same range in cortical but significantly higher in medullary homogenates when compared to the bioluminescence procedure, but agreed well with the results in microdissected nephron structures.
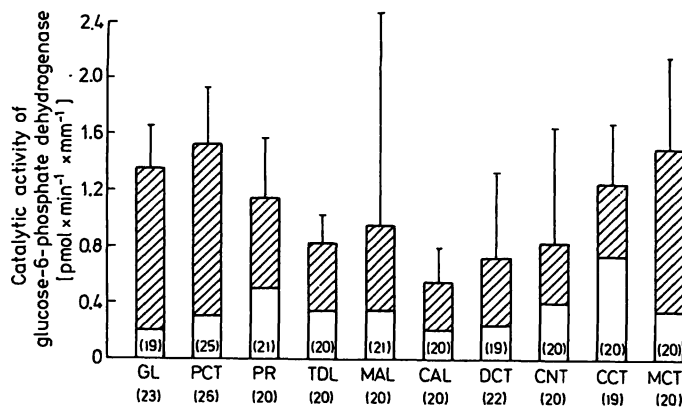

Fig. 7.   Catalytic activity of glucose-6-phosphate dehydrogenase in various rabbit nephron structures. NADPH formation was measured over 15 minutes in the absence (open bars) and presence (total bars) of 1 mmol/l glucose-6-phosphate and the activity related to tubular length and one glomerulus, respectively. Bars give the medians and 25 percentiles for total activities with the numbers of structures tested in parenthesis. For abbreviation of structures see Methods.

Measurement of 3-hydroxybutyrate dehydrogenase

The mitochondrial 3-hydroxybutyrate dehydrogenase has not yet been quantified in nephron structures. When optimal test conditions were evaluated in rabbit kidney cortex homogenates, the luciferase assay proved to have similar characteristics to the spectrophotometric assay (fig. 7, (11)). Its activity was stimulated by dithioerythritol with an optimum at 1 mmol/l (fig. 8a). NAD and substrate saturation was reached at 3 mmol/l (fig. 8b, c). When assayed

in 50 mmol/l tris buffer, enzyme activity exhibited a pH optimum between 8.0 and 8.5 (fig. 8d). In contrast to spectrophotometric procedures blanks omitting 3-hydroxybutyrate were dependent on NAD-concentration (fig. 8b), dithioerythritol (fig. 8a) and pH (fig. 8d) and had to be subtracted. Under otherwise optimal conditions the enzyme was measured at different temperatures (not shown). 30 °C resulted in twice the activity of 25 °C, 37 °C giving no further increase. Recovery of NADH added to the incubation was better at 25 °C. Therefore this temperature was chosen as standard for 3-hydroxybutyrate dehydrogenase.

When this enzyme was tested in isolated nephron segments, freezing and thawing, as used in previous studies (8), turned out not to be sufficient to give optimal enzyme release. Lyophilization at −20 °C was found to be optimal and therefore chosen as standard procedure. Under these conditions, enzyme activity was linear with time for up to 60 min and tubular length up to 5 mm, corresponding to 1.1 µg protein (fig. 9). The results obtained in various nephron structures from rabbit nephron are summarized in figure 10. In contrast to glucose-6-phosphate dehydrogenase and studies on 3-hydroxybutyrate dehydrogenase in mouse nephron (12), endogenous NADH formation obscured any significant catalytic activities in TDL, MAL, and collecting tubule structures. In addition large internephron heterogeneity made differences between endogenous and substrate mediated NADH formation insignificant in distal convoluted tubules. When the differences between endogenous and substrate mediated activities were related to tubular protein (tab. 2) the highest catalytic activities were found in cortical ascending limb and proximal convoluted tubules. Moreover the activities in diluted homogenates were in the same range as those calculated from the dissected segments (tab. 2).

Determination of 3-hydroxybutyrate dehydrogenase in human nephron samples

In the course of our studies on the distribution of 3-hydroxybutyrate dehydrogenase we had the opportunity to study a human kidney, obtained at nephrectomy for malformation, which histologically turned out to be normal (fig. 1). The preliminary results are given in table 3. Due to the lack of protein values for human kidney and the small numbers of samples the data can only show that the procedure described can be applied to human tissue and that activities of 3-hydroxybutyrate dehydrogenase are in the same range as measured in rodent and dog kidneys (12, 13). In addition the enzyme was measured in human glomeruli (tab. 3).
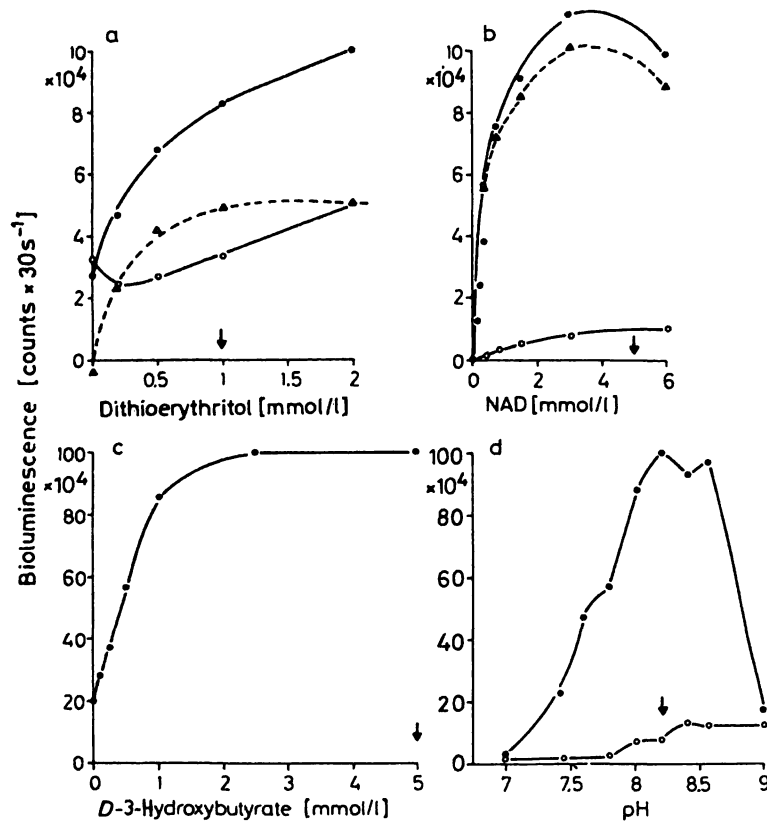
Fig. 8. Assay conditions of 3-hydroxyburyrate dehydrogenase in rabbit kidney cortex homogenates. 3-Hydroxybutyrate dehydrogenase was assayed as described in the methods section in the absence (O) and presence (⊙) of 5 mmol/l D-3-hydroxybutyrate. a. Dithioerythritol, b. NAD, c. 3-hydroxybutyrate, d. pH-dependence of enzyme activity. Arrows indicate the conditions used in the standard procedure (see methods).
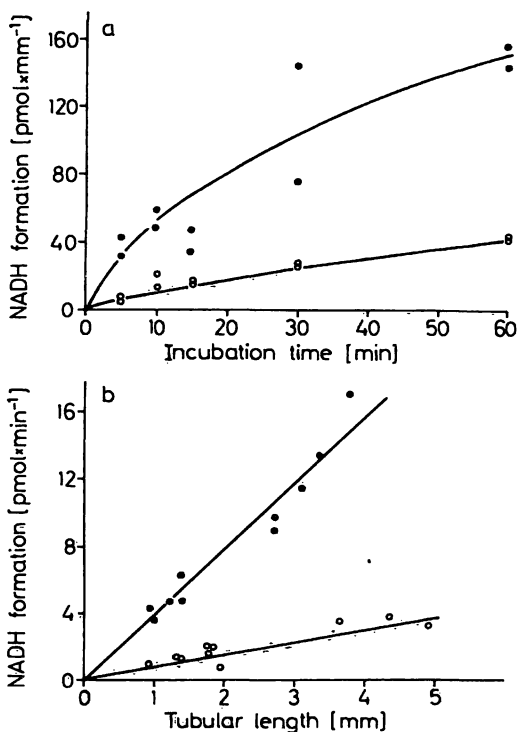

Fig. 9. Time (a) and tubular length (b) dependence of 3-hydroxy-butyrate dehydrogenase in rabbit proximal convoluted tubules. 3-Hydroxybutyrate dehydrogenase was measured in pooled microdissected segments of proximal convoluted tubule of rabbit nephron as described in the methods section. Rates of NADH formation in the absence (O) and in the presence (●) of 10 mmol/l D,L-3-hydroxybutyrate.

Tab. 3. Catalytic activities of 3-hydroxybutyrate dehydrogenase in human nephron structures.

A. Dissected structures	3-Hy-droxy-butyrate present	3 Hydroxybutyrate dehydrogenase	
		(pmol/ 15 min)	(pmol/mm × min) (pmol/glomerulus × min)
1. 1 Gl	−	3.42	0.216
2. 1 Gl	+	17.4	1.16
3. 3 Gl	+	50.2	1.11
4. PCT	−	9.95	2.01
5. PCT	+	83.9	7.55
6. PR	−	5.14	0.56
7. PR	+	18.3	0.68
8. TDL	+	0	0
9. CAL	+	14.6	2.44
10. CNT	+	0	0

B. Homogenates		μmol/min × g protein
Cortex	−	4.86/ 3.14
	+	13.67/13.06
Outer medulla	−	4.71/ 3.06
	+	15.46/12.16
Papilla	−	2.96/ 4.29
	+	3.49/ 8.9

Discussion

Applicability of bioluminescence procedures

The determination of enzyme catalytic activities in submicrogram quantities of tissue has previously been performed with ultramicro procedures using enzymatic cycling of NAD(P)H in combination with fluorimetry (3, 14) or radiochemical procedures (5—7). The present study shows that the same sensitivity can be obtained with bioluminescence assays. The availability of pure enzymes and cofactors gives a sufficiently stable light signal to measure NADH and NAD(P)H in the picomolar range. These procedures have several advantages over previously used methods. They can be performed in usual reaction vessels and with standard laboratory equipment. Reaction volume can be 20—100 μl thus avoiding the need for oil wells and antistatic treatment of samples (3). However, as in cycling procedures, internal standardization is necessary, because buffers, sample and other assay constituents largely modify the light signal of NAD(P)H. One drawback is that the luciferase from Photobacterium fisheri can not differentiate between NADH and NAD(P)H (9). In addition various blanks are needed to correct for impurities of reagents and endogenous NAD(P)H forming enzyme activities in samples. In spite of these difficulties, enzyme catalytic activities of samples as small as 50—100 ng tissue protein can be measured with sufficient precision. Reproducibility may be further increased by the use of thermoconstant chambers.

Glucose-6-phosphate dehydrogenase

In the present study glucose-6-phosphate dehydrogenase has been mapped along the rabbit nephron for the first time. This enzyme was speculated to be of physiological significance in renal drug metabolism (15), tubuloglomerular feedback (16) and renal acidification (17).

The distribution of enzyme activity along the rabbit nephron differs in several respects from that found in rat nephron by Schmidt (14) and in human nephron by Dubach (18). In both species catalytic activities of the distal convoluted tubule were as high as that of the glomeruli and higher than that of proximal tubules in rat studies (see l.c. (14) for references). The lower activity of glucose-6-phosphate dehydrogenase in the pars recta compared to the convoluted portion of the proximal tubule (tab. 1) contrasts with previous findings in the rat nephron. This species difference points to the possible role of glucose-6-phos-
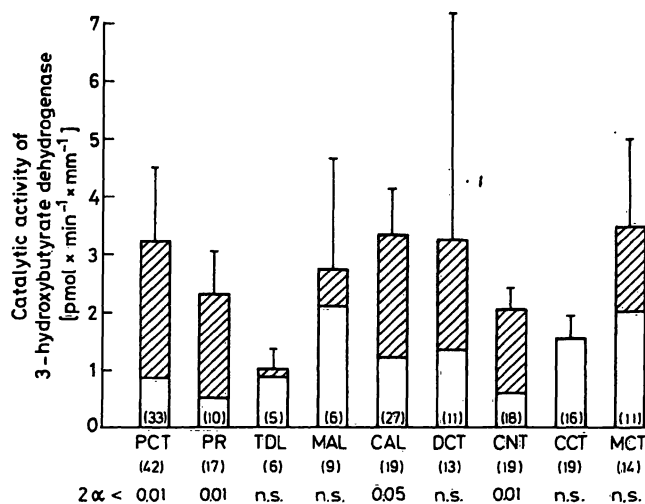

Fig. 10. Distribution of 3-hydroxybutyrate dehydrogenase along the rabbit nephron.
This was measured in the absence (open bars) and presence (total bars) of 10 mmol/l D,L-3-hydroxybutyrate and activities related to mm tubular length. Results are medians and upper 25 percentiles of total catalytic activities with the number of segments tested in parentheses. For abbreviation of structures see Methods. Significance of difference (hatched area) was tested with the signed rank test of Wilcoxon, Mann & Whitney (10) NS = not significant.

phate dehydrogenase in the proximal tubule, since cytochrome P_{450} exhibits the same behaviour (19, 20). This system was found to be highest in the second part of the proximal convoluted tubule in rabbits and in the pars recta of the rat nephron (20). Since the end proximal convoluted and straight tubule is the only site of renal mixed function oxidation (20) and contains high amounts of enzymes of peroxide metabolism (4) and millimolar concentrations of glutathione (4), glucose-6-phosphate dehydrogenase may function to provide NADPH for the reduction of glutathione. The high activity in glomeruli was also found in rat (14) and human nephron (18) and may be ascribed to the presence of phagocytic mesangial cells in this structure (21). In the distal nephron the enzyme may provide pentoses for nucleotide synthesis, since the distal nephron has a high RNA turnover (Vandewalle, personal communication) and may be a site of adenosine release (22), which has been thought to mediate tubuloglomerular feedback. Norgaard (21) found a higher activity in the macula densa region compared to the remainder the distal tubule. The possible involvement of the pentose phosphate shunt in distal hydrogen secretion (17) remains speculative.

When the distribution of glucose-6-phosphate dehydrogenase is compared with that of hexokinase (8) it becomes evident that glucose may be the main pre-

cursor of glucose-6-phosphate in the distal nephron, whereas gluconeogenic substrates are most likely to provide this metabolite in the proximal tubule. This is confirmed by the findings of *Klein* et al. (23) that distal but not proximal tubules form relatively more $^{14}CO_2$ from [1-^{14}C]glucose as compared to [6-^{14}C]glucose.

3-Hydroxybutyrate dehydrogenase

In contrast to glucose-6-phosphate dehydrogenase, 3-hydroxybutyrate dehydrogenase has to our knowledge not been localized in mammalian nephron structures. Observations with qualitative enzyme histochemistry in rat kidney demonstrated high catalytic activities of this enzyme in ascending limbs of *Henle*'s loop (24). This mitochondrial enzyme is present in relatively low activities in kidney compared with liver tissue (25). In the rabbit kidney the enzyme was found in the cortical part of the thick ascending limb of *Henle*'s loop, the proximal tubule and the distal tubule. With regard to the distribution of this enzyme along the proximal tubule, 3-hydroxybutyrate dehydrogenase follows the distribution pattern of mitochondria, providing the data given for rats apply for the rabbit (26, 27). Interestingly, this is different in the mouse nephron where a several fold higher catalytic activity was found in the pars recta of the proximal tubule (12). In accordance with the lower mitochondrial density, 3-hydroxybutyrate dehydrogenase decreases towards the medullary structures. With the present method no significant activity could be detected in the thin limbs of *Henle*'s loop and the collecting tubule. The high activity found in medullary collecting tubules in the absence of 3-hydroxybutyrate is probably due to endogenous substrates (probably glycogen) which may lead to NADH formation during in vitro incubation of the tubule.

The presence of 3-hydroxybutyrate dehydrogenase in proximal and distal nephron structures provides evidence that these segments can use 3-hydroxybutyrate as metabolic fuel. In fact, rabbit tubules in vitro metabolize 3-hydroxybutyrate to acetoacetate (28). Interestingly the rabbit in contrast to the mouse (12) and rat (29) kidney lacks the enzyme needed to further metabolize acetoacetate, i.e. 3-oxoacid transferase. This confirms the in vitro finding that 3-hydroxybutyrate metabolized by rabbit tubules is quantitatively released as acetoacetate (28).

Although preliminary, the results obtained from human kidney provide evidence that 3-hydroxybutyrate dehydrogenase is present in cortical and outer medullary structures of human nephron with activities similar to those in rat (13), dog (13) and rabbit kidney. The activity measured in human glomeruli, however, must be interpreted with caution since in this study glomeruli were not washed free from blood. However, similar activities were detected in mouse kidney glomeruli (12).

In summary, bioluminescence procedures can be applied to analyze NAD(P) dependent enzyme activities in minute amounts of tissue. The procedures are sensitive enough to perform enzyme assays from biopsy specimen homogenates or microdissected single nephron segments. These techniques may help to increase our understanding of the biochemical architectures of complex tissues and may stimulate studies on the changes of tissue enzymes in human diseases.

Acknowledgements

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