The Turnover Rate of Rabbit Urinary Tamm–Horsfall Glycoprotein

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1. The turnover rate of urinary Tamm–Horsfall glycoprotein in rabbits was determined by two different methods. The first involved measurement of the pool size of the glycoprotein in rabbit kidney and the daily urinary excretion rate by a radioimmunoassay from which the turnover rate was calculated. 2. The second method made use of the incorporation *in vivo* of $\text{Na}_2^{14}\text{CO}_3$ and sodium $[^{14}\text{C}]\text{acetate}$. After a single intramuscular injection of one of these compounds, urine collections were made every 24 h and the glycoprotein was isolated and its specific radioactivity was determined. 3. Incorporation of the label into urinary HCO_3^- , urea and plasma fibrinogen was also examined. The specific radioactivities of the *O*-acetyl, sialic acid, aspartic acid and glutamic acid residues isolated from the Tamm–Horsfall glycoprotein were compared and their half-lives were compared with that of the intact glycoprotein. The two methods gave results in quite close agreement and indicated a half-life for the glycoprotein of approx. 9 h. 4. An attempt was made to localize the glycoprotein within the kidney and within the cell. It is present throughout the kidney, but was not detected in the brush-border fraction isolated from the proximal tubules. From differential cell-centrifugation studies, the glycoprotein seemed to be predominantly present in the soluble fraction (100 000g supernatant). This suggests that it is either largely a soluble cytoplasmic component or is very loosely bound to a membrane, being readily released under the gentlest homogenization procedure. 5. The half-life of Tamm–Horsfall glycoprotein in human kidney was found by the radioimmunoassay method to be approx. 16 h. The similarity between the composition of Tamm–Horsfall glycoprotein and human erythropoietin is discussed.

T–H glycoprotein (Tamm–Horsfall glycoprotein) has been shown to occur normally in the urine of several species including man (Tamm & Horsfall, 1950) and rabbits (Cornelius *et al.*, 1965). It is almost certainly synthesized in the renal tubules (Cornelius *et al.*, 1965; Keutel, 1965; McKenzie & McQueen, 1969). A radioimmunoassay was developed for the measurement of very small amounts (approx. 0.15–5 μg) of T–H glycoprotein from humans and rabbits (Grant & Neuberger, 1973). Investigation of the excretion rate in certain pathological conditions showed that T–H glycoprotein excretion per functioning nephron unit in humans was elevated in two disorders of tubular function, cadmium nephropathy and Lignac–Fanconi cystinosis (Grant *et al.*, 1973). We therefore decided to investigate the rate of turnover of T–H glycoprotein to see if it turned over independently of the renewal of the cell. The life-span of the proximal and distal tubular cells of mouse kidney has been reported as 190 days (Cameron, 1971).

In the present paper the radioimmunoassay was applied to the measurement of both the pool size of T–H glycoprotein in rabbit kidney and the daily excretion rate. A rough estimate could be made of the rate of turnover of T–H glycoprotein in this way. An

alternative approach was also used. Rabbits were injected with $\text{Na}_2^{14}\text{CO}_3$ and sodium $[^{14}\text{C}]\text{acetate}$ and the fall in specific radioactivity of the excreted glycoprotein was measured. In addition, the specific radioactivities of two of its constituent amino acids, as well as sialic acid and *O*-acetyl residues were examined. In this way an independent estimate of the half-life of the excreted glycoprotein could be made.

Experimental

Materials

Sephadex G-200 was obtained from Pharmacia (G.B.) Ltd., London W5, U.K.; Triton X-100 (purified), 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; *N*-acetylneuraminic acid and collagenase (Type I) were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; sodium dodecyl sulphate was obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.; crystallized bovine serum albumin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; Nembutal was obtained from Abbott Laboratories Ltd., Queenborough, Kent, U.K.; and

neuraminidase was obtained from Behringwerke A.-G., Marburg/Lahn, Germany.

Methods

Human T-H glycoprotein was isolated as described by Fletcher *et al.* (1970a). Rabbit T-H glycoprotein was isolated and antisera were raised against these two glycoproteins as described by Marr *et al.* (1971). The radioimmunoassay for their estimation was performed as described by Grant & Neuberger (1973).

Total protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a reference standard.

New Zealand White rabbits were used for the radioisotope experiments.

Isotopes. The $\text{Na}_2^{14}\text{CO}_3$, sodium [^{14}C]acetate and ^{125}I were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

^{14}C -labelled samples were counted for radioactivity in a 1:2 (v/v) mixture of Triton X-100 and a toluene-based scintillation fluid [2,5-diphenyloxazole (5g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.2g) in 1 litre of toluene] in a Nuclear-Chicago mark 1 liquid-scintillation counter. Where necessary, samples were corrected for quenching by the external standard channels-ratio method. The efficiency of the counter was 81%. ^{125}I -labelled samples were counted for radioactivity in a Packard auto-gamma counter (model 578).

Urinary $\text{H}^{14}\text{CO}_3^-$. The radioactivity was released from urinary HCO_3^- by acidifying 1 ml of urine and trapping the $^{14}\text{CO}_2$ released on filter paper soaked with 0.1M-NaOH (Tait, 1970).

Urinary ^{14}C -labelled urea. Urinary urea was measured by the xanthidrol method of Lee & Widdowson (1937) and counted for radioactivity by filtering the precipitate on to a Millipore filter (0.65 μm), which was dried and counted for radioactivity in 10 ml of toluene-Triton X-100 (2:1, v/v) scintillation fluid.

Plasma ^{14}C -labelled fibrinogen. This was isolated by the method of Cohen *et al.* (1956) with the use of thrombin (Parke, Davis and Co. Ltd., Detroit, Mich., U.S.A.). The fibrinogen concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard and the radioactivity of the aqueous samples was determined as described above.

O-[^{14}C]Acetate. In preliminary experiments the method of Ludowieg & Dorfman (1960) involving liberation of the O-acetyl groups by alkaline hydroxylamine was used. After neutralization with acetic acid and subsequent dialysis, the acetyl hydroxamate could be determined but not easily counted for radioactivity, owing to high salt concentration. Decreasing the amount of alkaline hydroxylamine used gave a decreased yield and desalting the diffusate on a mixed-bed resin was not quantitative because of the minute

amounts being handled. A satisfactory alternative method involved liberation of the O-acetyl groups as the volatile methyl acetate (Whistler & Jeanes, 1943.) This was measured quantitatively by the Ludowieg & Dorfman (1960) method and counted for radioactivity in the toluene-Triton X-100 (2:1, v/v) scintillation fluid described above.

N-[^{14}C]Acetylneuraminic acid. The deacetylated T-H glycoprotein fractions were treated with neuraminidase overnight at 37°C. After dialysis the diffusate was freeze-dried and the N-acetylneuraminic acid content was determined by the procedure of Warren (1959). The fractions were then counted for radioactivity as above.

[^{14}C]Aspartic acid and [^{14}C]glutamic acid. A sample (3.8 mg or more) of each of four T-H glycoprotein fractions was hydrolysed in 1.2 ml of constant-boiling HCl at 110°C in a sealed evacuated ampoule for 24 h. The acid was removed on a rotary evaporator in the presence of NaOH pellets and the neutral hydrolysate was dissolved in 1.0 ml of water. This was then chromatographed on a column (8.6 cm $\times$ 1 cm) of Dowex-1 (X2; acetate form). All the amino acids were washed off with water, with the exception of glutamic acid and aspartic acid, which were then sequentially eluted with 0.5M-acetic acid (Hirs *et al.*, 1954). After freeze-drying, the samples were each quantitatively determined on the 23 cm column of a Locarte Mini amino-acid analyser and shown to be free of any other amino acids. They were then counted for radioactivity as above.

Results

Determination of the concentration of T-H glycoprotein in kidney tissue

Preliminary studies were carried out to try to establish a partial separation of T-H glycoprotein from the rest of the protein in a total kidney homogenate, to permit its subsequent assay. Rabbit T-H glycoprotein labelled with ^{125}I was added to the rabbit kidney homogenate and the distribution in the various fractions was examined. Of all the protein precipitants used, none was successful in precipitating protein without T-H glycoprotein. Similarly, dialysis of the homogenate caused precipitation of approx. 60% of the total protein and co-precipitation of approx. 54% of the T-H glycoprotein. A poor recovery (49%) was obtained in the peak eluted at the void volume of Sephadex G-200. Good recoveries of T-H glycoprotein from Sephadex G-200 were obtained when urea or sodium dodecyl sulphate were included in the eluting buffer.

These methods were abandoned when it was found that the whole homogenate could be assayed without a preliminary fractionation, with interference from non-specific protein only at concentrations

greater than approx. 100 µg/ml (Grant & Neuberger, 1973). The problem then became one of solubilizing the maximum amount of kidney tissue without degrading or denaturing the T-H glycoprotein.

Methods of solubilizing kidney tissue

Inclusion of 0.5% sodium dodecyl sulphate or 0.375% collagenase (pH 7.0, 4.5 h, 22°C) in the homogenization procedure were both unsatisfactory. Degradation of T-H glycoprotein in the latter procedure could possibly have been due to non-specific proteinases and peptidases known to be present in the collagenase preparation. These methods were discontinued.

The following procedure was found to give the best recoveries for T-H glycoprotein. The same species of rabbit was used as that from which urinary T-H glycoprotein had been isolated (New Zealand White). The rabbit (no. 16) was killed by an intravenous injection of Nembutal (5 ml) and the kidneys were excised and stored at -25°C. When required, the kidney was thawed overnight at 4°C, decapsulated and weighed (9.64 g). Three segments were removed, a cortical (2.21 g), medullary and pelvic (1.58 g) and whole kidney cross-section (2.07 g). Each segment was cut into small pieces with scissors and homogenized in a glass tube with a motor-driven glass pestle (fraction 1). Each homogenate was sieved through a 100-mesh sieve and the residue rehomogenized two or three times and refiltered until there was scarcely any residue remaining. All the filtrates and washings for each segment were combined (fraction 2) and sonicated in 7 ml portions in an MSE 50 W ultrasonic disintegrator operated at maximum output for 2 min in ice (fraction 3). Samples were removed at each stage for measurement of T-H glycoprotein and total protein content (Table 1). It was shown in subsequent

experiments that deep-freezing the intact kidney did not alter the apparent T-H glycoprotein concentration, although freezing the homogenate gave artificially high values. Slow freezing at -25°C affects the assay of urines in a similar way (Grant & Neuberger, 1973). Assay of the kidney homogenate on the day of preparation was essential, otherwise artificially high values were obtained.

Effect of sonication on T-H glycoprotein

To show that sonication of the kidney homogenate did not affect the behaviour of the T-H glycoprotein in the assay, 4 ml of an aqueous sample of pure rabbit T-H glycoprotein (1 mg/ml) was sonicated for five consecutive 2 min periods with 2 min intervals to permit cooling. A sample (20 µl) was removed at the beginning (control) and then after each subsequent sonication period. A final 12 min continuous sonication was performed. Radioimmunoassay of these samples showed that sonication for 2 min periods up to 10 min did not alter the apparent concentration of the T-H glycoprotein, within the limits of experimental error. The final 12 min sonication, however, did alter the behaviour of the glycoprotein, and gave lower results than the rest. A 2-4 min sonication period was used in subsequent experiments on the kidney homogenate. The results (Table 1) indicate that T-H glycoprotein occurs throughout the kidney, with perhaps slightly more in the cortex than in the medullary-pelvic fraction, although this was not always the case. Comparison of the T-H glycoprotein concentration in the three sonicated homogenates shows that the value for the whole kidney cross-section (0.73 mg/g of tissue) lies in between those of the cortex and the medulla (1.19 and 0.53 mg/g of tissue respectively). This would seem to be a reasonable value on which to base the calculations of pool size. The combined weights of both kidneys (19.23 g)

Table 1. *T-H glycoprotein pool size in rabbit kidney*

For a description of how these fractions were obtained see the text. The means ± s.d. were calculated from the values obtained at two or more dilutions within a single assay.

Tissue	Fraction	T-H glycoprotein (mg/g of tissue)	Protein (mg/g of tissue)	T-H glycoprotein as % of protein
Cortex	1. Homogenate	0.45 ± 0.07	156 ± 14	0.29
	2. Filtrate	0.78 ± 0.11	158 ± 0	0.49
	3. Sonicated material	1.19 ± 0.10	178 ± 21	0.67
Medulla and pelvis	1. Homogenate	0.53 ± 0.01	145 ± 19	0.37
	2. Filtrate	0.65 ± 0.0	153 ± 18	0.42
	3. Sonicated material	0.53 ± 0.12	123 ± 0	0.43
Whole kidney cross-section	1. Homogenate	0.39	169 ± 25	0.23
	2. Filtrate	0.74 ± 0.18	154 ± 6	0.48
	3. Sonicated material	0.73 ± 0.07	161 ± 12	0.43

Table 2. *Subcellular fractionation of human renal-cortical homogenate*

The preparation of the human renal-cortical homogenate is described in the text. It was centrifuged at the speeds indicated on an MSE Magnum model 18 and a Spinco model L centrifuge.

Fraction	T-H glycoprotein		Protein (mg)	T-H glycoprotein as % of total protein
	(μ g)	(% of total)		
1. Total homogenate	1040 $\pm$ 177	100	316 $\pm$ 14	0.33
2. Unhomogenized material (low-speed pellet)	133 $\pm$ 0	12.8	44.5 $\pm$ 0.3	0.30
3. 800g pellet (cells, nuclei, membranes)	123 $\pm$ 20	11.8	45.0 $\pm$ 3	0.27
4. 9000g pellet (mitochondria)	63 $\pm$ 9	5.8	23.2 $\pm$ 0.8	0.27
5. 15000g pellet (lysosomes and damaged mitochondria)	35 $\pm$ 1	3.4	10.3 $\pm$ 0.1	0.34
6. 100000g pellet (microsomes)	71 $\pm$ 14	6.8	14.4 $\pm$ 1.3	0.49
7. 100000g supernatant (soluble fraction)	456 $\pm$ 35	42.6	144 $\pm$ 20	0.31
Recovery		83.2%		89%

would therefore give a total T-H glycoprotein pool size of approx. $19.23 \times 0.73 = 14.0$ mg of T-H glycoprotein.

Complete 24h urine collections were made on 12 consecutive days for the purposes of the ^{14}C radioisotope experiment described later in the paper. These fractions were assayed for T-H glycoprotein and the excretion rate over the 12-day period was 26.7 ± 3.7 mg/24h (mean $\pm$ S.D., $n = 12$). Thus, the total kidney pool (14.0mg) would be excreted in 12.6h or half the pool would be excreted in approximately 6h.

Cellular location of T-H glycoprotein

Purified brush-border fraction from rabbit kidneys. A purified and characterized brush-border fraction whose preparation and composition was described by Quirk & Robinson (1972) was obtained from these authors and assayed for T-H glycoprotein. The brush-border fraction inhibited in the radioimmunoassay only at total protein concentrations greater than $95 \mu\text{g/ml}$, at which concentration non-specific protein is known to interfere in the assay (Grant & Neuberger, 1973). Therefore T-H glycoprotein was not detectable in this brush-border fraction by the radioimmunoassay.

Differential cell centrifugation. To try to discover the intracellular location of the glycoprotein, a sample of human kidney cortex (stored frozen) was cut into small pieces with scissors and then gently homogenized by hand with a Teflon pestle at 4°C in pH 7.0 buffer (1.7mM- Na_2HPO_4 , 0.52mM- KH_2PO_4 , 44mM-NaCl) containing 0.25M-sucrose. The homogenate was centrifuged at different speeds and the pellet was removed at each stage. These were suspended in the pH 7.0 buffer containing 0.0011% sodium dodecyl

sulphate and assayed for T-H glycoprotein and total protein content (Table 2). Of the 1.040mg of T-H glycoprotein measured in the total homogenate, 83.2% was recovered in the individual fractions and 89% of the total protein was recovered. The fraction containing by far the greatest proportion of T-H glycoprotein was the soluble fraction (100000g supernatant, 42.6%). The only other fractions that contained a significant amount were those containing whole cells (fractions 2 and 3). These fractions, together with the soluble fraction, represented 67.2% out of the total 83.2%. The fraction that contained the highest percentage of T-H glycoprotein relative to total protein (0.49%) was the microsomal fraction, where the glycoprotein is presumably synthesized. All the other fractions contained between 0.27 and 0.34% of T-H glycoprotein relative to total protein.

Incorporation of ^{14}C into rabbit urinary glycoprotein

It must be emphasized first of all that the turnover rate of T-H glycoprotein refers, in this paper, to the rate of removal of the glycoprotein from the kidney, not due to catabolism, but as a result of excretion in the urine. With the two substances used, $\text{Na}_2^{14}\text{CO}_3$ and sodium [^{14}C]acetate, there is very little re-utilization because of the rapid turnover of their large metabolic pools (Millward, 1970). This therefore permits more accurate determinations of the turnover rate of the glycoprotein.

Preliminary experiments were performed by injecting $\text{Na}_2^{14}\text{CO}_3$ intravenously into a rabbit and collecting the urine every 24h, from which T-H glycoprotein was isolated (Marr *et al.*, 1971). No incorporation of radioactivity was observed in the T-H glycoprotein and it was considered possible that if the

Fig. 1. Radioactivity excreted/h in total urine, urinary urea and urinary HCO_3^- after injection of $\text{Na}_2^{14}\text{CO}_3$

The radioactivity (c.p.m./h) for each fraction was plotted at the mid-point of the collection period. The horizontal bars denote the total period over which the urine was collected. ○, Urine; ●, urinary urea; △, urinary HCO_3^- .

label was given via the intravenous route the body may dispose of the label as expired CO_2 or excreted NaHCO_3 before it had time to enter the metabolic pool. Intramuscular injection was found to be satisfactory and the presence of ^{14}C was followed in the urinary HCO_3^- , urinary urea and plasma fibrinogen as well as urinary T-H glycoprotein to see whether the HCO_3^- was entering the metabolic pool.

Radioisotope experiment 1. The $\text{Na}_2^{14}\text{CO}_3$ (1.5 mCi) was injected into the right gluteus muscle of a rabbit (no. 17). After 3 h a small volume of urine was produced and the urine was then collected at approx. 24 h intervals for 9 days. The radioactivity per ml of urine due to HCO_3^- and to urea was measured. Blood samples were collected in heparinized tubes at intervals over the 9 days and fibrinogen was isolated and the specific radioactivity was determined.

Within 3 h of injection, radioactivity was present in the urine (Fig. 1). Approximately 74% was present in the urinary HCO_3^- , but none had been incorporated into the urea at this stage (Fig. 1).

The total urine radioactivity and the urinary urea and HCO_3^- radioactivity reached a maximum in the first 24 h after injection. Thereafter the amount of

radioactivity in the urinary HCO_3^- fell fairly rapidly, only 3.4% being excreted in the second 24 h period compared with the first. In most of the fractions, other than the first, more than 70% of the radioactivity could be accounted for by the urea. That the bulk of the radioactivity was in low-molecular-weight material was supported by the fact that dialysis of the urine samples against water removed all but about 13% or less of the total radioactivity.

The fall in the specific radioactivity of the plasma fibrinogen gave a half-life of approximately 70 h when plotted on a semi-logarithmic scale. This is within the range of half-lives (52–75 h) quoted by Cohen *et al.* (1956) who used iodinated fibrinogen. There is no re-utilization with iodinated proteins, since the iodinated amino acids are not recycled (Cohen *et al.*, 1956). This result indicates that the use of $\text{Na}_2^{14}\text{CO}_3$ probably gives a reasonably good estimate of the true turnover rate of a protein.

It was shown therefore that $\text{Na}_2^{14}\text{CO}_3$ given by intramuscular injection entered the metabolic pool and some was incorporated into protein and carbohydrate, part of which was later degraded and excreted as urea. After 24 h less than 2% of the total injected radioactivity was excreted in the urine.

The specific radioactivity of the urinary T-H glycoprotein was very erratic and the values did not lie on a smooth curve as did those for the fibrinogen. In the light of later experiments, it was concluded that other radioactive urinary components were probably adsorbed to T-H glycoprotein and were not removed even by prolonged dialysis, thus giving high erroneous specific radioactivities. There was too little material to pursue the investigations at this point.

Radioisotope experiment 2. The experiment was repeated with the difference that the penultimate stage in the isolation of urinary T-H glycoprotein was a dialysis against a solution containing 1% urea, 1% NaHCO_3 and 1% EDTA for at least 2 days before a final dialysis against water. This was to displace any radioactive components that may still have been present.

As described above, $\text{Na}_2^{14}\text{CO}_3$ (2.0 mCi) was injected intramuscularly into a rabbit (no. 16). A urine sample was obtained within 4.5 h of injection and the urine collections were then made approximately every 24 h for 11 days with intermittent blood sampling over the same period. The radioactivities of the urinary HCO_3^- , urea and plasma fibrinogen were measured and gave a picture similar to experiment 1. The half-life of the fibrinogen was 58 h (Fig. 2). By comparison it was obvious that the turnover rate of T-H glycoprotein was very much faster (7 h), the radioactivity in the second 24 h period only containing approx. 10% of that in the first 24 h collection (Fig. 2). Determination of the pool size by radioimmunoassay, as mentioned above, gave a value of approx. 6 h.

Fig. 2. Decrease in specific radioactivity (c.p.m./mg) with time of T-H glycoprotein and plasma fibrinogen after injection of $\text{Na}_2^{14}\text{CO}_3$

To isolate T-H glycoprotein the pH of the rabbit urine was adjusted to 5 with 3M-HCl and any insoluble material still remaining was removed by centrifugation (2000 rev./min, 5 min). NaCl (0.58M) was added and the urine was left overnight at 4°C. The precipitate was removed by centrifugation (2000 rev./min, 20 min, 4°C) and dialysed for at least 24 h against water. Two additional precipitations with 0.58M-NaCl each followed by dialysis were performed. The third precipitate was dialysed against a solution of 1% EDTA, 1% urea and 1% NaHCO_3 (or 1% sodium acetate in expt. 3). The final dialysis against water was followed by freeze-drying. All procedures up to the final dialysis were conducted in the presence of 0.02% NaN_3 . For explanation of horizontal bars see legend to Fig. 1. Δ , Fibrinogen; $\circ$, T-H glycoprotein.

To examine the possibility of incorporation of the radioisotope into either the O-acetyl groups on the sialic acid residues or the sialic acid residues themselves, these were selectively removed from each T-H glycoprotein fraction from days 2 to 8.

Isolation of O-acetyl groups and sialic acid residues from rabbit T-H glycoprotein

In this experiment the O-acetyl content ranged from 0.16 to 0.33 $\mu\text{mol}/\text{mg}$ of T-H glycoprotein, but the specific radioactivities were too low to be counted with any accuracy. However, there was significant incorporation of radioisotope into the sialic acid residues (Fig. 3). The half-life derived from a semi-logarithmic plot of specific radioactivity against time was 11 h.

Fig. 3. Decrease in specific radioactivity (c.p.m./ μmol) with time of sialic acid residues isolated from T-H glycoprotein after injection of $\text{Na}_2^{14}\text{CO}_3$

For explanation of horizontal bars see legend to Fig. 1.

Radioisotope experiment 3. In this experiment sodium [^{14}C]acetate was used. It is likely that the substitution of sialic acid by acetate is, if not the last, then one of the last steps in the biosynthesis of the glycoprotein (Schauer & Wember, 1971). The acetate pool is very large and has a very rapid turnover (Bloch & Rittenberg, 1945). The likelihood of re-utilization of [^{14}C]acetate itself is therefore small, although other products formed from the [^{14}C]acetate may be utilized. The decrease in specific radioactivity of the O-acetyl groups isolated from the rabbit T-H glycoprotein fractions would probably give a more accurate estimate of the half-life of T-H glycoprotein.

Approximately 0.3 mCi of sodium [$\text{U-}^{14}\text{C}$]acetate was injected into the gluteus muscle of each of three rabbits. Daily urine collections were pooled on each of 12 consecutive days and T-H glycoprotein was isolated from each fraction. The penultimate stage in the purification involved dialysis against a solution containing 1% urea, 1% sodium acetate and 1% EDTA. The samples were finally dialysed against several changes of water for 3 days, centrifuged at 9000 rev./min and the supernatant was freeze-dried.

The half-life of the intact T-H glycoprotein obtained from a semi-logarithmic plot was 9 h. The O-acetyl content of the glycoprotein ranged from 0.14 to 0.21 $\mu\text{mol}/\text{mg}$ of T-H glycoprotein and the specific radioactivity of these fractions is shown in Fig. 4. The half-life was obtained graphically (5 h).

To see if the polypeptide moiety was turning over at the same rate as the carbohydrate, two amino acids,

Fig. 4. Decrease in specific radioactivity (c.p.m./ μ mol) with time of O-acetate, aspartic acid and glutamic acid isolated from T-H glycoprotein after injection of sodium [14 C]acetate

For explanation of horizontal bars see legend to Fig. 1. Δ , Glutamic acid; $\square$, aspartic acid; $\bullet$, O-acetate.

aspartic acid and glutamic acid, were isolated from each of the first four fractions and their specific radioactivities were determined (Fig. 4). The half-lives were shown graphically to be 8h and 9h for glutamic acid and aspartic acid respectively.

Turnover rate determined by radioimmunoassay

Daily urine samples were collected from one of the three rabbits used in this experiment. The urines were assayed for T-H glycoprotein content (Grant & Neuberger, 1973) on four consecutive days and gave a mean value of 52 ± 13 mg/24h. One of the kidneys was homogenized and assayed as described under 'Methods' for rabbit no. 16. The mean value for T-H glycoprotein concentration in the whole kidney was 0.44 ± 0.15 mg of T-H glycoprotein/g of wet tissue. The combined weights of both kidneys (21.67g) therefore provided a pool of T-H glycoprotein of 9.5mg. The half-life by this method was therefore approx. 2h.

Pool size of T-H glycoprotein in human kidney

The kidneys from two patients were examined *post mortem*, one from a woman (79 years old) and the other a man (41 years old), both of whom died of heart attacks. A segment of cortex was homogenized and sonicated as described above and concentrations of T-H glycoprotein of 276μ g/g of cortex and 149μ g/g of cortex respectively were obtained. The combined weights of the two kidneys in each case were 242 and 447g respectively. Assuming the concentration of T-H glycoprotein was approximately the same throughout the kidney as in the cortex, as was the case with rabbit kidney, the total T-H glycoprotein pool was about 67mg in both cases. The average daily excretion of T-H glycoprotein for adults was shown to be 50mg/24h (Grant & Neuberger, 1973). This gives a half-life of the order of 16h for T-H glycoprotein in human kidney.

Discussion

Life-span of T-H glycoprotein

The turnover rate of rabbit T-H glycoprotein has been measured by two completely different methods, the results of which are in relatively close agreement. However, the limitations of each of the methods and the inherent assumptions made should be discussed.

In calculating the turnover rate from a measurement by radioimmunoassay of the total amount of the T-H glycoprotein present in both kidneys, and by determining the amount excreted daily in the urine, we have made the following assumptions: the renal T-H glycoprotein has the same immunological behaviour in the assay as the urinary T-H glycoprotein; none of the glycoprotein is catabolized in the kidney, but the total amount synthesized is excreted in the urine; an insignificant proportion of the glycoprotein is catabolized during the assay procedure; the total amount of T-H glycoprotein was solubilized in the homogenization procedure finally selected. Certain observations are relevant to these assumptions. Dilutions of rabbit kidney homogenate show parallelism to both the rabbit T-H glycoprotein standard and dilutions of rabbit urine in the radioimmunoassay; this suggests that they are all immunologically identical. It is not known whether some of the glycoprotein is normally catabolized in renal tissue, but it was apparent that if the kidney homogenates were kept at 4°C overnight before being assayed for T-H glycoprotein the values were usually significantly elevated. The immunological activity of T-H glycoprotein is preserved if the disulphide bonds remain intact, even after treatment with high concentrations of protein-dissociating agents such as 8M-urea or 1% sodium dodecyl sulphate (Marr, 1970). The relatively high cystine content of the glycoprotein represents about one half-cystine in every

Table 3. *Turnover time of T-H glycoprotein and of some of its constituent parts*

For details see the text.

Radioisotope expt. 2 (Na ₂ ¹⁴ CO ₃)	<i>t</i> _½ (h)
Intact T-H glycoprotein	7
Sialic acid	11
Radioimmunoassay of renal tissue and urine	6
Radioisotope expt. 3 ([¹⁴ C]acetate)	
Intact T-H glycoprotein	9
Glutamic acid	8
Aspartic acid	9
O-Acetate residues	5
Radioimmunoassay of renal tissue and urine	2

11 amino acid residues, so there may be a number of 'loop' peptides containing immunological determinants. It may therefore be possible for the glycoprotein to undergo mild proteolytic digestion in the renal homogenates with preservation of some of the 'loop' peptides, as with lysozyme (Arnon, 1968). These would probably react more effectively with the antibody than the intact glycoprotein because of their diminished size, thus giving artificially elevated values in 1-day-old homogenates. It was therefore essential to assay the homogenates on the day of preparation. However, other explanations for the apparent increase of T-H glycoprotein on storage are possible, and it cannot be asserted with complete certainty that our radioimmunoassay determines all the T-H glycoprotein present in both kidneys.

It is not known whether all the T-H glycoprotein was solubilized in the homogenization procedure, although several methods were tried and the one which gave the best results was selected, resulting in an ultimate solubilization of 15% of the total kidney weight as protein. The radioimmunoassay procedure gave half-lives of 6 and 2 h for T-H glycoprotein in two different rabbits (Table 3).

Although it was not possible to obtain a kidney and a urine sample from the same human subject, the T-H glycoprotein pool size was determined by the radioimmunoassay in human kidneys from two individuals. The average daily excretion rate in a group of normal subjects had previously been determined and a half-life of the order of 16 h for human T-H glycoprotein was estimated.

Determination of the turnover rate by a radioisotopic method was open to criticisms of a different kind. Incorporation of radioisotope *in vivo* is subject to re-utilization, both of the injected label and, to a lesser extent, of radioactive products formed from it, and this will obviously vary for different compounds. For this reason Na₂¹⁴CO₃ and sodium [¹⁴C]acetate were selected, since their large, freely diffusible,

rapidly turning-over pools will result in a very rapid fall in the specific radioactivity of the injected label, and therefore lead to minimum re-utilization. The half-life of the HCO₃⁻ pool in rats after a single intraperitoneal injection was shown to be approx. 15 min (Gould *et al.*, 1949), and, more recently, about 12 min (Millward, 1970). The half-life of the urea pool in rats was found to be about 3.6 h (Swick, 1958) and in rabbits about 3 h (McFarlane, 1963). The difference in the radioactivity of the urea and the HCO₃⁻ excreted (Fig. 1) may thus reflect the large difference in turnover rates. In addition, it should be emphasized that these curves do not represent specific radioactivity but the total amounts of radioactive HCO₃⁻ and urea excreted/h, so that the precursor-product relationship would not be expected to apply.

The essential correctness of our assumptions is shown by the fact that the half-life of fibrinogen measured with Na₂¹⁴CO₃ compared closely with that of iodinated fibrinogen (Cohen *et al.*, 1956), in which instance re-utilization of iodinated amino acids is known not to occur.

The infrequency with which rabbits pass urine made it possible to collect urine only once every 24 h. As can be seen from the graphical procedure used to calculate the turnover rate of T-H glycoprotein, only two points could be used in the extrapolation. This will lead to a certain degree of error. However, several constituents of the glycoprotein were isolated and the half-lives calculated, namely O-acetate residues, sialic acid, aspartic acid and glutamic acid residues, and the values obtained were in fairly close agreement. The non-linearity of the semi-logarithmic graph for sialic acid specific radioactivity suggested that some re-utilization may have occurred, giving rise to a slightly elevated turnover rate (11 h). However, all the other results which range from 5–9 h are of the same order and indicate that, despite the inaccuracies and assumptions inherent in these methods, the essential agreement of all the results suggests that the half-life of rabbit T-H glycoprotein is approx. 9 h or less. The results also show that the carbohydrate and polypeptide components of T-H glycoprotein turn over at the same rate. The life-span of the tubular epithelial cells in mouse kidney is 190 days (Cameron, 1971). If it is of the same order in rabbits, and if these are indeed the cells from which T-H glycoprotein originates, it suggests that the T-H glycoprotein is released into the tubular lumen independently of the renewal of the cell.

Location of T-H glycoprotein

Differential centrifugation experiments in this paper have shown that most of the glycoprotein is recovered in the soluble fraction (100000g supernatant). The only fraction which showed a slightly higher than average proportion of T-H glycoprotein

relative to the total protein was that containing microsomal material, and this is where the glycoprotein is presumably synthesized. These findings agree with those of Keutel (1965) that the entire cytoplasm of tubular cells, but not the nucleus, stained with fluorescent antibody to T-H glycoprotein.

It was previously reported by the use of ferritin-antibody conjugate that T-H glycoprotein was present in the brush border of the proximal tubule and also in the smooth endoplasmic reticulum (Pape & Maxfield, 1964). The electron micrographs shown did not appear quite conclusive. In addition, the possibility of a non-specific adsorption of a similar ferritin antibody conjugate had not been eliminated. In the present work a purified and characterized brush-border fraction (Quirk & Robinson, 1972) was assayed for T-H glycoprotein, but none was detected. For T-H glycoprotein to be present but not detectable by the radioimmunoassay, it would have to comprise less than 0.1% of the total protein in the membrane fraction. As it was easily detectable in whole kidney homogenate, it seems unlikely that it could be a major brush-border membrane component. It is, however, conceivable that the gentle homogenization and washing procedures used in its isolation may have removed very loosely bound glycoprotein. Our negative result agrees with that of Stevenson (1972) who reported that no T-H glycoprotein was found in the purified brush-border fraction isolated from rabbit kidney by a different procedure, involving digestion with collagenase.

Use of the radioimmunoassay to determine the kidney pool size indicated that the glycoprotein was distributed throughout the kidney in both the cortex and medullary fractions. There are two possible explanations for this finding. The glycoprotein may be synthesized throughout the nephron or it could be synthesized high up in the proximal tubule and then washed down the tubule lumen to the collecting ducts. Fluorescent-antibody studies are in some disagreement as to the exact location within the kidney, although it definitely seems to be absent from the glomerulus (Keutel, 1965; McKenzie & McQueen, 1969).

Possible relation of T-H glycoprotein to erythropoietin

No function has been attributed to T-H glycoprotein. However, it is interesting to note the general similarity in amino acid and carbohydrate composition between human erythropoietin (Espada *et al.*, 1972) and both human and rabbit T-H glycoprotein (Fletcher *et al.*, 1970a; Marr *et al.*, 1971). In particular, the proline content reported for erythropoietin is considerably higher than that of either of the T-H glycoproteins investigated, and no cysteine or tryptophan determination was performed for erythropoietin, although cystine and tryptophan were shown to be present (Keighley & Lowy, 1971). The

molecular weight has not been satisfactorily established, but is probably less than 70000 (Keighley & Lowy, 1971) compared with the subunit molecular weight of 79000 for human T-H glycoprotein (Fletcher *et al.*, 1970b). Both glycoproteins are present in kidney and urine. The sialic acid content of human T-H glycoprotein varies slightly between preparations (approx. 5.5%; Neuberger & Ratcliffe, 1972) but is consistently lower than that cited for erythropoietin (7.5%; Espada *et al.*, 1972). The presence of sialic acid is essential for erythropoietin activity when tested in the assay system *in vivo* (Keighley & Lowy, 1971). However, the asialoglycoprotein was shown to retain its biological activity in the bone-marrow-cell-culture test system (Goldwasser, 1966).

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