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De novo pyrimidine nucleotide biosynthesis in isolated rat glomeruli

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***De novo* pyrimidine nucleotide biosynthesis in isolated rat glomeruli.** Uracil ribonucleotide–sugars and aminosugars are required for glomerular basement membrane (GBM) biosynthesis. Since these nucleotides are metabolic derivatives of uridine 5′-triphosphate (UTP), we have studied the cellular pools of uridine 5′-diphosphoglucose (UDPG), uridine 5′-diphosphoglucuronic acid (UDPGA), uridine 5′-diphospho-N-acetyl glucosamine (UDPAG) and UTP, and measured UTP synthesis *de novo* in isolated glomeruli incubated in vitro. Improved techniques for nucleotide quantitation were established and the optimal conditions for glomerular isolation and incubation determined. Substantial quantities of uracil ribonucleotide coenzymes and an active utilization of orotate for the synthesis of pyrimidine nucleotides were demonstrated. UTP synthesis and the pools of UDPG and UDPGA varied markedly with changes in the experimental conditions. The adverse effects of suboptimal conditions were more apparent in glomeruli from diabetic animals than in controls. The use of suboptimal conditions could provide misleading information on GBM metabolism in isolated glomeruli since uracil ribonucleotide coenzyme availability might be reduced.

The formation rate of different glomerular basement membrane (GBM) components has been studied in isolated glomeruli of the normal rat [1–3]. Since experimental and human diabetic nephropathy are both characterized by accumulation of mesangial GBM material and thickening of the peripheral GBM [4], use of isolated glomeruli of the diabetic rat has been model for study of alterations in the biosynthesis of GBM material [5–7].

Biosynthesis of the collagenous moiety of GBM has been evaluated in isolated glomeruli by measuring the incorporation rate of radiolabeled lysine or proline [1–3, 5–7], and that of the non-collagenous portion by determining the incorporation of radiolabeled glucosamine, galactose or sulfate into GBM glycoproteins and glycosaminoglycans [3–8]. While these investigations have provided information on the rate of incorporation of exogenous substrates into the various GBM components, no studies have been carried out on the glomerular metabolism of uridine nucleotide coenzymes which are necessary for glycoprotein formation.

Following peptide chain synthesis, carbohydrate attachment represents an essential step in the synthesis of all GBM components. Glycosylation proceeds through the action of specific enzymes and in the presence of uridine 5′-diphospho sugars (UDP-sugars) and uridine 5′-diphospho aminosugars (UDP-aminosugars) which are donors in the carbohydrate transfer reaction. Since these uridine nucleotide coenzymes are uniquely metabolic derivatives of uridine 5′-triphosphate (UTP), their bioavailability will be related to the size of the cellular pool of UTP and the activity of the *de novo* pathway for uracil ribonucleotide formation (Fig. 1) [9]. In addition to being the source of coenzymes, UTP and cytidine 5′-triphosphate (CTP), its metabolic derivative, are also precursors for RNA biosynthesis (Fig. 1).

Glomerular pyrimidine nucleotide metabolism cannot be studied in freshly isolated tissue obtained in in vivo radiolabeling experiments by analyses of uracil ribonucleotide concentrations, because ribonucleoside triphosphates are likely to break down rapidly to more stable compounds during glomerular isolation [10]. This results in markedly altered tissue concentrations of specific nucleotides. Recent methodological improvements developed in our laboratory have permitted the investigation of the metabolism of nucleotides in isolated glomeruli during their incubation in vitro. This report describes the effects of variation in the experimental conditions on glomerular uracil ribonucleotide synthesis *de novo* in control and diabetic animals.

Methods

Tissue preparation and incubation technique

Male Fischer rats (Harlan Industries, Inc., Madison, Wisconsin, USA) weighing 160 to 200 g were used in all experiments. Diabetes was induced by the i.v. administration of streptozotocin (U-9889) at a dose of 50 mg/kg, 48 hr before the studies were performed [11]. Animals were stunned by a blow on the head and their kidneys rapidly removed and immersed in either cold 0.15 M NaCl containing 0.05 M Tris buffer or cold Hanks' balanced salt solution [12] containing 20 mM Hepes (Hanks-BSS), both solutions having their pH adjusted to 7.40 at 2°C. After separation from the medulla, pooled minced renal cortices were forced through a 120-mesh sieve, and glomeruli separated by graded sieving and differential centrifugation [13] using a solution similar to that in which the kidneys were initially placed. The glomerular concentration and purity of each isolate was assessed by microscopic examination of the final suspension. A purity of 98% or greater was obtained. The yield of the

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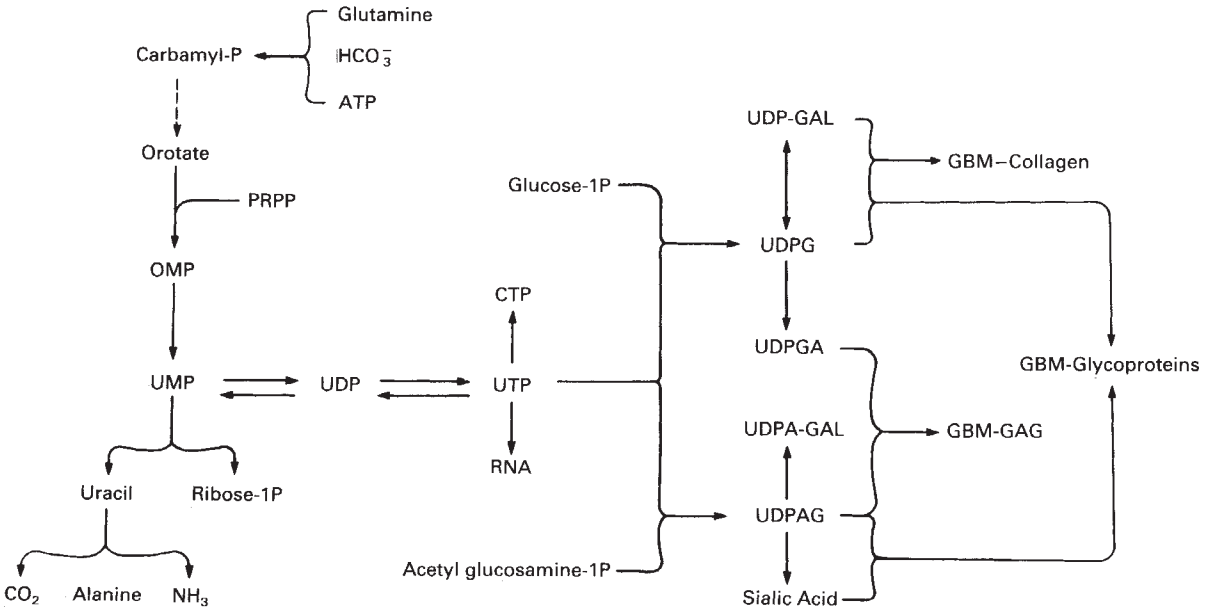

Fig. 1. *Simplified schema of the biosynthetic and catabolic pathways of uracil ribonucleotide metabolism.* The schema emphasizes the relationship between the sugar and aminosugar derivatives of uracil ribonucleotides and the formation of glomerular basement membrane (GBM) components. Abbreviations are: PRPP, 5-phosphoribosyl 1-pyrophosphate; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UDP-GAL, uridine 5′-diphosphogalactose; UDPG, uridine 5′-diphosphoglucose; UDPGA, uridine 5′-diphosphoglucuronic acid; UDPA-GA, uridine 5′-diphospho-N-acetyl galactosamine; UDPAG, uridine 5′-diphospho-N-acetyl glucosamine; GAG, glycosaminoglycans.

method used was about 20% (6200 glomeruli/renal cortex), which is comparable to that previously reported using similar procedures [1, 6, 14, 15]. The isolated glomeruli were resuspended in ice–cold incubation media containing 1 mM glutamine, 5 or 20 mM glucose, 1-80 μM sodium orotate, 11.6 μCi/ml of [^{3}H]orotic acid, and 10% (vol/vol) of dialyzed fetal calf serum in either Krebs–Ringer bicarbonate buffer [16] or Hanks-BSS with pH adjusted previously to 7.40 at 37°C. Total tissue preparation time was defined as the interval between initiation of glomerular isolation by mincing of the renal cortices and the start of the glomerular incubation.

To minimize variability between samples within the same experiment, a group of studies was carried out using glomerular pools derived from 260 to 300 renal cortices per experiment. From these, 10 to 12 samples were obtained for incubation, each sample containing 150 ± 18 × 10^3 SD glomeruli in 2 ml of incubation media. In these experiments total tissue preparation time for incubation was 180 to 240 min. In a second group of experiments, designed to shorten the time necessary for the preparation of glomeruli, only 32 to 40 renal cortices were used to rapidly obtain one glomerular pool, which was divided into two samples. These samples, containing 114 ± 22 × 10^3 glomeruli in 2 ml of media, were incubated immediately, reducing the total preparation time to a minimum of 40 to 45 min.

Glomerular samples were placed in polyethylene incubation vials. In all experiments one vial was immediately frozen in liquid N_2. The remaining samples were placed in a Dubnoff metabolic incubator at 37°C, equilibrated with 5% CO_2: 95% O_2, and incubated for periods of between 5 and 240 min. Incubations were terminated by immersion of the samples in liquid N_2. Since tissue nucleotide composition is rapidly altered after impairment of oxidative metabolism [10], media and tissues were not separated at the completion of the incubation.

Extraction of nucleotides and nucleic acids

The frozen contents of the incubation vials were mixed with frozen 1 N perchloric acid after pulverization in a stainless steel mortar pre-cooled in liquid N_2, and the mixture allowed to melt at 2°C. After addition of 0.04 μCi of [^{14}C]UTP, and 0.03 μCi of [^{14}C]uridine 5′-diphosphoglucose (UPDG) as internal standards, the samples were homogenized at 2°C in a final acid concentration of 0.24 N. The tissue fraction soluble in cold perchloric acid was separated and rapidly neutralized to pH 7.0 at 2°C with 1 N KOH. The neutralized samples were centrifuged at 20,600 × g for 30 min and the supernatant lyophilized for later chromatographic analysis of its nucleotides.

RNA and DNA were extracted from the acid insoluble fraction by alkaline and acid hydrolysis, respectively [17]. Nucleic acids were measured according to the quantity of RNA-associated pentose [18] and to the DNA ultraviolet absorption by a two–wavelength ratio method [19] using calf–thymus DNA (type I, Sigma Chemical Co., St. Louis, Missouri, USA) and d-ribose as reference, respectively. RNA results were expressed according to its ribose content, 0.6 mg of ribose being equivalent to 1 mg of yeast RNA (type XI, Sigma). The renal cortical content of RNA and DNA was determined in rapidly frozen tissue specimens in vivo [11] utilizing methods identical to those used for glomerular analysis. Total protein was measured by the method of Lowry et al [20].

Chromatographic analyses

The lyophilized samples were resuspended in 140 to 160 μl of distilled water and 20 to 30 μl of sample used in separate high pressure liquid chromatographic analyses. Ribonucleotide triphosphates were separated in 0.46 × 25 cm columns of Partisil-10 strong anion exchanger (Whatman Chemical Separation Inc., Clifton, New Jersey, USA) using a Varian-8500 chromatographic system (Varian Associates, Palo Alto, California, USA) [11]. Peaks were detected by the simultaneous monitoring of light absorption at 254 and 280 nm with a Waters 440 detector (Waters Associates Inc., Milford, Massachusetts, USA). Nucleotides were quantitated according to their absorbancy at their optimal wavelength. The amount of UTP contained in the sample injected for analysis varied between 0.15 and 1.2 nmole, which was well above 0.08 nmole, the estimated lowest limit of accurate quantitation. Average deviation from the mean of duplicate UTP measurements performed in the same glomerular sample was 3.1 ± 3.4% SD.

Quantitation of UDP-sugars, UDP-aminosugars, UDP-glucuronic acid (UDPGA) and UDP was carried out on a bonded phase amine column (Zorbax-NH_2, 0.46 × 25 cm; DuPont Co., Wilmington, Delaware, USA) used as a weak anion exchanger. Analyses were performed at 28°C in a Beckman 332 chromatographic system (Beckman Instruments Inc., Fullerton, California, USA). Samples were eluted at 1 ml/min using a concave gradient formed by a) an initial mobile phase consisting of 0.06 M KH_2PO_4, pH 2.8 to which 35% (vol/vol) acetonitrile and 0.05% (vol/vol) triethylamine were added, and b) 0.6 M KH_2PO_4, pH 3.6, containing the same amount of triethylamine, as the final mobile phase. Nucleotide peaks were identified and examined for purity as previously described [21]. The separation of UDPG from an unidentified compound which was not a uracil derivative was variable in different chromatographic columns obtained from the same source. Only columns demonstrating complete resolution of the UDPG peak were used in nucleotide analyses. Nucleotides were quantitated according to their absorbancy at 262 nm using a Spectroflow-773 detector (Kratos Analytical Instruments, Ramsey, New Jersey, USA). The amounts of uridine 5'-diphospho-N-acetyl glucosamine (UDPAG) and UDPG found in the samples injected for analysis were between 0.2 and 0.45 nmole. These amounts exceeded the lower limit of quantitation, which was estimated to be 0.05 nmole. Average deviation from the mean of duplicate UDPG and UDPAG measurements performed in the same glomerular sample was 5.3 ± 3.8% SD.

Column effluents were collected as 0.2 to 0.3 ml fractions for determination of ^{14}C and ^{3}H activity. The final recovery of the UTP and UDPG used as internal standards was 58.5 + 10.9%, and 87.3 ± 2.9% SD, respectively. These recoveries account for losses during the extraction and analytical procedures.

The radiolabeled compounds used as standards or as substrates for incubation were obtained from New England Nuclear Corp. (Boston, Massachusetts, USA). Their radiochemical purity was evaluated by high pressure liquid chromatography at the end of each experiment. The purity results were as follows: [5-^{3}H]orotic acid (20 Ci/nmole), 76 ± 13% SD; [2-^{14}C] UTP (46.8 mCi/nmole), 90 ± 6% SD; [Glucose-^{14}C (U)]UDPG (334 mCi/mmole), 77%.

Expression of results and statistical analysis

Since, except for cyclic nucleotides, there is no transport of nucleotides from cells into the incubation medium [22], the pyrimidine nucleotides found in the whole sample were considered to be exclusively intracellular. Values for UDP and UDP derivatives were the average of duplicate chromatographic analyses. The quantitation of UDP and UDP derivatives was corrected for the recovery of [^{14}C]UDPG. UTP and CTP values were corrected according to the recovery of [^{14}C]UTP. In assessing recoveries of these internal standards and incorporation of [^{3}H]orotate, results were adjusted according to the radiochemical purity of these radiolabeled compounds. The small amounts of ^{3}H activity detected in the chromatographic location of UTP in the extracts of non-incubated samples was subtracted from the amount found incorporated into this compound in the incubated samples. DNA content in the whole lyophilized glomeruli was approximately 25 mg/g.

The data were analyzed using multiple regression [23]. Quadratic terms were added to some models where noted. All results are presented as mean ± SD. The statistical significance of the differences between means was determined by Student's *t*-test for non-paired variables. Significance was determined from the distribution of *t* in a two–tailed test. Statistical analyses were carried out using the Statistical Analysis System (SAS Institute, Cary, North Carolina, USA).

Results

Effect of tissue preparation time on UTP synthesis following in vitro incubation

Preliminary experiments, carried out to compare UTP synthesis in glomeruli from control and diabetic rats, resulted in marked inconsistencies. In these initial attempts, samples of control and experimental tissue were obtained from single glomerular pools derived from 120 to 152 renal cortices. While results were not influenced by the order in which control and diabetic animals were killed, contradictory findings were obtained according to the sequence in which control and experimental glomeruli were isolated. At all concentrations of orotate in the media (1 to 80 μM), its incorporation into UTP in diabetic glomeruli was 10 to 30-fold the control value if glomerular isolation was carried out last in the diabetic group (Fig. 2). However, if the order was reversed, diabetic glomeruli incorporated only half the amount shown in controls (Fig. 2). Differences between groups were not influenced by media radiolabel concentration (5 to 23 μCi/ml), incubation time (20 to 180 min) or removal of glutamine from the incubation media. Because the length of time between glomerular isolation after mincing of cortical tissue and initiation of incubation was the apparent cause for the marked differences in the results, the effects of tissue preparation time were studied further. The metabolism of UTP was studied in rapidly isolated glomeruli after being kept in cold buffered 0.15 M NaCl for different periods of time to simulate varying periods of glomerular preparation. In addition, to elucidate whether the duration of tissue preparation time would affect UTP metabolism to the same degree in normal glomeruli and in glomeruli with possibly altered GBM synthesis, identical studies were carried out in glomeruli from control and 48 hr diabetic rats.

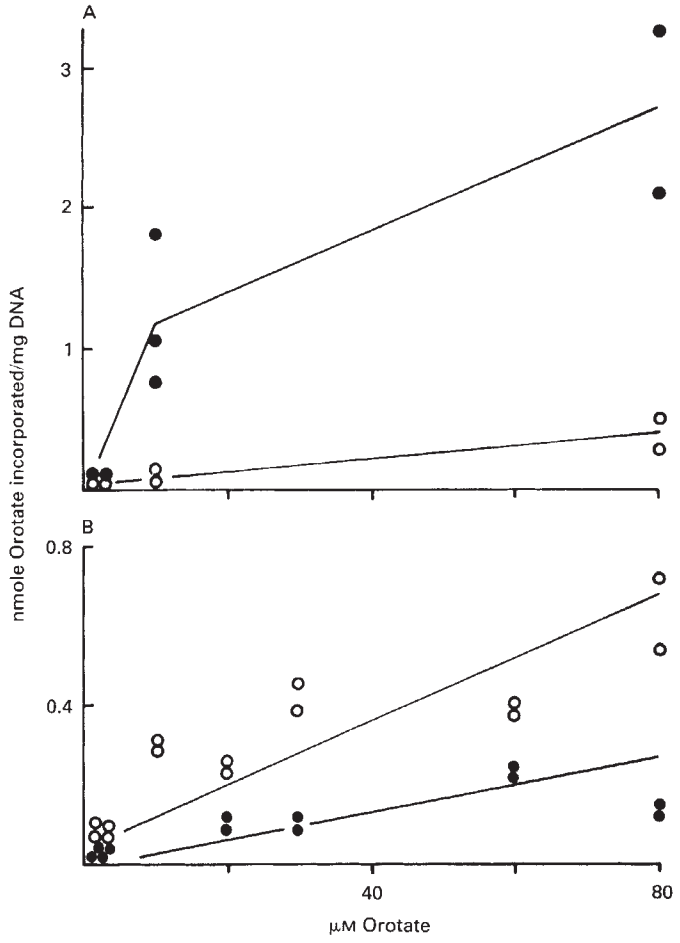

Fig. 2. *Glomerular incorporation of exogenous orotate into uridine 5'-triphosphate in diabetic and control rats.* Tissue samples of similar size were obtained from single glomerular pools derived from renal cortices of diabetic and control animals. Glomerular isolation was carried out at 2°C in 0.15 M NaCl containing 0.05 M TRIS buffer, pH 7.4. Orotate incorporation was measured following three hr incubations in Krebs–Ringer bicarbonate media with 20 mM glucose. Each data point represents the value for one diabetic (●) or control (○) sample. Results presented were derived from four separate experiments in which control glomeruli were isolated before diabetic glomeruli (A) or in which the order of tissue processing was reversed (B). Groups of 60 to 76 diabetic or control animals were used in each experiment.

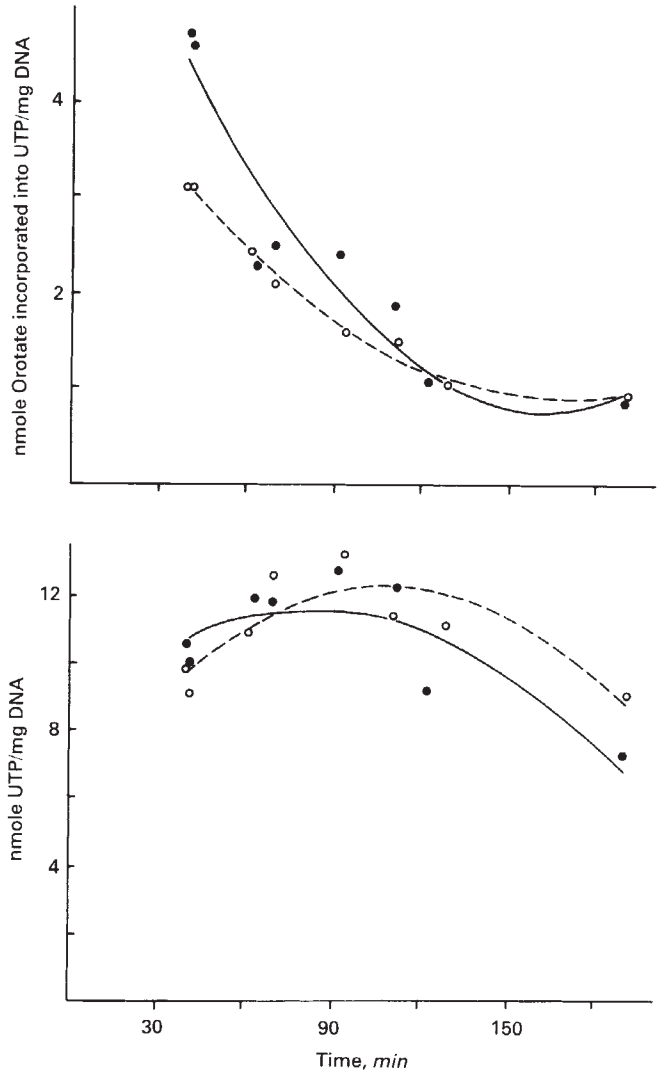

Fig. 3. *Effect of total tissue preparation time on uridine 5'-triphosphate (UTP) metabolism in glomeruli from control and diabetic rats incubated in vitro.* Glomeruli were obtained in 40 min in separate batches from kidneys of control (○———○) and 48-hour diabetic (●———●) animals using 0.15 M NaCl containing 0.05 M Tris. To mimic longer times for tissue preparation separate glomerular pools were rapidly isolated and kept in the solution used for isolation for 0 to 150 min at 2°C prior to incubation. Measurements were carried out following 3-hour incubations in Krebs-Ringer bicarbonate media with 10 μM orotate and 20 mM glucose. Results presented were derived from two separate experiments.

The *de novo* synthesis of UTP was assessed by the quantity of exogenous orotate incorporated into UTP and by the size of the cellular UTP pool at the end of a three hr incubation. The amount of orotate incorporated showed a significant curvilinear relationship with total tissue preparation time ($P < 0.001$), decreasing rapidly as the period was increased from 40 to 90 min (Fig. 3). There were no time group interactions. There was a significant group effect suggesting a differing effect of tissue preparation time on orotate incorporation ($P < 0.03$) for diabetic as compared to control glomeruli. It appears this difference was due to a more rapid decline in incorporation in diabetic glomeruli prepared between 40 and 90 min (Fig. 3).

Tissue preparation time also affected UTP cell content on subsequent glomerular incubation. The relationship between preparation time and UTP pool size was curvilinear ($P < 0.04$) with greater values at about 110 min than at shorter or longer intervals (Fig. 3). There was a tendency for lower UTP values in diabetic glomeruli prepared in 90 min or longer, however the differences were not statistically significant ($P > 0.4$) (Fig. 3).

Effect of buffer composition on UTP synthesis

In experiments in which tissue preparation time was 40 to 45 min, control glomeruli were isolated in isotonic, Tris-buffered NaCl, or in Hanks-BBS. At the completion of a three hr incubation, orotate incorporation into UTP and UTP pool size were greater in glomeruli isolated in Hanks-BBS than in those isolated in the NaCl solution (Fig. 4). However, replacement of

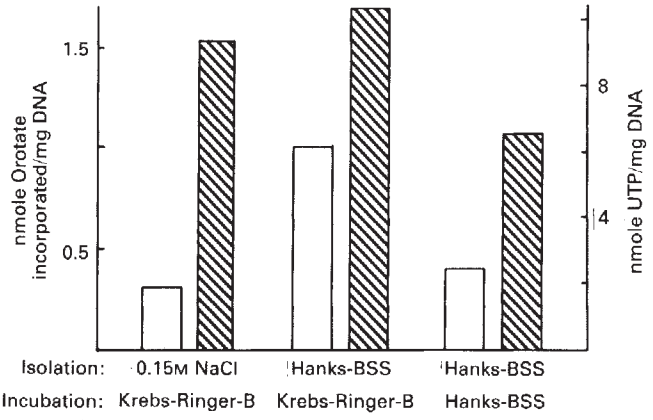

Fig. 4. *Effect of the composition of buffer solutions used for tissue isolation and incubation on uridine 5'-triphosphate (UTP) metabolism in incubated glomeruli.* Glomeruli were obtained in 40 min in separate batches using 0.15 M NaCl containing 0.05 M Tris or using Hanks balanced salt solution containing 20 mM Hepes (Hanks-BSS). Incubations were carried out for three hr in Krebs–Ringer bicarbonate buffer or in Hanks-BSS media with 20 mM glucose and 10 μM orotate. Results are mean of duplicate samples obtained in two separate experiments. Symbols are: ▨ UTP content; □ orotate incorporated into UTP.

Krebs–Ringer bicarbonate by Hanks-BBS as the buffer solution in the incubation media resulted in lower precursor incorporation and smaller UTP pool (Fig. 4).

Differences in UTP metabolism in control glomeruli prepared under optimal and suboptimal conditions for orotate incorporation

The accretion rate of UTP and the incorporation rate of orotate into UTP during glomerular incubation were determined in tissue prepared and incubated under conditions optimizing orotate incorporation into UTP. Results were compared to those obtained in glomeruli which, although viable and metabolically active, were believed to be prepared under less favorable conditions as judged by the experiments above.

In optimally prepared tissue (glomerular isolation in Hanks-BSS, 40 min tissue preparation) there was a group time interaction ($P < 0.01$), therefore it was necessary to fit a separate model for each group. Orotate incorporation into UTP increased linearly with incubation time at a rate of 5.82 ± 0.36 pmole/min/mg DNA. Increase in media glucose concentration from 5 mM to 20 mM decreased this rate significantly to 4.53 ± 0.2 ($P < 0.01$) (Fig. 5). In tissue suboptimally prepared (glomerular isolation in isotonic NaCl, 210 min tissue preparation), orotate incorporation increased at a slower rate reaching a maximum value at about 180 min of incubation (Fig. 5). In these glomeruli, an increase in media glucose concentration from 5 to 20 mM appears to enhance orotate incorporation into UTP ($P < 0.005$).

At the start of the incubations, the UTP pool was 3.5 times greater in glomeruli prepared to optimize orotate incorporation than in those prepared under suboptimal conditions (Fig. 5). However, regardless of the method of tissue preparation, UTP pool increased gradually as the time of incubation was prolonged.

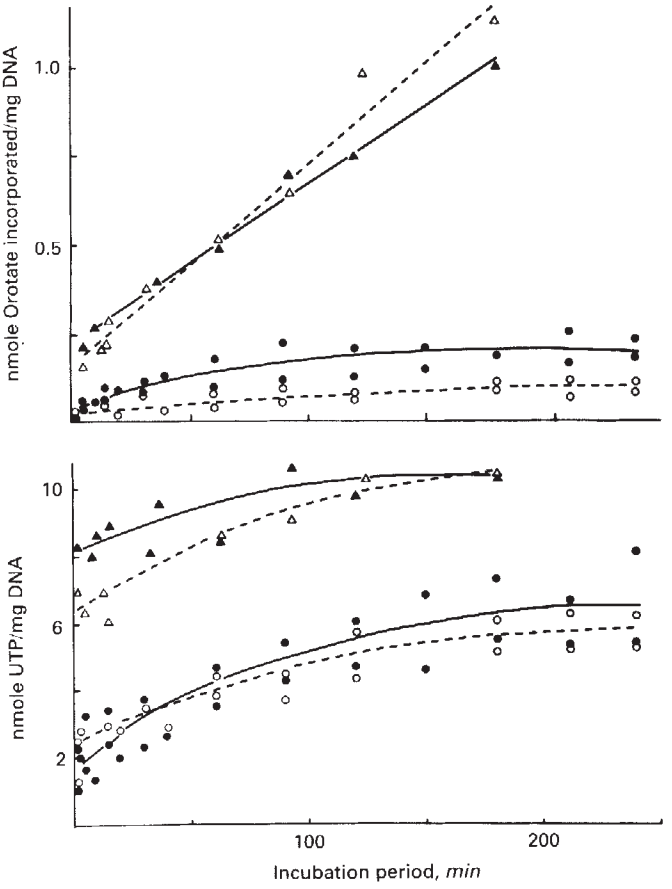

Fig. 5. *Effect of glomerular preparation conditions on the incorporation rate of exogenous orotate into uridine 5'-triphosphate (UTP) and on the rate of UTP pool generation in incubated glomeruli.* Isolated glomeruli were suspended in Krebs–Ringer bicarbonate media containing 20 μM orotate and its incorporation measured at different incubation times. Studies were carried out in glomeruli prepared in 40 min in Hanks-BSS and incubated in 5 mM (△———△) or 20 mM (▲———▲) glucose, and in glomeruli prepared in 210 min in 0.15 M NaCl containing 0.05 M Tris and incubated in 5 mM (○———○) or 20 mM (●———●) glucose. Each data point represents the value for one sample. Results obtained in five separate experiments are presented.

Cellular pools of metabolic derivatives of UTP in control glomeruli prepared under optimal or suboptimal conditions for orotate incorporation into UTP

In rapidly isolated glomeruli, protein/DNA ratio and mean cellular content of RNA were 2.8 and 2.3 times lower, respectively, than in whole renal cortex (protein/DNA, cortex: 21.8 ± 1(10), glomeruli: 7.7 ± 0.7(5), $P < 0.001$), (RNA, cortex: 137 ± 7(10), glomeruli: 59.6 ± 7.9(5) μg/mg DNA, $P < 0.001$).

Quantitation of the major metabolic derivatives of UTP in each glomerular nucleotide extract was obtained simultaneously in a single analysis by weak anion–exchange chromatography (Fig. 6). Except for uridine 5'-diphosphogalactose which coeluted with an unidentified compound, this method provided a complete resolution of all compounds in a purified standard mixture of 10 pyrimidine ribonucleoside diphosphates and orotidine 5'-monophosphate. However, only UTP, UDP,

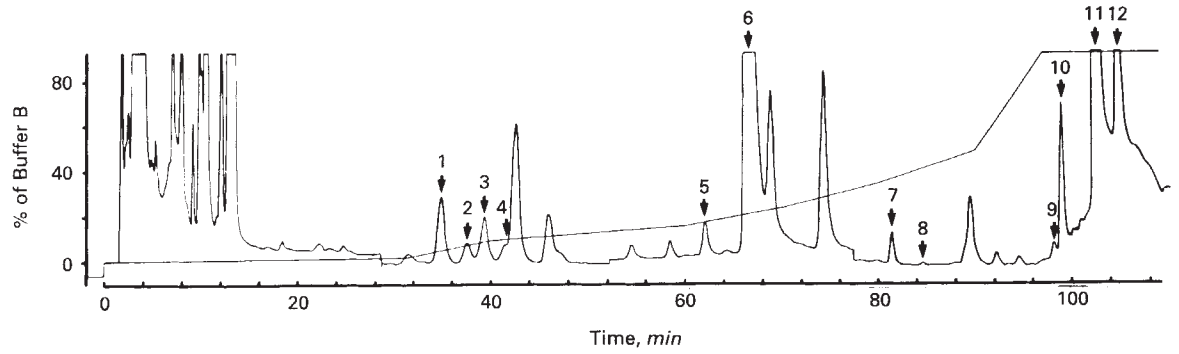

Fig. 6. *Chromatographic separation elution profile of ribonucleoside phosphate contained in a glomerular perchloric acid extract.* Weak anion–exchange high pressure liquid chromatography was carried out by gradient elution with a 0.06 M KH_2PO_4/acetonitrile as initial eluent and a 0.06 M KH_2PO_4 buffer as a final eluent (buffer B). The configuration of the gradient used is plotted. Represented is the ultraviolet absorption of the eluent at 262 nm at a detector sensitivity of 0.01 absorbance units full scale. Peaks are: 1, uridine 5′-diphospho-N-acetyl glucosamine; 2, uridine 5′-diphospho-N-acetyl galactosamine; 3, uridine 5′-diphosphoglucose; 4, uridine 5′-diphosphogalactose; 5, uridine 5′-diphosphate; 6, adenosine 5′-diphosphate; 7, uridine 5′-diphosphoglucuronic acid; 8, uridine 5′-diphosphogalacturonic acid; 9, cytidine 5′-triphosphate; 10, uridine 5′-triphosphate; 11, adenosine 5′-triphosphate; 12, guanosine 5′-triphosphate.

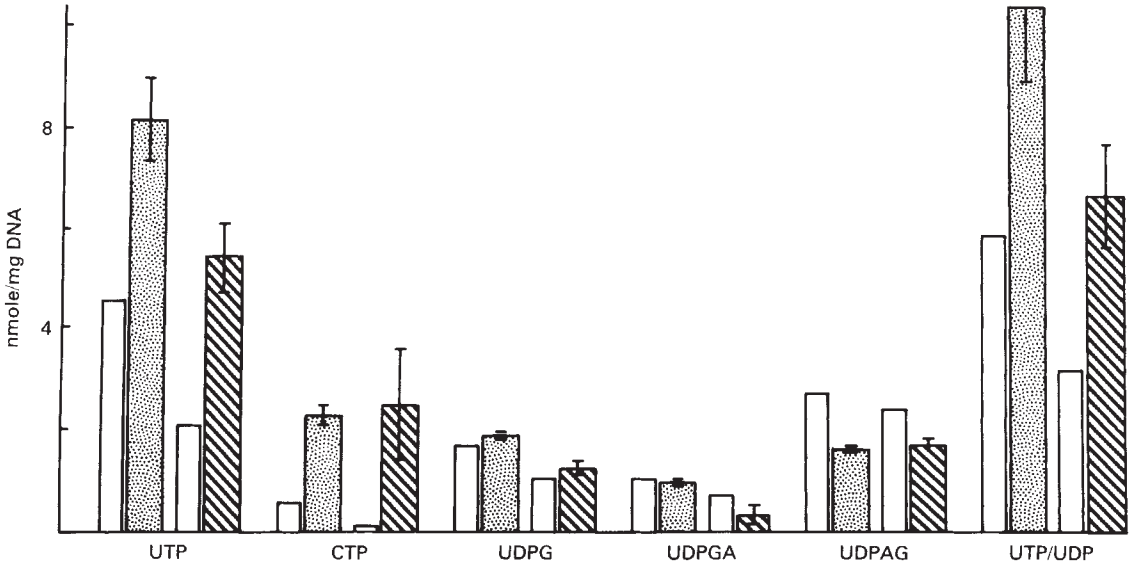

Fig. 7. *Effect of glomerular preparation conditions on the pre- and post-incubation cellular pools uracil 5′-triphosphate (UTP) and its metabolic derivatives cytidine 5′-triphosphate (CTP), uridine 5′-diphosphoglucose (UDPG), uridine 5′-diphosphoglucuronic acid (UDPGA) and uridine 5′-diphospho-N-acetyl glucosamine (UDPAG).* Isolated glomeruli were immediately frozen or incubated for three hr in Krebs–Ringer bicarbonate media containing 20 μM orotate and 20 mM glucose. Pre-incubation values represent the mean of two samples. Post-incubation values are mean ± SD of 6 to 8 samples. Symbols are: □ pre-incubation; ▨ 40 min tissue preparation (isolation in Hanks-BSS); ▩ 210 min tissue preparation (isolation in 0.15 M NaCl).

CTP, UDPG, UDPAG and uridine 5′-diphosphoglucuronic acid (UDPGA) were found in sufficient amounts for quantitation.

Substantial amounts of uridine nucleotide coenzymes were found in freshly isolated glomeruli before incubation. As compared to glomeruli isolated in Hanks-BSS and rapidly prepared, those obtained using isotonic NaCl and after long tissue preparation time contained smaller cellular pools of UDPG, UDPGA and UDPAG in addition to lower UTP/UDP ratios and UTP and CTP content (Fig. 7).

To elucidate whether changes in UTP metabolism, resulting from different tissue preparation methods, altered the formation of CTP and uridine nucleotide coenzymes, the pool size of these compounds was measured in incubated glomeruli prepared using the optimal and suboptimal conditions described above. Studies were carried out after the incubation period when maximal UTP pool sizes were expected to be attained. Compared to glomeruli prepared under optimal conditions, suboptimally prepared tissue showed significantly smaller pools of UDPG and UDPGA ($P < 0.001$) associated with impaired generation of UTP and a significantly lower UTP/UDP ratio ($P < 0.001$) (Fig. 7). However, the maximal size of the CTP and UDPAG pools was unaffected. The changes in UDPAG pool size were unique, in that it was the only pyrimidine nucleotide with a reduction in pool size following incubation (Fig. 7).

Discussion

Measurements of the pools of uracil ribonucleotides have been made possible in this study by their regeneration during in vivo incubation followed by rapid freezing of the tissue. Optimization of the procedures for nucleotide extraction and quantitation have resulted in improved recoveries and greater discrimination than in methods previously described for the analysis of small samples which have dealt only with erythrocytes [24].

Comparison of pre- and post-incubation results suggests that marked alterations in the cellular contents of pyrimidine nucleotides occur during glomerular isolation, even if this is carried out under optimal conditions. These alterations are primarily related to continuing breakdown while formation ceases, as shown by depletion of UTP, CTP and UPDG pools and by low UTP/UDP ratios. However, the greater pool of UDPAG before glomerular incubation indicates that the contents of UDP-aminosugars are altered also by additional inhibition of utilization.

These studies also demonstrate how important the tissue preparation conditions are in altering glomerular nucleotide metabolism. The time required for glomerular preparation critically changed the characteristics of UTP metabolism in incubated glomeruli. Prolongation of the tissue preparation time caused a rapid decrease in the incorporation of orotate into UTP, suggesting a marked decline in its synthesis. However, UTP pool size at the termination of incubation was larger in glomeruli prepared in 90 min than in those prepared in 40 min, indicating a greater impairment in UTP utilization than in its formation.

Glomerular preparation may affect nucleotide metabolism to a variable degree in tissues of same origin but different metabolic states. For example, in glomeruli obtained from rats 48 hr after the induction of diabetes, the adverse effects of prolongation of tissue preparation time on the synthesis of UTP were more intense than in control glomeruli. In rapidly prepared tissue, glomerular UTP synthesis was enhanced in diabetes, while if tissues were prepared in 120 min or longer, no differences were apparent or results were reversed.

Additional variables which were found to influence uracil ribonucleotide synthesis in isolated glomeruli were the composition of the solution used for glomerular isolation and the composition of the buffer present in the incubation media. The present results suggest that the UTP/UDP ratio at the completion of the incubation is a valuable index for assessing the integrity of uracil ribonucleotide metabolism. A ratio of about 10 was obtained in experiments performed under the best conditions.

The adverse effects of unsuitable conditions were not limited to the synthesis of UTP. Glomeruli studied under suboptimal circumstances achieved only a fraction of the maximal pool size of UDPG generated under optimal conditions. The bioavailability of UDP-sugars during in vitro incubation might influence the rate of GBM synthesis, since depletion of the pools of UDPG and UDPAG in the liver of the rat is associated with decreased glycosylation of secreted and tissue glycoproteins [9, 25]. Furthermore, in fibroblast cultures, insufficient glycosylation of newly formed procollagen and fibronectin results in decreased net secretion of these compounds [26, 27]. It is thus probable that an optimal degree of glycosylation of newly formed GBM material is required for its extracellular deposition in normal amounts. Studies on GBM metabolism in isolated glomeruli prepared and incubated in conditions adverse for uracil ribonucleotides synthesis are likely to underestimate GBM synthesis rate.

The effects of extracellular glucose concentration on glomerular UTP synthesis in vitro were also influenced by the experimental conditions. While orotate incorporation rate was greatly reduced in studies carried out in suboptimal conditions, this rate was improved significantly by increasing glucose concentration in the media, a response which was opposite to that obtained in glomeruli studied under optimal conditions.

Even with optimal experimental conditions, the cellular pools of uracil ribonucleotides in incubated glomeruli are significantly smaller than in the renal cortex in vivo [21, 28]. Comparisons of cellular contents between different renal cell populations is possible by selecting tissue DNA as reference since the amount of DNA in diploid mononuclear cells is constant in all tissues [29]. Renal cortex cellular content of UTP and UDP-sugars is eight to 25-fold the amount in glomeruli if pools are related to DNA, when expressed in the same units as in the present study [21]. This difference is partially due to a disparity in the mean cell size between glomerular and cortical cells as suggested by the two to threefold lower protein/DNA and RNA/DNA in isolated glomeruli as compared to cortical tissue. Most of the latter are tubular cells which, in the rat, are a total volume per whole kidney of about eightfold larger than that of glomerular cells [30].

The effects of specific conditions for study of glomerular metabolism appear to be most prominent in diabetes. The synthesis rate of uracil ribonucleotides seems to be highly dependent on optimal tissue preparation in glomeruli from diabetic animals. Further, under conditions associated with increased synthesis of GBM, concomitantly enhanced bioavailability of UDP-sugars could be crucial to maintain normal glycosylation of proteins resulting in the characteristic excessive deposition of GBM material. It is possible therefore, that differences in the preparation and incubation of glomeruli could influence results on GBM metabolism in diabetes and its alteration by extracellular glucose concentration [5, 6, 31, 32].

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