# Differential Renal Protein Clearance in Diabetes

*G. Jerums, M.D., R. S. Post, M.D., M. Miller, M.D., and E. Barzellato, Cleveland*

SUMMARY

**Measurements of differential renal protein clearance (ratio of the renal clearance of immunoglobulin-G to that of transferrin) have been performed in long-standing diabetics with varying amounts of proteinuria. In seventy-one diabetics the IgG-transferrin clearance ratio (0.44 ± 0.04, mean ± S.E.M.) was significantly lower than in seventeen normal subjects (1.85 ± 0.24). The mean IgG-transferrin clearance ratio was significantly lower in fourteen of these diabetics with normal amounts of proteinuria than in normal subjects. A further decrease in clearance ratio was apparent when nonproteinuric (125 mg./24 hr.) and minimally proteinuric (500 mg./24 hr.) diabetics were compared. Deterioration of renal function in proteinuric diabetics was associated with an increase in the IgG-transferrin clearance ratio. The results taken overall suggest that an initial fall and a delayed rise in the clearance ratio occurs in individuals who progress to terminal renal failure. The initial fall in the IgG-transferrin clearance ratio may reflect the earliest renal changes of diabetic nephropathy. The relationship of this fall to the onset of carbohydrate intolerance remains to be clarified.** DIABETES *22*:104-10, February, 1973.

Recently it has become apparent that changes in the normal pattern of urinary protein excretion accompany most renal disorders. In some cases, as in minimal change nephrotic syndrome ("foot process disease"), these changes in protein pattern are relatively characteristic and, therefore, assume diagnostic and prognostic importance. The impetus for such studies of renal protein excretion was first provided by measurements of the renal clearance of dextrans of varying molecular weight.[1-3] It was found that the renal clearance of dextrans normally decreases with increasing molecular size, and little dextran of 50,000 mol wt or greater appears in the urine. However, in subjects with nephrotic syndromes of certain types, the renal clearance of high molecular weight dextran usually increases so that no sharp cut-off point remains.[4] Measurements of the renal clearance of endogenous plasma proteins in nephrotic subjects have shown a similar increased excretion of the higher molecular weight proteins.[5-7] From clearance measurements of two or more proteins, investigators have derived indices of differential protein clearance (selectivity index) by relating clearances of higher molecular weight proteins to that of albumin or transferrin. While the derivation of such indices of differential protein clearance has no proven link with specific anatomical or functional changes in the kidney, these indices have nevertheless been found useful in classifying nephrotic syndromes.[5,8]

A hiatus in the understanding of differential protein clearance studies has persisted, however, because these measurements have not been performed serially in subjects at risk for renal disease before the onset, during the earliest stages of disease, and as the disease progressed. Hyperglycemia is usually detected in diabetics several years before renal impairment secondary to glomerulosclerosis is detectable. The prime object of this study was to investigate renal protein clearances in diabetics with and without proteinuria and with various degrees of renal functional impairment. In this population we have examined the relative renal clearances of two proteins of differing molecular size, transferrin (mol wt: 60,000) and immunoglobulin-G (mol wt: 160,000).

## MATERIALS AND METHODS

This study was performed on seventeen normal subjects with normal creatinine clearances, normal urinalyses and less than 125 mg. of urine protein per day (Shevky-Stafford), a group of seventy-one ambulatory diabetic subjects and four hospitalized diabetics in terminal renal failure. The ambulatory diabetics had a known duration of diabetes of at least five years, the mean being 13.8 ± 6.5 years (mean ± S.D., range 5 to 30 years). Forty-six of these seventy-one subjects had abnormal proteinuria (over 250 mg. total protein excreted per twenty-four

From the Department of Medicine, University Hospitals of Cleveland, Cleveland, Ohio.

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hours) and in this group the clinical features were compared with the results of the protein clearance measurements. Of these forty-six patients, seventeen (37 per cent) were known to have proteinuria for longer than five years. Two-thirds had known diabetes for longer than ten years. Retinopathy was detected in thirty (65 per cent), of whom sixteen had background and fourteen the proliferative type. Arterial disease of the extremities was present in twenty-seven of these forty-six patients as evidenced by one or more of the following: (1) intermittent claudication; (2) amputation of any part of an extremity; (3) inability to feel at least three out of four pedal pulses or documented loss of one previously palpable pedal pulse. The presence of recurrent urinary tract infections, identified by at least three positive urine cultures (over $10^5$ organisms of the same species per milliliter) was found in seven patients.

In each patient a venous blood sample was drawn and the separated serum stored at 4° C. until assay. A matching, carefully timed urine collection (three to twenty-four hours) was also obtained. The urine samples were centrifuged at 2,000 rpm x 500 g for twenty minutes, filtered through Whatman no. 1 paper, and merthiolate was added to a final concentration of 1:1,000. The urine was then concentrated for twelve to twenty-four hours at 4° C. with appropriate amounts of polyacrylamide gel.* The gel was found to be reusable after washing in running tap water overnight and drying at 60° C. For subjects with normal or trace proteinuria, a full twenty-four-hour collection was used for the concentration procedure which was generally performed in two or three stages. After concentration, all urine samples were passed through a 0.22 $\mu$ millipore filter and stored at 4° C.

In this study the ratio of the renal clearance of immunoglobulin-G (IgG) to that of transferrin was used as an index of differential protein clearance by the kidney.[9]

No attempt was made to document quantitatively the exact degree of concentration of the urine samples. No selective gain or loss of IgG or transferrin could be documented during the concentration process. Since the urine volume term is common to the clearance of both proteins, it and the degree of artificial concentration of both proteins in the urine need not be known to determine the final index used. This index will be called the IgG-transferrin clearance ratio. Individual protein clearances were not determined. The levels of IgG and transferrin in serum and concentrated urine were assayed by radial immunodiffusion[10,11] using commercially available antisera.* The radial immunodiffusion technic was adapted for the assay of proteins in dilute solutions containing as little as 1 to 2 mg./L. of IgG or transferrin, by the use of agar containing appropriately low antibody concentrations. After incubation for eighteen to twenty-four hours at 4°, drying and staining with amido black, the diameters of the immunodiffusion rings were measured along two axes, using a magnifier calibrated to 0.1 mm. (Hyland Laboratories). All samples were assayed in duplicate against a standard derived from pooled normal serum which was applied in at least eight serial dilutions to each slide.

The concentration of creatinine in serum and urine was determined by automatic colorimetry (Technicon AutoAnalyzer Method N-11b). Urinary protein concentration was estimated in unconcentrated samples by phosphotungstic acid precipitation (Shevky-Stafford).[12] In thirty urine samples with the lowest amounts of protein, a more sensitive estimation of protein content was made using the modified Lowry Method.[13,14] Before the latter assay, urinary proteins were precipitated by the addition of trichloracetic acid to a final concentration of 3 per cent (v/v). The supernatant was discarded and the precipitate was redissolved in 0.5 M sodium hydroxide. Protein concentrations were then assayed using crystalline bovine serum albumin as a standard.

The reproducibility of the method used to measure the IgG-transferrin clearance ratio was tested by replicate assays in duplicate. The result of twelve separate determinations of the clearance ratio for a standard serum and urine sample was 0.68 ± 0.03 (mean ± S.E.M.), with a coefficient of variation of 13.2 per cent. The coefficient of correlation for duplicate estimations of the clearance ratio in twelve subjects was 0.92 ($P < 0.01$) when all values for the clearance ratio were less than 0.5, and 0.90 ($P < 0.01$) when all values exceeded 1.0. Serial immunoelectrophoretic studies[15] showed no evidence of differential recovery of IgG and transferrin from the concentration procedure, and this was directly tested in twenty-one patients with heavy proteinuria by determining the clearance ratio of these two proteins before and after at least tenfold concentration. The clearance ratio was 0.48 ± 0.09 (mean ± S.E.M.) before concentration and 0.49 ± 0.07 after

*Lyphogel, Gelman Instrument Co.

* 'L' specific and 'H' specific antiserum to IgG and antiserum to transferrin (Hyland Laboratories, cat. nos. 071-201, 071-211 and 071-215).

concentration, with a correlation coefficient of 0.94 ($P < 0.01$).

To evaluate the possibility that light chain fragments of IgG could account for variation in the IgG-transferrin clearance ratio, 'H' chain specific antiserum was used for the assay of IgG levels in samples from a cross-section of normal and diabetic subjects. In thirty-five samples, the clearance ratio was 1.03 ± 0.15 (mean ± S.E.M.) with 'H' and 'L' chain specific antiserum and 1.06 ± 0.18 with 'H' chain specific antiserum (correlation coefficient 0.95, $P < 0.1$).

The effect of storage at 4° C. on the protein composition of serum and concentrated urine was assessed in a cross-section of samples by serial immunoelectrophoresis, radial immunodiffusion and double diffusion[16] against antisera to transferrin and IgG. No significant variation in protein concentration was observed until after samples were stored for over twelve months. All samples in this study were assayed within three months of collection. The variability of the IgG-transferrin clearance ratio in any one normal individual was tested by serial determinations in two subjects. In three measurements on each subject at intervals of three to four weeks, values for the clearance ratio were 1.25, 1.58, 1.25 and 0.50, 0.55, 0.60 respectively.

Statistical evaluation of differences between means was performed by Student's *t* test. In comparison of clearance ratios, Wilcoxon's rank test confirmed the results of the test. Chi-square analysis was used for comparing sex ratios.

## RESULTS

The IgG-transferrin clearance ratio in seventeen normal subjects was 1.85 ± 0.24 (mean ± S.E.M.) with a range of 0.50 to 3.86. Clearance ratios calculated from previous studies in normal subjects yield results which are widely scattered but generally in the same range as the above.[17-20] By contrast, the clearance ratio in the seventy-one ambulatory diabetics studied was 0.44 ± 0.04 (mean ± S.E.M.) with a range of 0.06 to 1.62, which was significantly lower than the values for the normal group ($P < 0.01$) (table 1). It is important to note that the diabetic group as a whole was older than the normal subjects. The normal subjects had a mean age of 32.8 ± 11.8 years (mean ± S.D.) (range 22 to 60) while the mean age of the seventy-one diabetics was 53.9 ± 14.7 years (range 18 to 80). No significant correlation was demonstrable between age and differential protein clearance in either normal ($r = -0.193$, $t = .763$) or diabetic subjects ($r = -0.054$, $t = .449$). Also, no sex differences were demonstrable in the IgG-transferrin clearance ratio in these subjects.

TABLE 1

Differential protein clearance and renal function of normal subjects and diabetics at least five years after diagnosis

| | IgG-transferrin clearance ratio* | Proteinuria‡ (gm./24 hr.) | Creatinine clearance* (ml./min.) |
|---|---|---|---|
| Normal subjects (n = 17)† | 1.85 ± 0.24 (0.50-3.86) | < 0.13 | 123 ± 8.0 (59-179) |
| Diabetics (n = 71) | 0.44 ± 0.41 (0.06-1.62) | 2.11 ± 0.41 (<0.125-23.15) | 85 ± 5.5 (9-183) |
| P | <0.01 | <0.01 | <0.01 |

* Results are expressed as the mean ± S.E. of mean with the range in parentheses.
† n = number
‡ As determined by Shevky-Stafford technic.

In the diabetic group with proteinuria not exceeding 500 mg./24 hr. (Shevky-Stafford) there was an inverse relationship between IgG-transferrin clearance ratio and quantity of total protein excreted (figure 1). This decrease in clearance ratio was apparent with minimal increases in proteinuria, and became pronounced after total proteinuria exceeded 250 mg./24 hr.

Furthermore, in fourteen diabetics who were indistinguishable from sixteen normal subjects on the basis of total proteinuria as determined by the Lowry Method, a significantly lower IgG-transferrin clearance ratio was observed (0.68 ± 0.1 vs. 1.89 ± 0.25, mean ± S.E.M., $P < 0.01$).

In the forty-six patients with proteinuria exceeding 250 mg./24 hr. there was no further decrease de-

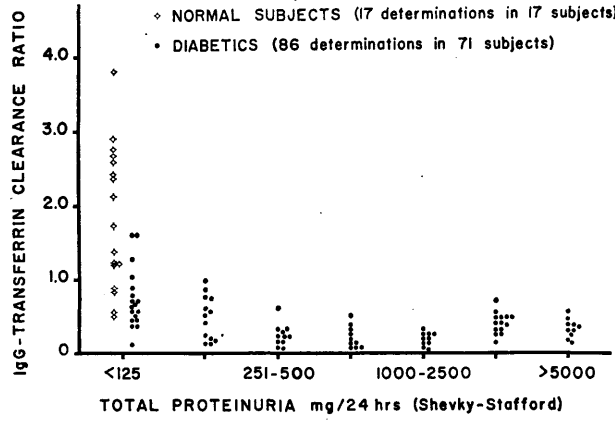


FIG. 1. Differential protein clearance in normal subjects and diabetics with varying degrees of proteinuria.

ectable in the clearance ratios for the group as a whole, s the quantity of protein excreted increased and all alues were between 0.06 and 0.70. When specific clinical findings were related to the clearance ratio in individual patients with abnormal proteinuria, a questionbly significant, higher clearance ratio was observed in he seventeen patients with occlusive arterial disease of he extremities than in the twenty-nine remaining paients (0.34 ± 0.03, and 0.24 ± 0.03, mean ± S.E.M., espectively, $P < 0.05$). However, no differences were ound when patients in this group were classified acording to presence or absence of hypertension, cardiac ailure, coronary artery disease, retinopathy, duration of iabetes or duration of proteinuria. When the IgGransferrin clearance ratios in diabetics with abnormal roteinuria were plotted against creatinine clearance ieasurements, a progressive increase in the clearance atio was evident as renal function (creatinine clearnce) deteriorated. The four patients in terminal renal ailure all had values exceeding 0.65 (figure 2).

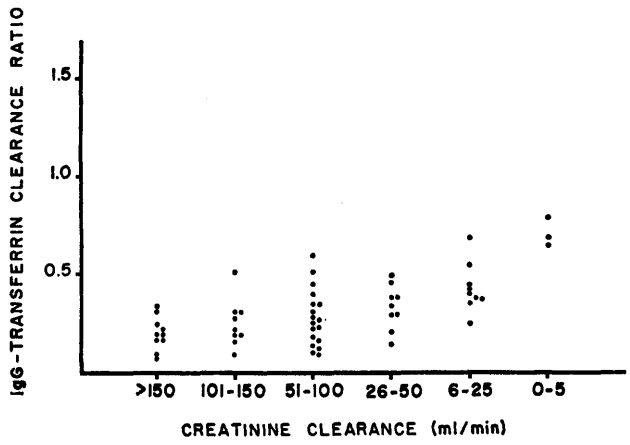


FIG. 2. Relationship between protein clearance and creatinine clearance in diabetics with proteinuria exceeding 250 mg./24 hr. (Four hospitalized diabetics with uremia are included.)

The IgG-transferrin clearance ratio in subjects with ondiabetic renal disease was similar to that observed 1 diabetics with proteinuria. For instance, the clearnce ratio was 0.15 and 0.17 in two subjects with lipoid ephrosis (established by renal biopsy) and 0.15 to .47 in five subjects with membranous glomerulonephritis. Each of the latter five patients had been shown by renal biopsy to have extramembranous subepithelial deposits of immunoglobulin.[21] By contrast, much higher values were obtained in two subjects with end-stage renal disease and in two nondiabetic subjects with long-standing vascular disease (table 2).

Serial determinations of the IgG-transferrin clearance

TABLE 2

Differential protein clearance in patients with intrinsic renal disease and long-standing vascular disease

| iagnosis | Age | Proteinuria (gm./24 hr.) | IgG-transferrin clearance ratio | Creatinine clearance (ml./min.) |
|---|---|---|---|---|
| lomerulonephritis* | | | | |
| ipoid Nephrosis) linimal change | 32 | 2.20 | 0.15† | 186 |
| ipoid Nephrosis) linimal change | 62 | 0.96 | 0.17 | 11 |
| lembranous | 34 | 12.85 | 0.16 | 96 |
| lembranous | 50 | 6.39 | 0.17 | 63 |
| lembranous | 40 | 3.23 | 0.21 | .167 |
| lembranous | 24 | 5.23 | 0.44 | 22 |
| lembranous | 54 | 7.83 | 0.47 | 4 |
| roliferative | 18 | 3.42 | 0.67 | 2 |
| embranoproliferative | 40 | 0.29 | 0.78 | 1 |
| ascular Disease | | | | |
| eneralized arteriosclerosis | 90 | 0.04 | 1.00 | 0.75‡ |
| ssential hypertension | 53 | 0.10 | 1.03 | 53 |

* The diagnosis of glomerulonephritis was based on renal biopsy findings after examination by light microscopy supemented by immunofluorescence studies of thin sections.[21]
† Proteinuria and edema appeared three months after diabetes was first diagnosed. All signs of renal disease disappeared ie month after adrenal corticosteroid therapy was initiated.
‡ Serum creatinine.

ratio were performed on thirteen diabetic patients. Their age at the time of the first assay was 54.3 ± 2.8 years (mean ± S.E.M.), the known duration of their diabetes 12.3 ± 1.7 years, and the duration of their proteinuria 2.7 ± 0.7 years. The second assay was performed after an interval of one to eight months (5.4 ± 0.5, mean ± S.E.M.), at which time no significant change had occurred in the amount of proteinuria, creatinine clearance, or the IgG-transferrin clearance ratio, considering the group as a whole. In five subjects, however, with initial proteinuria of less than 1 gm./24 hr., changes in the clearance ratio were evident. In two of these, an increase in the clearance ratio accompanied a fall in total proteinuria. In another two, a decrease in the clearance ratio accompanied a rise in total proteinuria. By contrast, subjects with greater amounts of proteinuria had relatively constant IgG-transferrin clearance ratios, despite marked variations in the degree of proteinuria in some cases.

Histological studies of renal tissue obtained at autopsy were possible in four subjects. Two subjects died with end-stage diabetic renal disease which was confirmed by light microscopy. In the two patients who died from intercurrent illness, diffuse or nodular glomerulosclerosis was not identified by routine light microscopy, yet they both had low IgG-transferrin clearance ratios (0.26 and 0.33) compared to values found in normal subjects.

## DISCUSSION

The finding of major significance in this study is that a change in renal protein clearance may precede the earliest clinical signs of diabetic renal disease. The decreased clearance ratio is more clearly evident when proteinuria exceeds 250 mg./24 hr., but a unique "diabetic pattern" is not discernible.

Studies of protein clearance in small groups of diabetics with pronounced proteinuria have similarly uncovered no specific pattern and have shown a wide range of results.[6-8,22,23] It is not surprising, therefore, that the comparison of protein clearances in proteinuric diabetics and nondiabetic subjects with other renal diseases has demonstrated some overlap in this study. This finding is also supported by previous studies of protein clearances in intrinsic renal disease, using a variety of technics for the concentration of urine, measurement of protein concentrations and expression of results.[5,6,8] It seems clear, for example, that protein clearance measurements cannot predict the presence of a second glomerular disease in the presence of diabetic glomerulosclerosis: the patient listed in table 2 as having steroid responsive lipoid nephrosis illustrates this point. The fact that a nephrotic syndrome was present soon after the documented onset of hyperglycemia, that renal function was good and that a history was available to document a childhood nephrotic syndrome with spontaneous remission strongly suggested a glomerular disease aside from diabetic glomerulosclerosis. The IgG-transferrin clearance ratio of 0.15 did not clearly distinguish her from other diabetics in the study. Others have documented the occurrence of potentially treatable nondiabetic glomerular disease in diabetic patients.[24]

The association observed in this study between differential protein clearances and decreasing renal function in long-standing diabetics with established proteinuria is in accord with the results of one previous study which suggested a link between the excretion of large proteins such as haptoglobins and the presence of severe diabetic nephropathy,[25] although this latter study did not differentiate between changes in protein pattern and changes in total proteinuria. These changes in protein clearance appear to represent an effect of increasing renal damage, since similar findings have been described in patients with nondiabetic renal disease. By analysis according to functional criteria, it has been found that the renal clearance of the larger plasma proteins is relatively increased in patients whose inulin clearances are reduced.[7,26] By analysis according to renal structural changes on light microscopy, higher IgG-transferrin clearance ratios have usually been associated, also, with the most severe histological lesions, regardless of the disease process.[8]

On the basis of the cross-sectional study presented here, it seems possible that a biphasic variation in the IgG-transferrin clearance ratio occurs over several years in those subjects in whom reduction of renal function progresses to terminal renal failure. According to this hypothesis, the initially high protein clearance ratio (normal) would fall before, coincident with, or shortly after the onset of clinically overt diabetes and this eventually would be associated, though not always, with some increase in the amount of proteinuria. Subsequently, a much more gradual rise in the clearance ratio would occur if and when renal function deteriorated. Whether such changes in differential protein clearance occur in the time course of other renal diseases is not known.

The data which relate to the finding of a decreased clearance ratio in those diabetic patients with normal proteinuria may be explained by at least three hypo-

theses none of which can be excluded by the present study, but which appear worthy of further investigation. One would suggest that if the decreased clearance ratio antedated the carbohydrate defect, the hypothesis proposed by Siperstein[27] based upon studies of muscle capillaries may be correct and that a vascular lesion produces the eventual carbohydrate defect. If the decreased clearance ratio appears concomitant with the carbohydrate defect then they may or may not be causally related. If the decrease in clearance ratio follows the appearance of the carbohydrate defect, then it would appear possible that some alteration in basement membrane metabolism resulting from the carbohydrate defect leads to a decrease in clearance ratio as suggested by Spiro.[28] Studies of families or populations with a high incidence of overt diabetes should permit the answering of this question.

Since the initial changes in protein clearance indices in this study were in general not related to the presence of clinically recognizable vascular complications of diabetes, it may be inferred that vascular factors are not the primary cause of the decreased ratios. Pyelonephritis is an unlikely cause, since no relationship could be demonstrated between the clearance ratio and past or present urinary tract infections in this study and in one previous study.[29] The glomerulus seems the most obvious site for a process which could explain the present results.

It has been suggested that urinary protein composition is determined exclusively by glomerular filtration,[5,30] but more recent studies have demonstrated that the renal clearance of proteins may be independent of molecular size and, largely because of this, have led to the classification of proteinuria into glomerular, tubular and physiological types.[22,31,32] According to this classification, the present results probably represent a transition from the "physiological" to predominantly "glomerular" proteinuria. However, definition of the exact roles of glomerular and tubular factors in the renal clearance of individual proteins in disease states must await more extensive direct measurements of protein concentrations in the glomerular filtrate.

Physiologic proteinuria (high clearance ratios) may conceivably be the result of preferential back diffusion of albumin (transferrin) since recent tubular puncture studies clearly indicate that in the normal rat, albumin does pass out of the more distal portion of the nephron.[33,34] No data are available which effectively rule out inward diffusion of albumin at the same site, the result of peritubular capillary damage, as a cause for decreased clearance ratios in this or other renal disease states. Therefore, while the glomerular changes of diabetic nephropathy, particularly those in and around the glomerular capillary basement membrane, appear to be the most obvious potential cause for alterations in the renal clearance of large proteins such as IgG and transferrin, it is by no means proven that they account for the results observed in this study.

Individual protein clearance measurements were not performed in this study. By analogy from clearance studies in other disease states it would be expected that the renal clearance of a large protein such as IgG would normally be considerably less than that of transferrin: This expectation has not been fulfilled in other studies.[17-20] Moreover, a direct comparison in normal subjects between the differential clearances of proteins and dextrans of similar molecular weight has demonstrated marked disparities which remain unexplained.[35]

It might be expected that the onset of glomerular disease would be accompanied by a reduction of the barrier to the excretion of larger proteins and by the increasing filtration and excretion of proteins of progressively higher molecular weight. If this were so, then a relative increase in transferrin clearance would precede changes in IgG clearance. Such a sequence could account for the initial fall and subsequent rise in the IgG-transferrin clearance ratio which appears to occur in diabetics as they progress toward terminal renal failure. Recent investigations of the urinary protein pattern in nondiabetic patients with trace proteinuria suggest that a relative increase in transferrin excretion is one of the earliest signs of glomerular damage.[36] This is supported by electrophoretic studies of urinary protein composition in diabetics in that recently diagnosed diabetics have shown a relative increase in transferrin excretion as has been found in diabetics with established proteinuria, in common with other causes of "glomerular" proteinuria such as glomerulonephritis, lupus nephritis and amyloidosis.[26] By implication, a decrease in the IgG-transferrin clearance ratio may precede clinically measurable increases in urinary albumin excretion reported to occur in older diabetics a short time after diagnosis.[37] Since a fall in the IgG-transferrin clearance ratio appears to be a constant feature of early diabetic renal disease, this study raises the possibility that differential protein clearance measurements may be of value in identifying subjects who will later develop overt diabetic nephropathy.

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