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CHARACTERIZATION AND ORIGIN OF IMMUNOREACTIVE GLANDULAR KALLIKREIN IN RAT PLASMA

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Abstract—A radioimmunoassay (RIA) for rat urinary kallikrein (RUK), capable of detecting 200 pg of antigen, has been developed and used to help characterize the nature and origin of immunoreactive glandular kallikrein in rat plasma. Although rat plasma did not cross-react in a parallel manner in the RIA, a highly significant ($P < 0.001$) 4-fold increase in antigen was seen after bilateral nephrectomy. To further investigate this finding, plasmas were gel filtered and three peaks of antigen were detected in both sham-operated and nephrectomized rats. The first two peaks appear to represent inhibitor complexes, and the antigenic contents of these peaks were similar in both nephrectomized and sham-operated animals. In contrast, the third peak showed immunological identity to RUK, and the antigenic content of this peak was increased about 6-fold in nephrectomized rats compared to sham-operated controls, hence supporting the data obtained with whole plasmas. In pancreatectomized rats, the antigenic content of whole plasma and the profile obtained after gel filtration did not differ from those of sham-operated controls. Bilateral submandibular/sublingual gland excision, however, caused significant reduction in whole plasma antigen levels and in the antigenic content of Peak 3 when compared to controls. Peak 3 was indistinguishable from purified RUK and rat submandibular kallikrein by gel filtration and behaved like a typical glandular kallikrein on ion-exchange chromatography. Purified Peak 3, however, had no activity against kininogen or the chromogenic substrate S-2266 and showed a consistently smaller molecular weight (M_r 29,500) on sodium dodecylsulfate-polyacrylamide gel electrophoresis than that of RUK (32,000). Hence, glandular kallikrein circulates in rat plasma in an inactive form. The submandibular and sublingual glands appear to be a major source of the plasma antigen, and the kidney plays an important role in the clearance and/or metabolism of glandular kallikrein from plasma.

Glandular kallikreins (EC 3.4.21.8) are serine proteinases that are found in extracts and secretions of all exocrine glands, including the kidney. All glandular kallikreins have similar physicochemical properties and, within a given species, the enzymes from all glands are immunologically identical [1–3]. Immunoreactive glandular kallikrein has been reported previously to exist in rat plasma [4–6]. This study utilizes a recently developed radioimmunoassay (RIA) for rat urinary kallikrein (RUK) in an attempt to characterize more thoroughly the nature of this antigen and to determine its origins.

MATERIALS AND METHODS

The following materials were obtained commercially: sheep immunoglobin and rabbit anti-sheep IgG double antibody (Cappel Laboratories, Downington, PA); ketamine hydrochloride (Parke-Davis, Detroit, MI); acepromazine (Ayerst, New York, NY); $Na^{125}I$ (Amersham/Searle, Arlington Heights, IL); Bio-Gel A-0.5m (Bio-Rad Laboratories, Richmond, CA); DE-52 Cellulose (Whatman Inc., Clifton, NJ); PM-10 membranes (Amicon Corp., Lexington, MA); [^{3}H]TAME (*n*-α-*p* tosyl arginine[^{3}H-methyl] ester (Amersham/Searle); Aprotinin (Sigma Chemical Co., St. Louis, MO); S-2266 (Kabi AB, Stockholm, Sweden); and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards (Pharmacia Fine Chemicals, Uppsala, Sweden). All other chemicals were reagent or ultrapure grade.

Rat renal lymph samples were provided by Drs. S. Nakamura and F. Carone, Northwestern University School of Medicine, Chicago, IL. Purified rat submandibular kallikrein was a gift of Dr. K. Nustad, Radium Hospital, Oslo, Norway. The rat trypsin used was that described by Proud *et al.* [2].

Kallikrein labeling. Pure RUK [7] was labeled via a modification of the method of Hunter and Greenwood [8]. Ten microliters of a 1 mg/ml solution of kallikrein was mixed on ice, with 60 μl of 0.1 M Tris–HCl/0.025% NaN_3, pH 7.4, and 5 μl (0.5 mCi) of $Na^{125}I$. Twenty-five microliters of a 2 mg/ml solution of chloramine T in water was added, with mixing, and the reaction was allowed to proceed for 2 min on ice. Fifty microliters of aqueous $Na_2S_2O_5$

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(10 mg/ml) and 50 μl of aqueous KI (10 mg/ml) were then added. Labeled protein was separated from free iodide by two successive gel filtrations on a Sephadex G-75 column (0.8 × 19 cm) equilibrated with 0.1 M Tris–HCl/0.025% NaN_3/0.1% bovine serum albumin (BSA), pH 7.4. The specific activity of iodinated RUK was about 15 μCi/μg, determined by measurements of known volumes with a calibrated well counter and by measurement of the areas under gel filtration peaks on a graph. The labeled protein was stored at −80° and was stable for a minimum of 6 weeks.

Radioimmunoassay (RIA). Assays were carried out in 0.1 M Tris–HCl/0.025% NaN_3/0.1% BSA, pH 7.4 (RIA buffer), and all dilutions were made in this buffer. Standard (200 μl) or unknown (200 μl), at a suitable dilution, was incubated with 100 μl of labeled RUK (7000–8000 cpm) and 100 μl of sheep antiserum to RUK [7] for 18–24 hr at 4°. Standards were assayed in triplicate, and unknown samples were assayed in duplicate at a minimum of two dilutions. Antigen–antibody complex was separated from free antigen by immunoprecipitation as follows: 10 μl of a 0.5 mg/ml solution of sheep IgG was added to each tube, followed by 300 μl of an optimum dilution of rabbit anti-sheep IgG, and all tubes were incubated in a shaker bath for 2 hr at 25°. Buffer (2 ml) was added to each tube and the antigen–antibody complex was sedimented by centrifugation at 3200 *g* for 10 min in a Sorvall RC2-B centrifuge. The supernatant fraction was removed to a fixed mark by aspiration. The buffer addition, centrifugation, and aspiration procedures were repeated, and radioactivity associated with the precipitated antigen–antibody complex was counted in a Beckman 8000 gamma-counter. Results were calculated using the computer program described by Faden and Rodbard [9].

Rat experiments. Male Sprague Dawley rats, 200–250 *g* (Taconic Farms, Germantown, NY), were placed in individual stainless-steel metabolic cages, and 24 hr urine samples were collected under toluene. Urine samples were filtered through coarse filter paper, sodium azide was added (final concentration = 0.025%), and urines were stored at 4°. For the surgical procedures, the rats were anesthetized with ketamine hydrochloride (70 mg/kg) plus acepromazine (0.7 mg/kg). Blood was collected in heparin (25 units/5 ml of whole blood), and centrifuged at 600 *g* for 15 min at room temperature. Plasma was removed, made to 0.1% NaN_3, and stored at −80° until used.

Subtotal (90–95 per cent) pancreatectomies were performed by the method of Scow [10], and plasma antigen levels were examined 1–5 days after surgery. Plasma was also drawn 5 days after bilateral submandibular/sublingual gland excision and 1–2 days after bilateral nephrectomy. In a combined experiment, bilateral submandibular/sublingual gland excision and bilateral nephrectomy were performed simultaneously, and plasma was obtained 24 hr later. Finally, in another experiment, bilateral submandibular/sublingual gland removal was performed on day 1, bilateral nephrectomy was performed on day 4, and plasma was obtained on day 5.

All animals were allowed food and water *ad lib.* except for the day before surgery and on the day prior to drawing blood. On these days food was removed, but animals were allowed free access to water.

Gel filtration studies. Pooled rat plasmas (5 ml) were gel filtered at 22° on a Bio-Gel A-0.5m column (2.6 × 99 cm) equilibrated with 0.01 M Tris–HCl/0.15 M NaCl, pH 7.4. Rat urinary kallikrein and rat submandibular kallikrein were used as reference proteins.

Molecular weight (M_r) determination. After gel filtration, the molecular weights of the major peak of the immunoreactive glandular kallikrein in plasma and of purified RUK were estimated by SDS-PAGE, according to the method of Weber and Osborn [11]. Unstained gels were frozen on Dry Ice and sliced into 2 mm segments with a Bio-Rad slicer system. Each slice was homogenized with a teflon pestle in 0.5 ml of 0.1 M Tris–HCl, pH 7.4, and left overnight at 4°. The supernatant fraction was assayed for immunoreactive glandular kallikrein.

Further purification of the major immunoreactive antigen peak in uremic plasma. Heparinized plasma was obtained from rats 24 hr after bilateral nephrectomy. The plasma (22 ml) was fractionated between 25 and 75% saturation of ammonium sulfate. The sediment was resuspended in, and dialyzed against, 0.01 M Tris–HCl/0.15 M NaCl, pH 7.4, for 24 hr at 4°. The dialysand was concentrated by ultrafiltration through a PM-10 membrane and 5 ml of the fraction was then gel-filtered on a Bio-Gel A-0.5m column as described above. Those fractions corresponding to the major antigen peak in plasma were pooled and concentrated by ultrafiltration. Two milliliters of this major antigen preparation was added to a DE-52 cellulose ion-exchange column (0.9 × 30 cm) equilibrated with 0.1 M NaCl/0.01 M sodium phosphate, pH 7.0. The eluents were 100 ml of this equilibration buffer followed by a 100 ml linear gradient from 0.1 M NaCl/0.01 M sodium phosphate, pH 7.0, to 0.5 M NaCl/0.01 M sodium phosphate, pH 7.0. Two-ml fractions were collected at a flow rate of 6 ml/hr.

Bioassay. Partially pure rat kininogen was prepared by 33–46% ammonium sulfate fractionation of rat plasma [12]. Human kininogen [13] was also used as substrate. Kininogenase activity of enzyme preparations was determined by their abilities to release kinin from these preparations. Generated kinin was measured by bioassay on the isolated rat uterus [14].

Plasma kallikrein assay. Rat plasma kallikrein was assayed as described previously for human plasma [14] using a reference lot of plasma protein fraction [15] as a source of Hageman factor.

RESULTS

Radioimmunoassay. Antiserum and double antibody titer curves were performed in the absence of non-labeled antigen. Antiserum to RUK was used at a final dilution of 1/400,000 to obtain about 40 per cent binding, whereas double antibody was used at an initial dilution of 1/30 for optimum precipitation of antigen–antibody complex. Complete precipita-

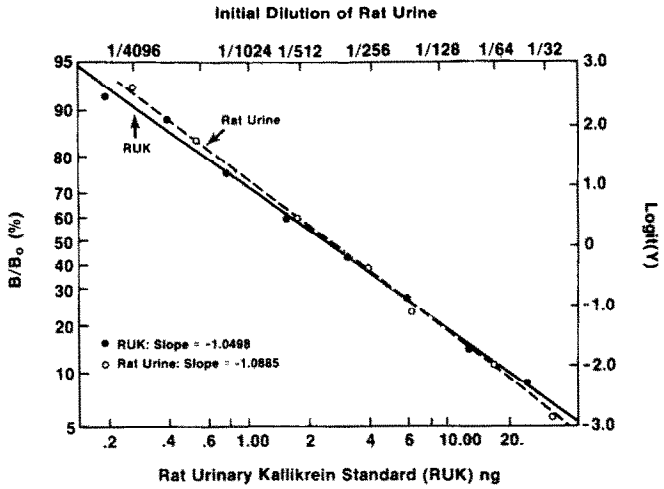

Fig. 1. Comparison of logit-log plots of RUK standard (●——●) and normal rat urine (○——○).

tion of the complex was achieved during a 2-hr incubation.

A typical standard curve for RUK, expressed in the form of a logit-log plot, is shown in Fig. 1. With a bound percent (B/Bo) of 96 ± 1 (mean ± S.D.) used as the limit of detectability, the assay could detect a minimum dose of about 200 pg. Serial dilutions of rat urine (Fig. 1), purified rat submandibular kallikrein, and rat renal lymph showed immunological identity to the RUK standard. Rat trypsin, lysyl-bradykinin, pepsin, and human urine showed no significant cross-reaction. Immunoreactive antigen in rat plasma was detected but did not cross-react in a manner parallel to the standard curve. When an individual urine sample was tested six times in the same assay, the intraassay coefficient of variation (CV) was 2 per cent (S.E.M./$\bar{x}$). Measurements of six different urines in eight assays gave an interassay CV of 5 per cent. Assays on normal rat urines (N = 6) detected 94 ± 12 μg antigen/24 hr (mean ± S.E.). Recovery of RUK added to plasma was 100 ± 3 per cent (N = 6).

Effects of bilateral nephrectomy. Heparinized plasma obtained 48 hr post-nephrectomy (bilateral) showed about a 4-fold increase in antigen compared to sham-operated controls (Table 1). Since the antigen in plasma did not cross-react in a manner parallel to the standard curve, the increase in antigen after nephrectomy was considered semiquantitative. Nevertheless, the results were highly significant ($P < 0.001$) and were not due to non-specific effects of uremia since dialysis of the plasma samples against RIA buffer for 30 hr decreased the total amount of antigen recovered but did not alter the ratio of antigen found in uremic compared to normal plasma (784/177 = 4.4 predialysis vs 251/55 = 4.6 postdialysis; N = at least six in each group).

Gel filtration of plasmas from sham-operated and nephrectomized rats revealed three peaks of antigen in each case (Fig. 2, A and B). A comparison of the profiles revealed that the first two peaks had similar antigenic levels in both control and nephrectomized animals. Peak 3, however, was increased dramatically in the plasma from nephrectomized rats. When

Table 1. Immunoreactive glandular kallikrein in rat plasma

Surgical procedure (N)	Antigen* (ng/ml)
Sham operation† (12)	177 ± 7
Pancreatectomy (6)	188 ± 11
Bilateral submandibular/sublingual gland resection (6)	111 ± 6‡
Bilateral nephrectomy (6)	784 ± 6‡
Bilateral submandibular/sublingual gland resection + nephrectomy (8)	238 ± 19§, ‖

* Values are $\bar{x}$ ± S.E.M.
† No difference between various types of sham operators.
‡ Significantly different from controls ($P < 0.001$).
§ Significantly different from bilateral nephrectomy ($P < 0.001$).
‖ Significantly different from bilateral submandibular/sublingual gland excision ($P < 0.001$).

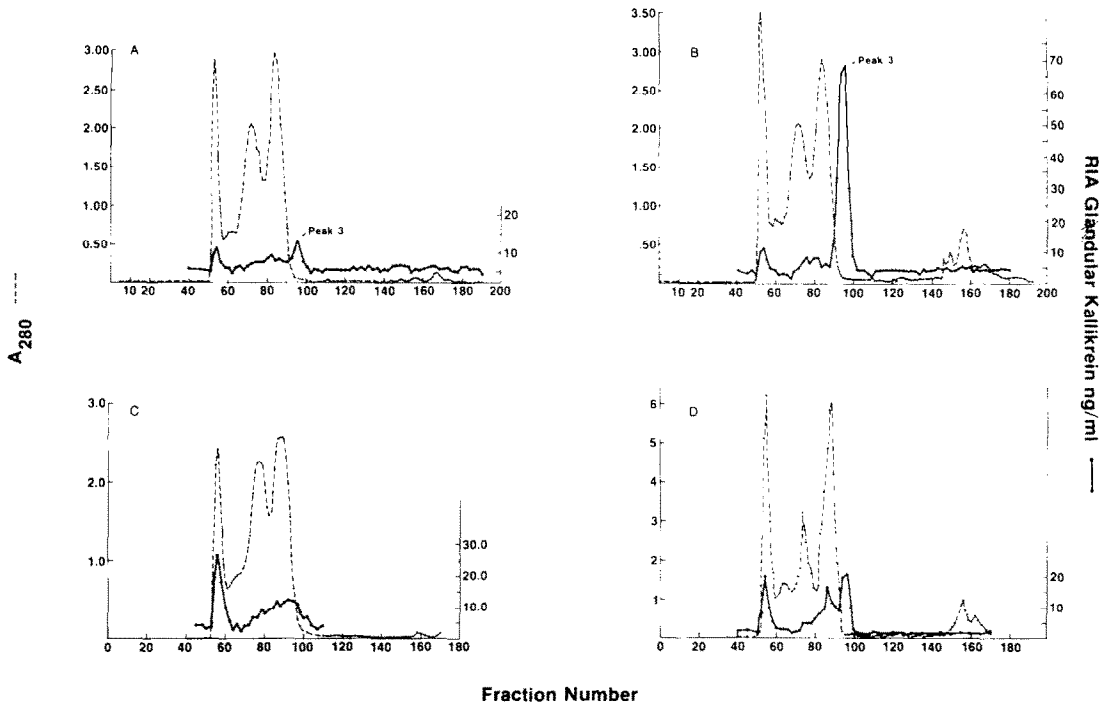

Fig. 2. Bio-Gel A-0.5m gel filtration of pooled rat plasmas. Column, 2.6 × 99 cm; sample volume, 5 ml; eluant, 0.01 M Tris–HCl/0.15 M NaCl, pH 7.4; fraction volume, 3 ml; flow rate, 18 ml/hr. Key (● - - - ●) A_{280}; and (●——●) ng antigen/ml. (A) Sham-operated animals; (B) bilaterally nephrectomized animals; (C) bilateral submandibular/sublingual gland excision; and (D) salivary gland excision + bilateral nephrectomy.

fractions from each peak were pooled, serially diluted, and assayed by RIA, only Peak 3 showed cross-reaction that was parallel to the standard curve. When plasma from either sham-operated or nephrectomized rats was gel-filtered, the total cumulative recovery in Peaks 1, 2 and 3 was 29 and 31 per cent, respectively, of the applied sample. No difference was seen between plasmas drawn 24 or 48 hr after bilateral nephrectomy. Addition of plasma protein fraction generated a peak of esterase activity at fraction 64 in both sham and nephrectomized plasmas. This corresponds to the expected position of plasma kallikrein and suggests that the antigen peaks in plasma are not due to plasma kallikrein.

Effect of pancreatectomy or salivary gland excision. Immunoreactive glandular kallikrein in plasma obtained from pancreatectomized rats did not differ from that obtained from sham-operated animals (Table 1). Gel filtration of plasma from rats after pancreatectomy gave a profile indistinguishable from that seen for control plasma. No difference was seen begween plasmas drawn 1 or 5 days after pancreatectomy.

Plasma drawn 5 days after bilateral submandibular/sublingual gland resection had antigenic levels that were significantly ($P < 0.001$) reduced to 70 per cent of the levels of sham-operated controls (Table 1). Gel filtration of plasma obtained after salivary gland resection showed a broadening of Peak 2 and an apparent decrease in Peak 3 (Fig. 2C).

When urines collected pre- and post-surgical procedures were analyzed by RIA, no difference was seen between urines from pancreatectomized or sham-operated animals or from rats that had undergone salivary gland resection. In all cases, however, pre-operative urinary kallikrein was about 1.5 times post-operative values (Table 2).

In rats that had undergone the staggered bilateral salivary gland resection/bilateral nephrectomy procedure, antigenic content of whole plasma was less than one-third the levels in plasma from nephrectomized animals but still one-third higher than the sham-operated controls (Table 1). Gel filtration of plasma after the staggered surgery showed a level of antigen in Peak 3 that was comparable to that in sham-operated plasma (Fig. 2D). Gel filtration of plasma obtained 24 hr after simultaneous bilateral submandibular/sublingual gland resection and bilateral nephrectomy gave an antigenic profile identical to that for the staggered experiment (Fig. 2D).

Characterization of Peak 3. Purified rat urinary kallikrein and rat submandibular kallikrein had gel filtration elution volumes indistinguishable from that of Peak 3. When trypsin was incubated with Peak

Table 2. Effects of surgical procedures on levels of urinary kallikrein

Surgical procedure (N)	Antigen* (μg/24 hr) Pre-operation	Post-operative
Sham operation (6)	94 ± 16	54 ± 15
Pancreatectomy (6)	98 ± 12	56 ± 10
Bilateral submandibular/sublingual gland resection (6)	92 ± 10	60 ± 11

* Values are $\bar{x}$ ± S.E.M.

3 antigen for 20 min at room temperature, and the mixture applied to the Bio-Gel column, only one peak of antigen was detected. This peak eluted at an elution volume identical to that of Peak 3 alone. When compared by SDS-PAGE, purified RUK had an M_r of 32,000 while Peak 3 antigen had an M_r of 29,500.

Further purification of Peak 3 using DE-52 ion-exchange chromatography gave the results shown in Fig. 3. Most of the antigen eluted at about 0.01 M sodium phosphate/0.25 M NaCl, pH 7.0. The fractions corresponding to this peak were pooled, concentrated by ultrafiltration to a final volume of 0.9 ml (2.5 μg antigen), and assayed for activity against kininogen and a synthetic substrate.

Both the ion-exchange fraction and the kininogen used contained negligible kininase activity and no detectable kallikrein inhibitor. Incubation of 200 ng of antigen with kininogen for 1 hr at 37° failed to generate detectable kinin using a muscle strip sensitive to 2 ng of lysyl-bradykinin. By comparison,

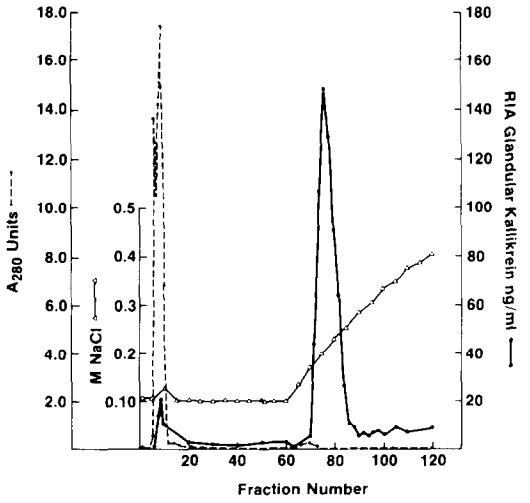

Fig. 3. DE-52 ion-exchange chromatography of Peak 3. Column, 0.9 × 30 cm; sample volume, 2 ml; eluents, 100 ml of 0.01 M sodium phosphate/0.1 M NaCl, pH 7.0, then a 100 ml linear gradient from 0.01 M sodium phosphate/0.1 M NaCl, pH 7.0, to 0.01 M sodium phosphate/0.5 M NaCl, pH 7.0; flow rate, 6 ml/hr; fraction volume, 2 ml. Key: (● - - - ●) A_{280}; (△–△–△) conductivity gradient; and (●——●) ng antigen/ml.

50 ng RUK generated about 100 ng lysyl-bradykinin under identical conditions. In addition, when assayed against the chromogenic substrate for glandular kallikrein (S-2266), the ion-exchange antigen showed negligible activity while one-fourth the amount of RUK could easily be detected. The ion-exchange fraction could not be activated by trypsin.

DISCUSSION

A sensitive, specific RIA for rat urinary kallikrein, capable of detecting 200 pg of antigen, has been developed. The assay gave estimates for the antigenic contents of rat urines that were in good agreement with those of Shimamoto *et al.* [16] and detected antigen in rat plasma at levels comparable to those first reported by Nustad *et al.* [4].

The source of the immunoreactive antigen in plasma has not hitherto been determined, although it has been suggested that several organs contribute kallikrein to the circulation. Roblero *et al.* [17] discovered kallikrein in the renal vein perfusate of the isolated rat kidney, while DeBono and Mills [18] found kallikrein in dog renal lymph. In addition, pancreatic kallikrein is absorbed across the intestinal wall [19, 20], and Ørstavik *et al.* [21] found that, if labeled kallikrein were injected into rat submandibular gland via the main duct or intraglandularly, radioactivity could be traced into venous blood. To obtain more definitive evidence we studied the effects of the removal of various glands on levels of immunoreactive kallikrein in plasma.

Bilateral nephrectomy caused an unexpected 4-fold increase in the antigenic content of whole plasma compared to sham-operated controls. The interpretation of these data was complicated by the fact that, in agreement with other reports [4, 6], plasma did not cross-react in a parallel manner in the RIA. Nevertheless, the difference seen was highly significant ($P < 0.001$) and warranted further investigation. Gel filtration of rat plasma on Bio-Gel A-0.5 m revealed three antigenic peaks corresponding to those described by Nustad *et al.* [22]. The first two peaks did not show immunological identity to RUK and probably represent kallikrein complexed to the two inhibitors of glandular kallikrein described by Hojima *et al.* [23]. In contrast, the third peak showed immunological identity to RUK. Comparisons of the gel filtration profiles of plasma from sham-operated and bilaterally nephrectomized rats showed no difference in the first two peaks but Peak

3 was dramatically increased after nephrectomy, supporting the data obtained with the whole plasma.

These results are compatible with two previous reports. MacFarlane *et al.* [24] showed that the increase in vascular permeability caused by intravenous injection of glandular kallikrein was enhanced by nephrectomy, while Mills *et al.* [25] reported that intravenously injected radiolabeled kallikrein was degraded by the kidneys, and the plasma half-life of the kallikrein was prolonged by nephrectomy. Hence, the results from this study support the concept that the kidney plays a role in clearing and/or metabolizing glandular kallikrein from plasma. Further evidence that the kidney is not a major source of glandular kallikrein in plasma comes from a collaborative study* between the present authors and F. Carone and S. Nakamura (Northwestern University School of Medicine, Chicago, IL). Measurements were made on rat renal lymph and on plasmas obtained simultaneously from a renal artery and a renal vein. Although these results cannot be regarded as definitive, due to the non-parallel cross-reaction of plasma, we were unable to detect any arterio-venous difference across the kidney, and renal lymph contained levels of antigen that were not significantly greater than those in plasma. By contrast, it has been reported that renin is found in much higher concentration in lymph than in venous blood [26].

Plasma from animals that had undergone subtotal pancreatectomy did not appear to differ from control plasma either in gel filtration profile or when antigen was measured in whole plasma. This suggests that the pancreas is not the major source of the antigen in plasma, although the 5–10 per cent of the organ left to prevent severe diabetic ketoacidosis [10] may potentially contribute antigen to the circulation.

After bilateral submandibular and sublingual gland excision, however, immunoreactive antigen in whole plasma was reduced significantly compared to sham-operated controls and, upon gel filtration, it appeared that Peak 3 was reduced in comparison to normal plasma. The level of Peak 3 in normal plasma, however, is fairly low and so decreases related to this level are not conclusive. It was decided, therefore, to perform combined experiments in which salivary gland resections were repeated with bilateral nephrectomies performed either simultaneously or 4 days later. In both combined experiments, antigen levels in whole plasma were greatly reduced compared to bilateral nephrectomy alone and Peak 3 was dramatically reduced compared to the large peak seen after nephrectomy alone. These results suggest that the submandibular and sublingual glands are a major source of immunoreactive glandular kallikrein in rat plasma. Nevertheless, antigen is still present after removal of these glands and may be derived from the parotid gland, pancreas, intestine, or other sources.

A comparison of 24 hr urine collections obtained pre- and post-operatively revealed no apparent differences between pancreatectomized, salivary gland resected and sham-operated animals. In all cases, however, pre-operative urinary kallikrein levels were higher than post-operative levels either 1 or 5 days after surgery. The causes for the reduction in urinary kallikrein are not known but may be related to anesthesia, changes in blood pressure, or reduced renal blood flow [27].

It had been suggested previously that Peak 3 may represent free glandular kallikrein [4]. In the present study it was decided to characterize the peak more thoroughly. Rat urinary kallikrein and rat submandibular kallikrein eluted from the gel filtration column at an elution volume identical to that of Peak 3. Treatment of Peak 3 with trypsin did not change its elution volume on gel filtration, so it seems unlikely that the antigen is complexed with any large molecular weight inhibitor. It is also unlikely that Peak 3 antigen represents plasma kallikrein, since treatment with plasma protein fraction generated activity at a much higher molecular weight but did not generate activity in the region of Peak 3. On DE-52 ion-exchange chromatography, Peak 3 antigen showed typical glandular kallikrein behavior, eluting at 0.01 M sodium phosphate/0.25 M NaCl. The antigen recovered from the DE-52 column, however, was not biologically active and had no action on the glandular kallikrein substrate, S-2266. On SDS-PAGE, RUK had an M_r of 32,000 while Peak 3 was consistently slightly smaller with an M_r of 29,500.

Hence, Peak 3 appears to represent glandular kallikrein that has been inactivated in such a way as to leave the molecule almost intact and immunologically unchanged. The submandibular and sublingual glands appear to be the major source of this antigen. It is not known if the antigen enters the venous effluents of the glands and is inactivated after entering the circulation or if the enzyme is swallowed in saliva, inactivated in the stomach, and then absorbed across the intestine. Ultimately, the antigen is cleared by the kidney where it may contribute to urinary antigen levels, but not to levels measured by esterase or biological activity.

* Unpublished results.

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