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QUANTIFICATION OF RAT T-KININOGEN USING IMMUNOLOGICAL METHODS

APPLICATION TO INFLAMMATORY PROCESSES

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Abstract—Antibodies raised in rabbits against rat T-kininogen (alpha$_1$-cysteine proteinase inhibitor) were used to develop a radioimmunoassay and a nephelometric quantification for T-kininogen. These assays were specific and analytically reliable. We also described a radioimmunoassay for kinin measurement. These immunological methods have been used to study the behaviour of T-kininogen during inflammatory processes and specify the two properties of this kind of kininogen: its inhibitory capacity towards cysteine proteinases and its activity as precursor of T-kinin.

Control plasma level of T-kininogen in male rats was lower than that of female rats. The maximum level was observed in plasma, liver, kidney and uterus of female rats during metestrus. After turpentine injection, T-kininogen level increased not only in plasma but also in liver and kidney. In carrageenan-induced peritoneal exudates, we found a large accumulation of T-kininogen and of immunoreactive kinins, these latter being identified by HPLC as bradykinin.

The plasma of most species contains two kinds of kininogen: low and high molecular weight kininogens, which are multifunctional proteins. They are precursors of kinins, pro-inflammatory peptides. High molecular weight kininogen is a non-enzymatic cofactor of the activation of Hageman factor by plasma kallikrein. Recently, a third property of kininogens has been described: by their heavy chain, they inhibit the enzyme activity of cysteine proteinases (review in Ref. 1). Simultaneously, Okamoto and Greenbaum [2, 3] discovered a second low molecular weight kininogen, T-kininogen, in rat plasma. Contrary to the two others, T-kininogen is not a substrate for tissue and plasma kallikreins, but it releases T-kinin, Ile-Ser-bradykinin, when incubated with large amounts of trypsin, of cathepsin D and of endopeptidase K [2, 4]. T-kininogen was found to be similar if not identical to alpha$_1$-major acute phase reactant of the rat, or alpha$_1$-cysteine proteinase inhibitor. The plasma concentration of this antiprotease increases largely during inflammatory processes [1].

We have previously purified T-kininogen from rat plasma [5]. In this work, we developed two immunological methods to quantify T-kininogen in plasma and in various organs of the rat. These methods will allow us to study the behavior of this acute phase protein in different inflammatory processes and by this way to evaluate the antiprotease role of kininogens.

MATERIALS AND METHODS

T-kininogen was purified from plasma of Wistar and deficient Brown Norway rats as described elsewhere [5]. This material was analytically homogeneous in SDS polyacrylamide gel electrophoresis and in analytical HPLC gel filtration on a Superose 12 column. The specific activity of this purified material incubated with trypsin was 13 μg of T-kinin/mg of total proteins and its calculated molecular weight was 68,000 D. In any case, the purified molecule did not release any kinin myostimulating activity or any immunoreactive kinin with plasma or glandular kallikreins [5].

Monoclonal antibodies and polyclonal antisera have been prepared, but at this stage of our work only polyclonal antisera have been used. These antisera were induced in rabbits by the immunisation scheme of Vaitukaitis *et al.* [6]. Fifty microgrammes of T-kininogen were dissolved in 0.5 ml of isotonic saline and homogenised with 0.5 ml of complete Freund adjuvant. This emulsion was injected intradermally, at multiple sites along the spinal column. Five sets of booster injections, about 50 each, were given over three months.

Radioimmunoassay of T-kininogen

Labelling of the T-kininogen. T-kininogen (10 μg) was radiolabelled with Na^{125}I (Amersham International, Amersham, Bucks, U.K.) using 20 μg of Iodogen (Pierce Chemical Co., Rockford, U.S.A.) as previously described [7]. To purify the radiolabelled kininogen, the reaction mixture was successively chromatographed through a 0.5 × 25 cm column of Sephadex G 50 (Pharmacia Upsala, Sweden) and through a 0.8 × 80 cm column of Sephadex G 100 The specific activity of the undegraded immunoreactive T-kininogen was 140 mCi/g of protein, corresponding to four iodinated tyrosine per molecule. The total number of tyrosine residues per molecule was previously calculated as equal to 13 [5].

One hundred microlitres of samples, increasing quantities of unlabelled kininogen or 100 μl of diluted plasma (10^{-3} to 4.10^{-5}) and antiserum (final dilution 10^{-4}) were incubated with the labelled kininogen (20000 cpm) at 4° in a total volume of 0.4 ml of incubation buffer $KH_2PO_4/Na_2HPO_4.2H_2O$ 200 mM, pH 7.5 containing per liter 5 g of bovine albumin, 0.1 g of NaN_3 and 0.37 g of EDTA.

After 4 hr, we stopped the reaction by adding 2 ml of isotonic saline containing sheep anti-rabbit gamma globulin antiserum (final dilution 100-fold), polyethylene glycol 6000 (40 g/L) and microcristalline cellulose (500 mg/L). After incubation at room temperature for 30 min and centrifugation at 1000 *g* for 15 min, the supernatant was decanted, and the radioactivity of the pellet counted.

The calibration curves were expressed in logit/log coordinates. The parallelism of the curves was tested according to Faden and Rodbard [8].

Measurement of T-kininogen by laser nephelometry

The assays were performed on the Behring nephelometer analyser (BNA Behringwerke, Marburg, F.R.G.) using the following parameter settings: sample dilution 1/20; sample volume 2.5 μl; antiserum dilution 1/3; antiserum volume 40 μl; reaction buffer 160 μl; reaction time 6 min. The standardization was performed using a pool of rat plasma previously calibrated by the RIA method. Plasma samples were previously cleared with Lipoclean using the instructions of the manufacturer (Behringwerke).

Samples for the quantification of T-kininogen

We used Wistar rats of both sexes weighing about 220 g. Vaginal smears were withdrawn from untreated female rats and examined by phase contrast microscopy for the determination of the time of the estrus cycle. In a first series of experiments, male rats were left intact or treated by a subcutaneous injection of turpentine (0.1 ml/100 g). In a second series, male rats were treated by an intraperitoneal injection of 5 ml of lambda carrageenan solution (1% in saline). We used also Brown Norway rats from the strain BN/May Pfd f. The plasma of these latter animals contains T-kininogen but is devoid of the two other kininogens [9].

The animals were anesthetized by exposure to ether. Blood was taken by cardiac puncture and additioned with sodium citrate (0.1 vol., 3.8%). The abdominal aorta was cannulated and the inferior vena cava was sectioned near the heart. The abdominal organs were perfused with 100 ml cold Tyrode solution and then the liver, the kidneys and the uterus were quickly excised. These organs were homogenized in cold Tyrode medium (1 g in 4 ml) with an Ultraturax. The homogenates were centrifuged at 750 g for 10 min, and the supernatants frozen at −25° until assay. Plasma was separated by blood centrifugation at 2500 *g* for 15 min and stored at −25°. All the animals were killed at about 10.00 a.m.

The peritoneal exudates were withdrawn through a midline incision and divided in three fractions. The first fraction was directly mixed with cold ethanol (final dilution: 80%). The second fraction was stored at −25°. The third fraction was used for the determination of the leucocyte level which was performed by phase contrast microscopy using a Thoma's plate. The fractions mixed with ethanol were centrifugated at 2500 *g* for 10 min. The supernatant was evaporated and the dried residue was dissolved in phosphate buffer for measurement of the kinin content.

The frozen samples were used for the immunological assays of T-kininogen and for the quantification of total proteins by the biuret method, allowing the expression of T-kininogen concentration in mg/g total proteins.

Measurement and identification of kinins

We used bradykinin and kallidin from UCB (Belgium), T-kinin, trypsin and *N*-tosyl-L-phenylalanine choloromethyl ketone (TPCK) from Sigma (St. Louis, MO). The radioimmunoassay of the kinins was performed using similar conditions as the ones described for T-kininogen, except that albumin was substituted by lysozyme (1 g/L) in the incubation buffer. The antiserum was obtained in rabbits against bradykinin coupled to swine thyroglobulin and used at a final dilution of 10^{-5}. Radioiodinated bradykinin was purchased from NEN (Bruxelles, Belgium) and the calibration curves ranged from 0.1 to 1000 ng of bradykinin (Sigma). In this system and on a molecular basis, kallidin, T-kinin and des-Arg^9-bradykinin showed a complete cross-immunoreactivity.

The identification of the kinins was obtained by separation of peptides by a reverse phase HPLC. Purified T-kininogen or plasma samples from a pool of plasma from five rats were hydrolysed with trypsin and the reaction was stopped by adding ethanol (80%). After centrifugation, the supernatant was lyophylized and the dried residue was dissolved in phosphate buffer. The kinins in the extract were separated by a reverse phase HPLC, using a column (0.46 × 25 cm) of TSK-gel ODS-120 T 5u (LKB). Peptides were eluted at a flow rate of 1 ml/min at ambient temperature, with 20% acetonitrile in phosphate buffer (0.05 M, pH 2.5), 20 sec fractions were collected and kinins detected by radioimmunoassay. The recovery of this assay was 72%.

Statistical analysis

The intra-assay variance was estimated by calculating the variation of 10 determinations of the same plasma run in the same assay and also of each point of the calibration curve. The inter-assay variance was assessed by measuring six different plasmas in six different assays.

Results are expressed as mean ± SEM either in μg/ml of plasma or in μg/mg of total proteins. The statistical evaluation was performed by analysis of variance. Results were judged to be significantly different when a value of $P < 0.05$ was obtained.

RESULTS

RIA of plasma T-kininogen: calibration curve and assay characteristics

Figure 1 shows the calibration curve for T-kininogen quantification when the final dilution of the antiserum was 2.5×10^{-5}. This calibration curve was linear between 0.025 and 1 μg/ml. The within run assay coefficient of variation (CV) at various points

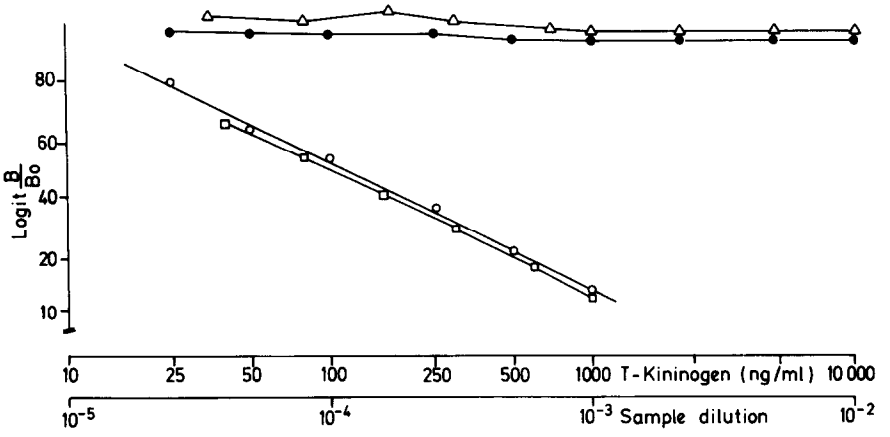

Fig. 1. Radioimmunoassay of T-kininogen. Calibration curve (○), animal plasma (△), Wistar and Brown Norway rat plasmas (□), high molecular weight kininogen, bradykinin and T-kinin (●).

of the calibration curve was < 5%.

When specificity was tested, none among the various animal plasmas (chimpanzee, horse, rabbit, mouse, goat, cat, dog and cow) displaced labelled T-kininogen from the antiserum. A lack of immunoreactivity was also observed for rat high molecular weight kininogen, T-kinin and bradykinin. However, complete and parallel reaction was obtained for progressive dilutions (1/1000–1/25000) of a pool of Wistar rat plasmas containing 800 μg/ml of T-kininogen. Moreover, an identical reaction was obtained for Brown Norway rat plasma.

Nephelometric quantification of T-kininogen

Figure 2 shows the calibration curve obtained on the Nephelometer Analyser, when scattered light intensity was expressed in dependence on antigen concentration. This calibration curve ranges from 120 to 3.75 μg of T-kininogen per ml allowing a determination of plasma samples between 2400 and 75 μg/ml (sample dilution 1/20). In this case, too, the precision was excellent. In fact, for each calibration value, the coefficient of variation was lower than 5% (Fig. 2). The correlation between the two immunological methods was excellent (Fig. 3). However, the relationship between the values obtained by the two methods indicated that the results obtained by nephelometry were higher than the values measured with the radioimmunoassay.

Immunoreactive T-kininogen in biological samples

T-kininogen in normal Wistar rats. In normal male Wistar rats, the plasma level of T-kininogen was 272 ± 40 μg/ml or 5.4 ± 0.6 μg/mg of total proteins (N = 12). In female Wistar rats, the plasma level of T-kininogen changed with the time of the estrus cycle. Minimum values were recorded during the proestrus. Levels remained relatively low during the estrus and then increased till the metestrus (Table 1). Comparatively, the plasma level of male Wistar rats was significantly different ($P < 0.05$ to $P < 0.001$) from the ones measured in the female rats except during the proestrus.

We detected the presence of immunoreactive T-kininogen in the homogenates of the liver, of the kidneys and of the uterus. During the estrus cycle, the levels of immunoreactive T-kininogen in the organs paralleled the changes affecting the plasma

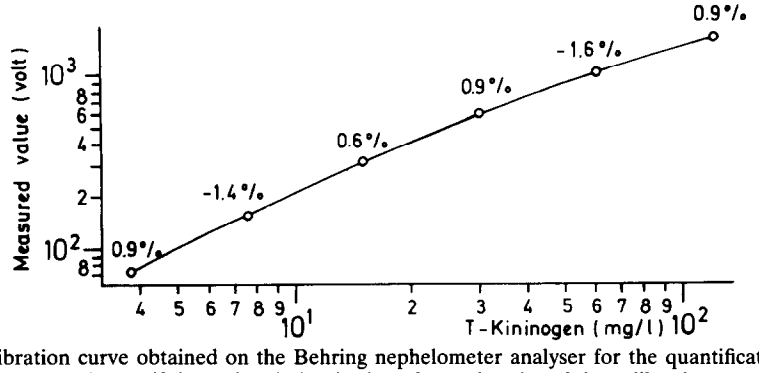

Fig. 2. Calibration curve obtained on the Behring nephelometer analyser for the quantification of T-kininogen. The coefficient of variation is given for each point of the calibration curve.

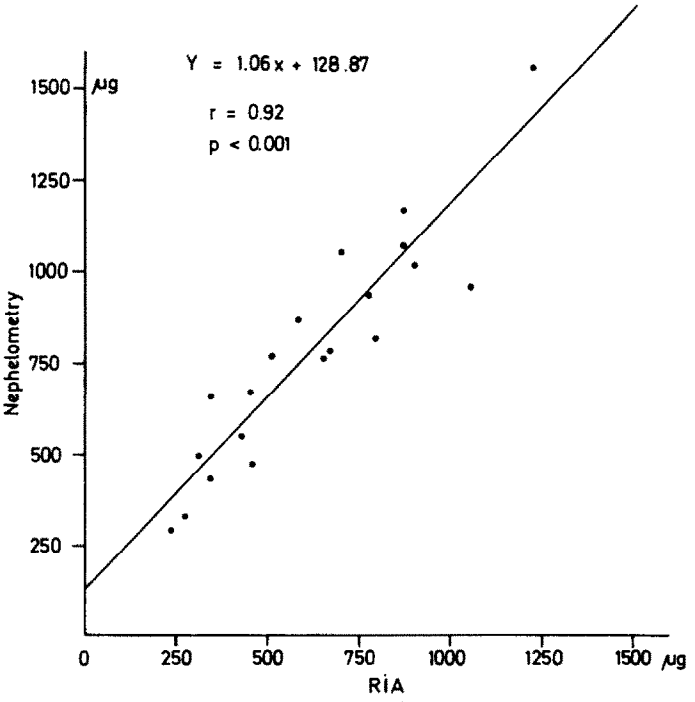

Fig. 3. Measurement of plasma level of T-kininogen. Correlation between the values obtained for different samples of plasmas by direct RIA and by laser nephelometry.

Table 1. T-kininogen level in male rats and in female rats during the estrus cycle

	Plasma (µg/ml)	Plasma (µg/mg)	Liver (µg/mg)	Kidney (µg/mg)	Uterus (µg/mg)
Male	272 ± 40	5.41 ± 0.65	0.12 ± 0.02	0.34 ± 0.06	
Female					
Estrus	454 ± 30*	9.43 ± 0.49*	0.33 ± 0.07*	0.52 ± 0.07	5.56 ± 0.37
Metestrus	589 ± 59* (1)	12.21 ± 1.69*	0.47 ± 0.11*	1.18 ± 0.33* (2)	10.92 ± 1.85 (3)
Diestrus	482 ± 73*	10.09 ± 1.3*	0.35 ± 0.1*	0.75 ± 0.22	7.54 ± 1.61
Proestrus	340 ± 20	7.9 ± 0.36*	0.17 ± 0.03	0.36 ± 0.05	3.17 ± 0.41

Each value represents the mean ± SEM of 6 determinations (12 for plasma in male rats) of T-kininogen level expressed in µg/ml of plasma or in µg/mg of total proteins. (1) Significantly different from proestrus at $P < 0.005$; (2) significantly different from proestrus at $P < 0.025$; (3) significantly different from proestrus and estrus at $P < 0.005$; * Significantly different at least at $P < 0.05$ from the corresponding values measured in male rats.

level: minimum values were recorded in the organs during the proestrus (Table 1).

T-kininogen after turpentine injection. In male Wistar rats, the plasma level of T-kininogen increased after turpentine injection and reached a maximum 1882 ± 95 µg/ml or 38.4 ± 2.5 µg/mg of total proteins (N = 6) at the 72 hr (Fig. 4). A similar increase of the content in immunoreactive T-kininogen was observed in the liver and the kidney. However, the increase in the liver was already significant at 3 hr and the maximum peak was observed at 24 hr (Fig. 4).

T-kininogen and kinins in peritoneal exudate. The intraperitoneal injection of 5 ml carrageenan solution induced the accumulation of T-kininogen in the peritoneal fluid. The level was maximum 4 hr after the injection. At this time, the peritoneal cavity contained 2.6 ± 0.3 ml of fluid (N = 12). In this exudate, we found 96 ± 20 µg/ml of T-kininogen (N = 6); 21.8 ± 1.1 mg/ml of total proteins (N = 6); 499 ± 111.10^3/ml of leucocytes (N = 6). The exudate contained also immunoreactive kinins which were eluted by a reverse phase HPLC at the same time as bradykinin (Fig. 5). The bradykinin level amounted for 9.3 ± 3.8 ng/ml (N = 6). In only one sample, we found a small quantity of T-kinin: 0.2 ng/ml. This value contrasted with the amount of bradykinin in the same sample, which equaled to 14.9 ng/ml. In these animals, the plasma level of T-kininogen was not significantly modified: 269 ± 66 µg/ml (N = 6).

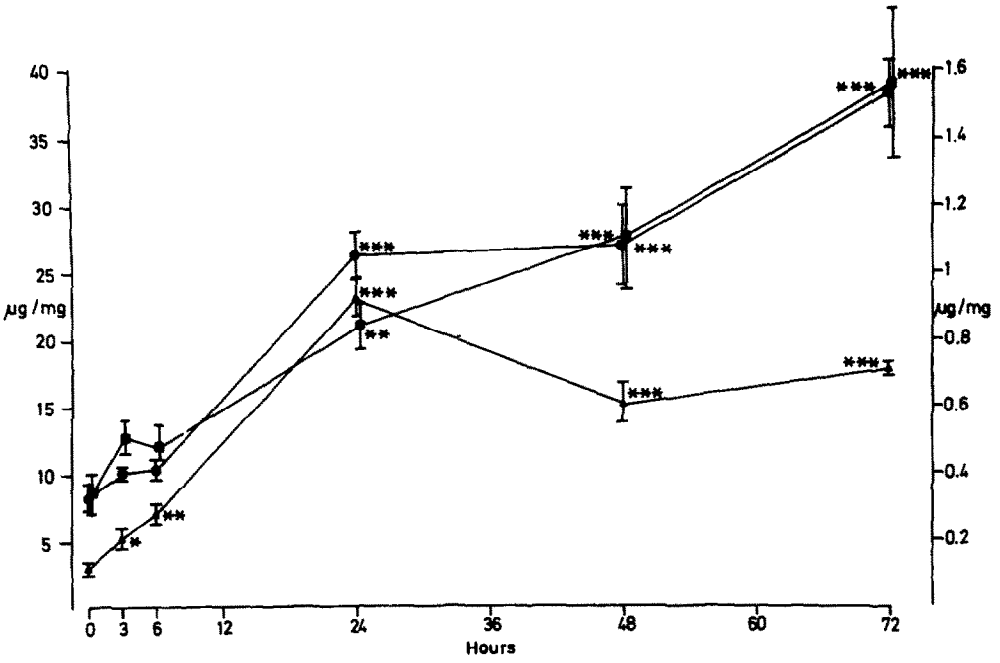

Fig. 4. Time-course of the effect of turpentine (0.1 ml/100 g) on the level of immunoreactive T-kininogen in plasma (●), in liver (▲) and in kidney (■). Plasma level left scale, organ level right scale. Each point is mean value for 6 rats. ** $P < 0.02$; *** $P < 0.005$.

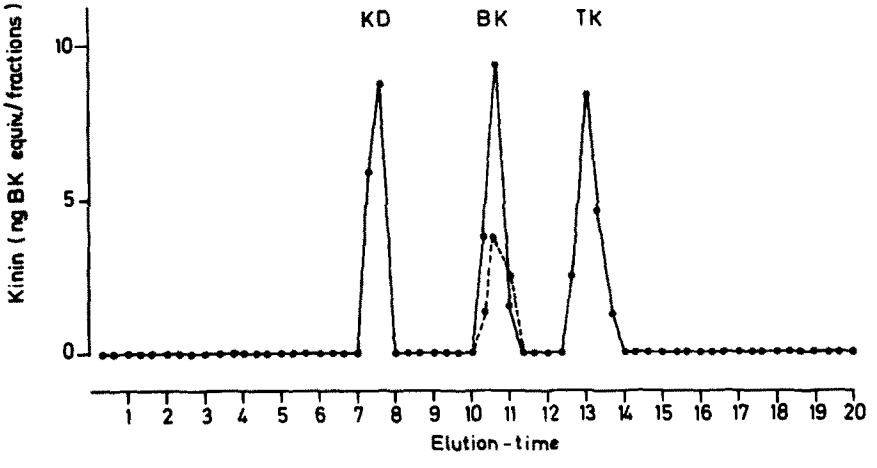

Fig. 5. Reverse phase HPLC of immunoreactive kinins present in peritoneal exudates after carrageenan injection. Kinins were measured by RIA using bradykinin as a standard. ———, control peptides (20 ng each); KD, kallidin; BK, bradykinin; TK, T-kinin. – – – –, peritoneal exudates. Elution time in min.

DISCUSSION

We developed two immunological methods for the quantification of rat T-kininogen in plasma and tissue extracts from injured and non-injured Wistar and deficient Brown Norway rats. The radioimmunoassay is specific for rat T-kininogen. In fact, we did not observe any cross reactivity of rat high molecular weight kininogen, bradykinin and T-kinin with the antiserum. Moreover, the plasma of kininogen-deficient Brown Norway rat showed an identical immunoreactivity to plasma of Wistar rat, this latter containing low and high molecular weight kininogens. Although the inter- and intrabatch reproductibility was excellent, the radioimmunological methods exhibited the disadvantage of the need of a high dilution of the plasma before quantification but suited perfectly for T-kininogen content in organ extracts.

To avoid the plasma sample dilution, we developed a nephelometric method for plasma quantification. Although, in this case too, the precision was good, the measured plasma T-kininogen showed higher values than the radioimmunoassay. This could

be explained, in part, by the presence of low molecular weight cysteine proteinase inhibitors [1]. On the other hand, we could not apply the nephelometric method to tissue extracts: the quantified values were too low and the blank values of tissue homogenates were too high interfering in the nephelometric quantification of this kind of kininogen.

These are the reasons why all the measurements in biological materials (plasma, exudates and organs) were carried out by the radioimmunological assay in spite of the high dilution of plasma.

Some reports have already given estimations for plasma level of T-kininogen measured indirectly by its ability to release T-kinin [9–11], by a radial immunodiffusion [12] or by radioimmunoassays [13–16]. Our results are in close agreement with those of Bouhnik *et al.* [14, 15] but lower than the values reported by Sakamoto and Yoshikawa [12] 432 μg/ml. According to Thomas and Schreiber [17], the plasma level of the alpha$_1$-major acute phase protein of the rat is about 0.46 g/L in adult rat. The use of different strains or of female rats could explain these differences. Previously, we measured the plasma level of the two other kininogens in Wistar rats: 140 μg/ml [9]. This result indicates that in these rats, T-kininogen represents 66% of total plasma kininogen, observation that perfectly fits with the measurements of 65% of Bouhnik *et al.* [14].

Changes in the plasma level of total kininogens have been observed in female rats during the estrus cycle. Smith and Perks [18] have carefully studied this evolution using the ability of kininogens to release kinins after hydrolysis by trypsin to measure the kininogen level. As T-kininogen has a poor sensitivity to trypsin hydrolysis [2] and represents the major fraction of total kininogens in normal rats, we examined the evolution of that molecule during the estrus cycle. We observed similar changes for T-kininogen as those reported by Smith and Perks [18] for total kininogens, although the order of magnitude of these changes was smaller in our study. A fall in plasma T-kininogen was observed just before ovulation while the maximal level was measured during the metestrus. This variation has to be taken into account when using female rats.

Plasma level of T-kininogen increased after inflammation with adjuvant [10], carrageenan [11–16], turpentine [9, 14, 15, 19] lipopolysaccharide [15] or after surgery [15]. Our results confirm and specify these previous observations of several laboratories among which our own [9, 19]. That increase depends on a stimulation of the liver synthesis of T-kininogen [16, 20]. Indeed, our results indicate a significant increase in the level of immunoreactive T-kininogen in liver homogenates at 3 hr after turpentine injection before the increase in the plasma level occurred. Thereafter, the plasma level increased till 72 hr after turpentine injection while the peak increase in the liver content was already reached at 24 hr. We observed also the presence of immunoreactive T-kininogen in kidney homogenates. This content varied directly with the plasma level. The same variation was observed in kidney homogenates during the estrus cycle. Large amounts of T-kininogen were also measured in homogenates of uterus. The presence of T-kininogen in homogenates of washed organs does not indicate that T-kininogen is present in the cells. However, Chao *et al.* [21] have recently described the presence of T-kininogen in various organs of the injured rat. A part of the T-kininogen content in washed organ could be explained by an extravasation from the blood vessels in the interstitial fluid. The accumulation of T-kininogen in uterus during the metestrus suggests that the inhibitory activity of kininogens towards cysteine proteinases [22] could take place during the involution of the tissue. Similar inhibitory activity of T-kininogen could occur during inflammatory processes as this molecule was found in peritoneal exudates as in other inflammatory fluids [11, 12, 23, 24]. In peritoneal exudates induced by carrageenan, we found large amounts of bradykinin but a trace of T-kinin in only one case. The lack of significant release of T-kinin would indicate that kinins during inflammatory processes arise mainly from the usual kininogens and not from T-kininogen, as previously proposed [25].

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