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STUDIES ON URINARY KALLIKREIN IN *TRYPANOSOMA BRUCEI* INFECTIONS OF THE RABBIT

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Abstract—Urine was collected daily from rabbits chronically infected with *T. brucei* and from uninfected controls. This urine was shown to contain a substance with both enzymatic, oxytocic, hypotensive and kinin-releasing activity and is considered to be urinary kallikrein.

Urine volume increased about 5 fold and kallikrein levels 4–8 fold during the first parasite peak. Urinary kallikrein levels remained elevated thereafter and again showed higher levels with each succeeding parasite peak. A unique property of the enzyme was its direct effect on isolated rat uterus, causing spontaneous rhythm. This could be inhibited by high levels of Trasylol, and by heating at 96° for 30 min, but not by SBTI or heating at 56° for 30 min. The hypotensive, kinin-forming and esterase properties were also similarly affected. The excretion of elevated levels of urinary kallikrein during chronic Trypanosomiasis is considered to be due to glomerular damage, and possibly to activation of plasma kallikrein by the parasite and by parasite/antibody complexes, resulting in increased glomerular blood flow and glomerular filtration rate.

The first indications that pharmacologically active substances contribute towards the pathogenesis of African trypanosomiasis was produced by Goodwin and Richards in 1960 [1] when they showed that elevated kinin levels were present in the urine of mice infected with *Trypanosoma rhodesiense*. Since that original observation several other studies have been undertaken both in acute and chronic trypanosomiasis.

Richards [2] found that histamine and kinin levels increased in the urine of mice acutely infected with *Trypanosoma brucei*. In addition raised kinin levels were detected in the blood as well as decreased kininogen concentrations. Elevated concentrations of urinary kinin in chronically infected rabbits with *T. brucei* was first demonstrated by Boreham [3]. The kinin levels increased during the first week of the infection reaching maximum values of about eight times pre-infection levels between 7 and 10 days after the initial infection. The levels thereafter fell but remained above pre-infection concentrations. A parallel rise in plasma was also noted with a corresponding drop in plasma kininogen concentrations. In rats acutely infected with *T. brucei* there was a daily increase in urinary kinin until death occurred. However, in rats infected with the non-pathogenic organism *T. lewisi* no changes in urinary or plasma kinins were observed.

These studies have been extended to cattle infected with *T. brucei* and man infected with *T. rhodesiense* [4, 5]. In both instances, there was a major release of kinin just after the first antigenic variant was produced approximately 10 days after the infection. It was suggested that an immune complex consisting of parasite antigen/antibody was responsible for the release of kinins. Evidence to support this was provided [6] when it was shown that plasma pre-kallikrein was activated during *T. brucei* infections of the rat. Boreham and Goodwin [7] later demonstrated conclusively that trypanosomes from rats when combined with immune sera liberated kinin from fresh rabbit plasma. Trypanosomes or immune sera alone were unable to cause kinin release. It was suggested that kinin activation was dependent upon Hageman factor since heating the serum at 65° to destroy Hageman factor also prevented the release of kinins whereas heating at 56° for 30 min which destroys complement did not inhibit the amount of kinin released. Further evidence to support this hypothesis has recently been provided [8] when it was shown that particulate complexes of trypanosomes and antibody injected intravenously into normal rabbits caused hypotension whereas trypanosomes or antibody alone had no such effect. This reaction was inhibited by Aprotinin (Trasylol). This suggests that plasma kallikrein may have very important pathological effects in rabbits infected with *T. brucei* and in order to investigate this further and to determine the mechanism of urinary kinin production a study has been undertaken on the role of urinary kallikrein in trypanosomiasis infections.

The first study on urinary kallikrein was undertaken by Frey [9] when he found a non-dialysable substance in the urine which acted as a kinin-forming enzyme which he thought originated in the pancreas. Later work, however, showed that this substance was probably not derived from this gland [10]. Urinary kallikrein will cause the release of kinin from its precursor in plasma protein [11], and also it has been shown to be derived from the kidney. Nustad [12] has shown that both the kidney and urine contain

a similar kininogenase enzyme and that urinary kallikrein is quite distinct from plasma kallikrein, and is derived from another source.

MATERIALS AND METHODS

Experimental infections. Adult New Zealand white rabbits of either sex weighing 3.5–4.5 kg were infected subcutaneously with *Trypanosoma brucei* strain 427, details of this strain are given by Boreham and Facer [13].

Collection of urine. Rabbits were housed in metabolism cages and urine collected via a siliconised glass funnel which separated urine from faeces. This apparatus is described by Boreham [14]. Twenty-four-hr samples were collected in polythene beakers and the vol. measured. The urine was centrifuged lightly and the supernatant stored at −20° until required.

Separation of trypanosomes. Blood was collected into heparinised tubes by heart puncture from rats infected 3 days previously, with *T. brucei*. The parasites were separated from the heavily infected blood by the method described by Lanham [15] on DEAE-cellulose chromatography columns.

Disintegrated parasite suspensions were obtained by repeated freezing and thawing of the parasites. Such suspensions were stored at −20° until required.

Parasitaemia. Parasitaemia was determined daily by examining fresh venous blood under the microscope. The parasitaemia was assessed on 0–4+ scale depending upon the number of parasites visible, at a magnification of ×400.

Urinary kallikrein esterase activity. Urinary kallikrein activity was estimated using a modification of the method described by Roberts [16]. 0.5 ml of urine was added to 3.5 ml of 0.05 M Tris–HCl buffer (pH 8.4) in a plastic tube. To this mixture was added an enzyme substrate consisting of 1 ml of 0.05 M Tris–HCl buffer, containing 0.05 M *N*-tosyl L-arginine methyl ester (TAME). The mixture was incubated at 37° for 1 hr. Duplicate samples were prepared and as a control 0.5 ml of urine and 0.5 ml of 0.05 M Tris–HCl buffer (pH 8.4) either containing Trasylol or soya bean trypsin inhibitor (SBTI) were incubated for 15 min at 37°. The enzyme solutions containing inhibitor were then added to 3.0 ml 0.05 M Tris–HCl buffer (pH 8.4) and 1.0 ml of 0.05 M Tris–HCl buffer (pH 8.4) containing 0.05 M TAME and assayed as above. Padutin (kallikrein, Bayer Ltd.) was used as a standard and was treated in a similar manner to urine. Enzyme Unit (EU) was defined as that amount of enzyme necessary to hydrolyse 1 μmole TAME/min at 37°.

Biological assay of kallikrein. The effect of urinary kallikrein on blood pressure was determined in rabbits anaesthetised with urethane 1.2 g/kg body wt. The anaesthetised rabbits were heparinised with 1000 i.u. heparin/kg i.v. and their body temp maintained at about 37°, using an electric heating pad. Arterial blood pressure was recorded with a Bell & Howell electronic pressure transducer from the left carotid artery. The right jugular vein was canulated for administration of test samples and drugs. Samples were administered in 0.1 or 0.2 ml vol and washed into the bloodstream with 0.2 ml sterile saline. Graded doses (2, 4, 6 and 8 enzyme units) of Padutin were used as a standard. As a control 0.5 ml urine was incubated with 0.5 ml saline containing either Trasylol or SBTI at 37° for 15 min and injected intravenously into the rabbits. As an additional control equal volumes of urine and normal saline were incubated at 37° for 15 min and then injected into the rabbit. Such an incubation had no effect on the response of blood pressure to the kallikrein. However it was noted that constant freezing and thawing of the urine did diminish its biological effect. Administration of Trasylol, SBTI or saline alone had no effect on blood pressure. Samples were always assayed in duplicate. One biological unit (BU) of urine kallikrein was defined as the amount of urine which caused a fall in blood pressure equal to that of one BU of Padutin. These results were then related to the urine volume produced in 24 hr and was expressed as BU/24 hr.

Enzyme samples were also assayed on the isolated rat uterus [4]. The following inhibitors were present in the physiological saline solution: atropine sulphate (10^{-6} g/ml); mepyramine maleate (10^{-7} g/ml) and methysergide bimaleate (10^{-6} g/ml). Maximum contractions of the uterus were usually obtained by addition of 0.8 ng of synthetic bradykinin (BRS-640). Padutin (up to 40 BU) had no effect on the muscle preparation. Enzyme samples were also incubated at 37° for 15 min with either Trasylol, SBTI or 10^8–10^9 disintegrated *T. brucei*. Under these conditions acetylcholine, histamine acid phosphate and 5-hydroxytryptamine had no effect on the isolated uterus preparation. In addition in some preparations indomethacin (an inhibitor of prostaglandin $F_2\alpha$ synthesis) [17] was also added to the organ bath at the rate of 3.5 mg/ml. Test samples were assayed before and after its addition.

Kinin-forming activity of the urinary kallikrein. Kininogen substrate from normal rabbit blood was prepared according to the method of Diniz and Carvalho [18]. A volume of 0.7 ml of substrate (containing 0.05 ml plasma) was incubated at 37° with 0.2 ml urinary kallikrein or 0.2 ml saline containing 50 μg trypsin or 4 or 8 BU Padutin. The reaction was terminated after 15 min by the addition of 2 ml of absolute alcohol at 90°. The suspension was further incubated for 10 min at 90° before evaporation to dryness in a rotary evapomix (Buchler Instruments) below 40°. Control samples of enzyme and inhibitor and inhibitor or saline alone were also incubated with the kininogen substrate. The released kinin was resuspended in 4 ml de Jalon solution and assayed on the isolated rat uterus, synthetic bradykinin being used as standard.

RESULTS

Urine volume

The urine volume increased above pre-infection levels during each parasite peak and fell to pre-infection levels between each peak. The level rose 4–5-fold during the first two peaks (days 3–12) and increased 2-fold during the subsequent peaks. Urine volume in control rabbits was relatively unchanged.

Urinary kallikrein

The results of urinary kallikrein estimations of two control rabbits taken over a 28-day period are given

in Fig. 1. It can be seen that at no time did the biological activity exceed 1600 BU/24 hr and the esterase activity 120 EU/24 hr.

The results for four rabbits infected with *T. brucei* are given in Fig. 1. It can be seen that soon after infection the amount of urinary kallikrein as estimated by either the biological activity or the esterase activity increases markedly reaching maximum levels 6 or 7 days after infection. A second peak of activity was noted 17 days after infection and a third peak 24 days after infection. During most of the infection urinary kallikrein concentrations were raised. Significant correlation coefficients of 0.87 existed between BU and EU, of 0.97 between BU/24 hr and EU/24 hr, and of 0.88 between BU and EU/24 hr. Significant correlation coefficients also existed between urine volume and BU/EU ratio (0.85), parasitaemia and BU/24 hr (0.88) and parasitaemia and EU/24 hr (0.82) (<0.81 n.s.).

Effect of inhibitors

(i) *Enzymatic activity.* In control animals 1000 inhibitor units (IU) of Trasylol/ml of urine caused at least 75 per cent inhibition of enzymatic activity. However, with infected urine 1000 IU Trasylol/ml inhibited less than 30 per cent of the activity whereas 3000 IU Trasylol ml inhibited 60 to 65 per cent. In control experiments 1000 IU Trasylol inhibited more than 80 per cent of the enzymatic activity of 10 BU Padutin. Soya bean trypsin inhibitor in concentrations up to 20 mg/ml of urine had no inhibitory effect. Likewise SBTI had no inhibitory effect on Padutin.

(ii) *Biological activity.* Two thousand to 3000 IU Trasylol/ml of control urine caused 100 per cent inhibition effect on blood pressure (approx 100 IU/BU). Five to ten thousand IU Trasylol/ml of infected urine produced 90 per cent inhibition of the blood pressure changes (approximately 100 IU/BU). In control experiments 750 IU Trasylol/10 BU Padutin inhibited 100 per cent of the blood pressure change. Soya bean trypsin inhibitor up to concentrations of 20 mg/ml had no effect on the hypotension caused by infected urine or by 10 BU Padutin. SBTI and Trasylol had no direct effects on blood pressure.

Properties of the esterase. The pH optimum for hydrolysis of TAME by urinary kallikrein using Tris–HCl buffer was 8.4. The optimum temperature was 37.5°. A plot of substrate concentration against velocity of reaction showed that the optimum substrate concentration was 0.05 M.

Biological properties of the urinary enzyme. Urine from infected and non-infected rabbits had hypotensive properties. The urine taken during parasitaemic peaks of infected rabbits was 4–8 times more active than the urine of uninfected animals. In addition to the hypotensive properties the urine also caused an increase in heart rate. With doses of urine equivalent to 4 KU (1 KU/kg) or less blood pressure usually returned to normal within 2–3 min. Urine heated at

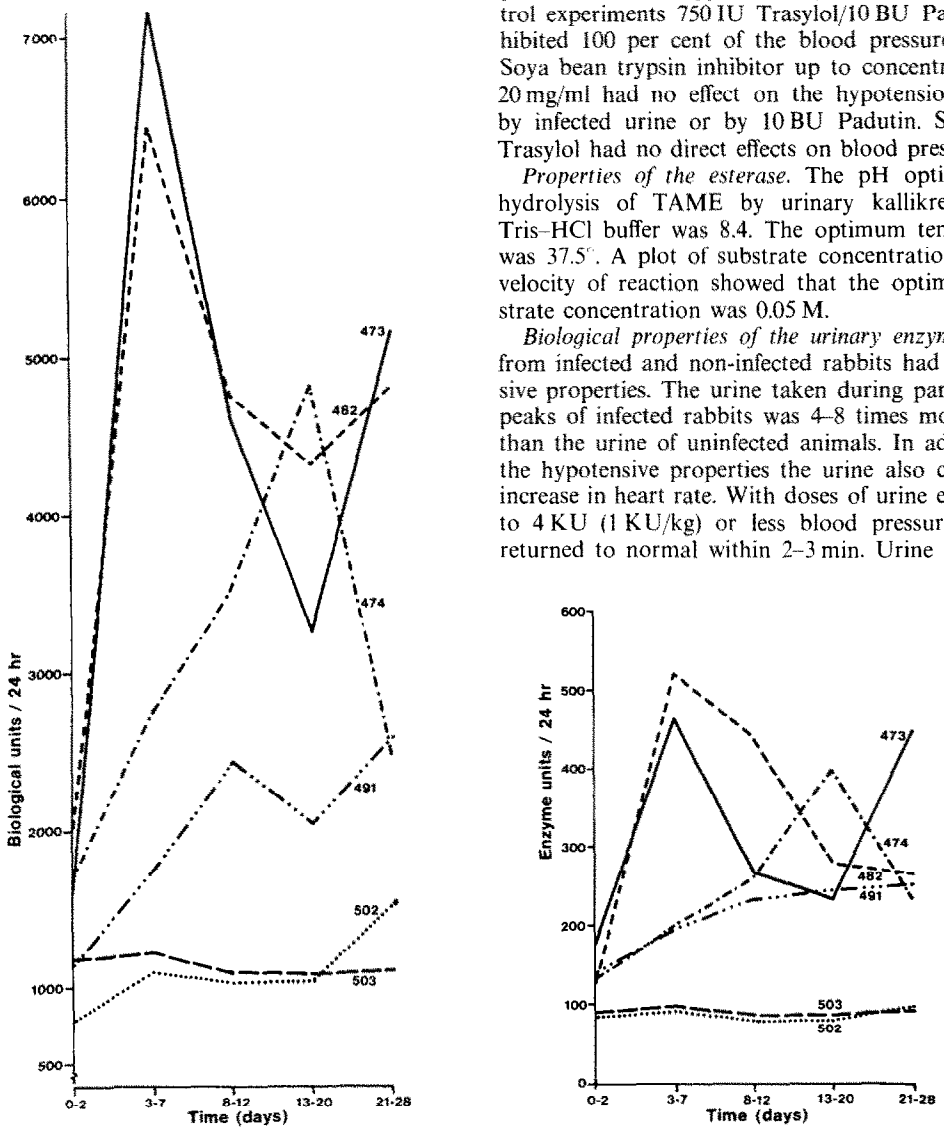

Fig. 1. Biological units (BU)/24 hr and enzyme units (EU)/24 hr of urinary kallikrein from four rabbits infected with *T. brucei* (Nos. 473, 474, 482, 491) and from two uninfected controls (Nos. 502, 503). Data was averaged during time periods listed to correspond with the appearance of detectable parasite peaks in infected animals, with the exception of days 0–2 which preceded the first parasitaemia peak.

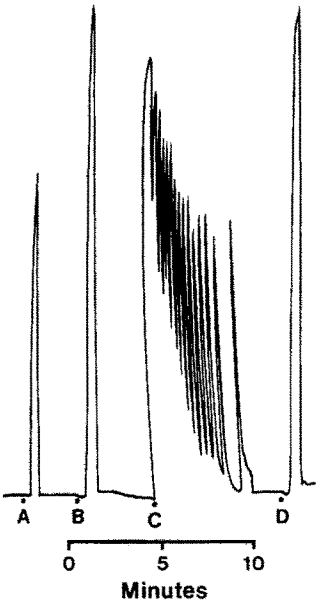

Fig. 2. Effect of 0.1 ml urine (UK) (C from animal infected with *T. brucei* (day 10), on isolated rat uterus. De Jalon solution with atropine sulphate (10^{-6} g/ml), mepyramine maleate (10^{-7} g/ml) and methysergide bimaleate (10^{-6} g/ml); 3 ml bath. Synthetic bradykinin (BK) was added to the bath at dots with doses as follows (A 0.6 ng; B 0.8 ng; D 0.8 ng).

96° for 30 min had no effect on blood pressure but urine heated at 56° for the same period had similar biological activity to unheated urine. Both normal and infected urine had potent oxytocic properties on the isolated rat preparation. Upon application of urine, the uterus contracted within 3–5 sec (Fig. 2). The infected urine was approximately 3–5 times more potent on the dose basis than normal urine. Padutin up to a concentration of 40 KU or 1 ml of serum had no such effect. Doses of 0.1 ml urine would cause the uterus to undergo a series of rapid spontaneous rhythmical contractions and relaxations (Fig. 2). Higher doses of 0.5 ml caused the uterus to go into continuous contraction lasting at least 30 min after which time the muscle was so fatigued that it no longer responded to either kallikrein or synthetic bradykinin. Urine heated at 96° for 30 min had no effect on muscle preparation but heating at only 56° for 30 min did not prevent this spontaneous activity. Disintegrated trypanosomes had no oxytocic properties. The contraction caused by the urine was not inhibited by methysergide, mepyramine, atropine or indomethacin. Urine (0.5–1.0 ml) also produced weak relaxation of the rat duodenum.

Urinary kallikrein from both infected and uninfected rabbits released kinin from rabbit kininogen substrate. Infected urine was 2–5 times more active than control urine. Urine heated to 96° for 30 min did not possess any kinin-forming or oxytocic properties but heating at only 56° for 30 min did not inhibit these properties (Fig. 3). Three thousand IU Trasylol/ml of normal urine completely inhibited both the kinin-forming and oxytocic effects (Fig. 3). Similarly 10000 IU Trasylol/ml of infected urine inhibited both kinin-forming and oxytocic effects while 5000 IU Trasylol/ml of infected urine produced between 40–60 per cent inhibition. Padutin had no direct oxytocic effect although it had strong kinin-forming properties. This kinin-forming activity was inhibited completely by 1000 IU Trasylol/10 BU Padutin. SBTI concentrations of 20 mg/ml of urine did not inhibit the kinin-forming or oxytocic properties of the urine (Fig. 3).

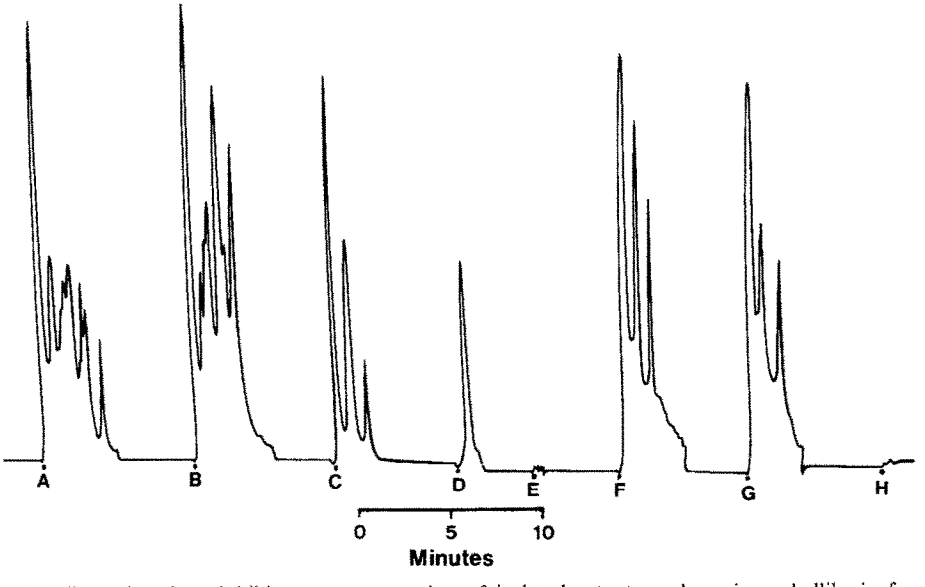

Fig. 3. Effect of various inhibitors on contraction of isolated rat uterus by urinary kallikrein from a rabbit chronically infected with *T. brucei*. De Jalon solution with atropine sulphate (10^{-6} g/ml), mepyramine maleate (10^{-7} g/ml) and methysergide bimaleate (10^{-6} g/ml); 3 ml bath. A; 0.1 ml urine; B: 0.1 ml urine + SBTI (2 mg) incubated at 37° 15 min; C: 0.1 ml urine + Trasylol (100 IU) incubated at 37° 15 min; D: 0.1 ml urine + Trasylol (500 IU) incubated at 37° 15 min; E: 0.1 ml urine + Trasylol (1000 IU) incubated at 37° 15 min; F 0.1 ml urine; G: 0.1 ml urine heated at 56° for 30 min; H: 0.1 ml urine heated at 96° for 30 min. Samples applied at dots.

neither did it inhibit the kinin-forming activity of 10 BU Padutin. SBTI and Trasylol alone had no direct kinin-forming or oxytocic properties.

DISCUSSION

In addition to providing useful information on the pathogenesis of trypanosomiasis this study has produced some interesting results on the pharmacology of urinary kallikrein. In 1966, Beraldo *et al.* [19] showed that the urine collected from rats contained a substance that had oxytocic, kinin-forming and esterase properties. It caused the contraction of the isolated rat uterus and lowered the blood pressure of the rat and dog. This substance had no effect on other isolated muscle preparations. If heated at 96° for 20 min both the oxytocic and kinin-forming properties were lost but heating at 56° had no effect. The action of this substance on the isolated uterus was inhibited by Trasylol but SBTI had no effect. It was concluded from this study that the substance present in rabbit urine was not oxytocin.

It is known that urinary kallikrein differs from plasma kallikrein in molecular dimension, electrophoretic mobility and the effect that inhibitors have on them [12]. Small amounts of Trasylol will inhibit both urinary and kidney kallikrein but large amounts of SBTI were needed for even partial inhibition. Werle and Maier [20] have shown that SBTI inhibits plasma but not glandular or urinary kallikrein whereas Trasylol affects the hypotensive, esterase and kinin-forming activities of most kallikreins including those from serum, glands and urine [21]. In his experiments Nustad [12] showed that the spectrum of inhibition of esterase and kinin-forming activity of urinary kallikrein were similar, Trasylol causing over 80 per cent inhibition, but SBTI having little effect. In the experiments described here similar results were found confirming that the substance excreted in chronically infected rabbits was urinary and not plasma kallikrein. Addition of normal or infected rabbit urine to the isolated rat uterus produced a sustained contraction which could not be inhibited by atropine, mepyramine, methysergide or indomethacin indicating that ACh, histamine, 5-HT and the prostaglandin $F_2\alpha$ were not involved. Inhibition of this effect by heating at 96°, but not at 56°, suggests that the substance was relatively heat stable and certainly excludes inorganic components such as KCl [22], or a calcium imbalance. These inorganic substances would be stable at 96°. The most likely explanation is that this effect is mediated directly by urinary kallikrein. Reports in the literature suggest that urinary kallikrein produces a single contraction when applied to the rat uterus [23]. These authors stated that the contraction caused by kallikrein was slow like that of bradykinin but was produced after a much longer lag period. Similar findings have also been reported [10]. Nustad and Pierce [23] concluded that the direct action on the isolated rat uterus was probably due to kinin liberation from kininogen indigenous to the uterus. However the rapid onset of spontaneous rhythm produced by the action of rabbit urinary kallikrein on the rat uterus in this study suggests that in this system at least, the kallikrein is acting directly on the muscle preparation.

It is known that kinins are formed in the kidney [24]. Since the kidney itself contains kininogen it is possible that kinin formation is independent in the kidney of plasma kininogens [25, 26]. Removal of the kidneys results in plasma kininogen concentrations increasing 3–5-fold within 72 hr indicating that there is a constant consumption of plasma kininogen by kidney kallikrein [27].

Local kinin formation in the cortex of the kidney may help regulate local blood flow and vascular permeability [28]. Direct renal infusion of kallidin into a dog produced a large increase in diuresis with a concurrent increase in renal blood flow without any change in the renal arterial or systemic blood pressures [29]. The glomerular filtration rate was increased suggesting that there was a direct relationship between urinary kinin levels (and presumably urinary or kidney kallikrein) and urine vol. In the early stages of these present studies such a relationship exists. However, working with normal rats, Nustad [12] found an inverse relationship between kallikrein production and urine vol.

The role of urinary kallikrein in the general regulation of blood pressure has also been suggested, since decreased levels of urinary kallikrein have been observed in humans and rats with essential hypertension [30–32].

Recent studies have shown that rabbits infected with *T. brucei* are profoundly hypotensive [8]. Evidence has also been produced that this hypotension is mediated by kallikrein. Intravenous injection of kallikrein usually produces a biphasic fall in blood pressure. A similar biphasic fall in blood pressure was noted when particulate complexes of trypanosomes and hyperimmune sera were injected intravenously into normal rabbits. Since this reaction could be inhibited by Trasylol it was concluded that it was mediated by kallikrein. The initial rapid fall in blood pressure by injection of kallikrein is probably due to the direct actions of this substance. Kallikrein is known to dilate coronary vessels and it is also thought to have a direct action on the myocardium [33]. The later, slower, prolonged fall was probably due to the release of kinin (Kallidin) by the direct action of kallikrein on kininogen [34].

In this study we have shown that the amount of urinary kallikrein produced in chronic trypanosomiasis of the rabbit is markedly increased. This is particularly true early in the infection and seems to be correlated with peaks in parasitaemia. The finding of large amounts of urinary kallikrein during each peak in parasitaemia correlates well with the hypothesis already proposed that the formation of immune complexes at this time activates Hageman factor with subsequent activation of prekallikrein. However, very large amounts of urinary kallikrein are produced early in the infection and by day 2 there are already increased amounts present in the urine. At this time it is very unlikely that sufficient antibody will have been produced for this reaction to have been mediated by immune complexes. It therefore would appear that at this stage trypanosomes are having a direct effect on the kinin system. It has been suggested that rabbits infected with *T. brucei* die in a state of vascular shock with renal failure which may have an immunological origin [35, 36]. Proteinuria

develops as the infection advances [36, 37]. The proteins in the urine are similar to normal plasma proteins.

During the initial period of the infection there was little change in the amount of protein produced in the urine, but by 3–4 weeks after infection large amounts were present. Kidney damage occurs very early on in the infection as indicated by the presence of urinary casts from day 14 onwards. Evidence for kidney damage in experimental trypanosomiasis has been accumulating recently. Boreham and Kimber [38] recorded the presence of immune complexes in the venules of rabbits infected with *T. brucei*. Nagle *et al.* [39, 40] have demonstrated IgG, properdin and complement deposits in the glomeruli of rhesus monkeys infected with *T. rhodesiense*. They concluded that a glomerulonephritis develops that is mediated by immune complexes which are probably derived from the circulation. More recently, Lambert and Houba [41] have demonstrated IgG, IgM and C'_3 deposits in the glomerulus of mice infected with trypanosomes. The antigen was shown to be of trypanosomal origin. It seems quite possible that the damage to the kidney is mediated via kallikrein. Nustad [12, 42] has shown that urinary and kidney kallikreins have many common properties and that most of the kidney kallikrein is contained in the cortical region, 85 per cent of which is composed of the proximal convoluted tubules [43]. Kallikreins have been shown to be synthesised in the kidney [44]. When the proximal tubules are damaged experimentally the amount of urinary kallikrein excreted is markedly decreased [44].

Since in this experiment the amount urinary kallikrein increased it would appear unlikely that damage occurred to the tubules but that any kidney damage originates in the glomerulus.

The actual stimulus for increased production of kallikrein in the urine of rabbits infected with *T. brucei* is not known. However, it seems probable that kidney glomerular damage occurs as a result of immune complex deposition, resulting in proteinuria. It appears that little of the kallikrein in the urine results from plasma kallikrein leakage. The diuresis seen early in the infection is probably due to elevated plasma kallikrein levels at that time. This would have the effect of increasing the glomerular blood flow with subsequent increased filtration rates.

Preliminary studies on plasma kallikrein support this hypothesis (Boreham and Parry, unpublished). Further studies are required to elucidate the exact mechanism of increased secretion of kallikrein in the kidney. *In vitro* studies of kallikrein production in the kidney using immune complexes would be valuable although some evidence has been produced that trypanosomes have a direct action on the kinin system early in the infection.

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