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SOLUBLE DEXTRAN COMPLEXES OF KALLIKREIN, BRADYKININ AND ENZYME INHIBITORS*

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Abstract—Pig pancreatic kallikrein, the protease inhibitor aprotinin (Trasylol), SQ 21541 (Arg-Pro-Gln-Ile-Pro-Pro, an angiotensin I converting enzyme or kininase II inhibitor), and bradykinin were each coupled covalently to soluble dextran with an average molecular weight of 500,000. Dextran had been activated either with cyanogen bromide (CNBr) or sodium meta-periodate (SMP). Of the reactants, 56 per cent of kallikrein, 35 per cent of aprotinin and 23 per cent of bradykinin had been bound to CNBr-activated dextran, while 38 per cent of SQ 21541 and 45 per cent of bradykinin had been bound to SMP-activated dextran. The activities of the complexes were determined *in vitro*. Kallikrein CNBr-dextran had 72 per cent of the esterase activity of non-coupled kallikrein. Aprotinin CNBr-dextran had 41 per cent of the kallikrein inhibitory activity of free aprotinin, and SQ 21542 SMP-dextran had 24 per cent of the converting enzyme inhibitory activity of the free peptide *in vitro*. The relative potencies of bradykinin CNBr–dextran and bradykinin SMP–dextran on the isolated rat uterus were 6 and 29 per cent those of native bradykinin. Their relative immunological potencies, however, were 92 and 80 per cent as determined by radioimmunoassay. Free and bound bradykinin inhibited the hydrolysis of hippuryl-glycylglycine by converting enzyme, but the coupled peptide inhibited less than the free kinin. Bradykinin coupled covalently to dextran was inactivated more slowly by converting enzyme than free bradykinin.

Although substances coupled to insoluble supports have been applied frequently in biochemistry, for example, in the purifications of enzymes, antibodies, and hormone receptors [1, 2], their use *in vivo* was limited because they are insoluble. Soluble high molecular weight (mol. wt) complexes of some biologically active materials such as insulin [3], heparin [4], hemoglobin [5] and kallikrein [6] have been prepared and some of their properties determined *in vitro*. The insulin and kallikrein complexes were also reported to be active *in vivo*. The properties of macromolecular complexes of some proteins can differ from the native molecules. Some enzymes covalently attached to soluble dextran are more resistant to heat inactivation and, in the case of trypsin, less susceptible to autodigestion than the native enzyme [7, 8]. The potential therapeutic usefulness of soluble dextran complexes of biologically active substances that are more active *in vivo* than their unbound counterparts, makes it desirable to prepare and evaluate such conjugates. Increased activity of the high mol. wt complexes could be due to several factors such as differences in excretion, inactivation, or interaction with receptors. With this thought in mind, we have prepared soluble dextran complexes of the following proteins and peptides: kallikrein, the enzyme which releases kinin from its precursor, kininogen; aprotinin (Trasylol), the trypsin and kallikrein inhibitor; SQ 21541, a synthetic hexapeptide inhibitor of kininase II or angiotensin I converting enzyme [9] (peptidyl dipeptidase); and bradykinin, a basic nonapeptide with vasodepressor activity.§

MATERIALS AND METHODS

Reagents. Aprotinin (mol. wt 6500) and purified pig pancreatic kallikrein (mol. wt 28,600) were supplied by Prof. G. L. Haberland of Bayer AG, Wuppertal Elberfeld, Germany. SQ 21541, Arg-Pro-Gln-Ile-Pro-Pro (mol. wt 779), was a gift from Dr. Z. Horowitz of the Squibb Inst. Med. Res., Princeton, NJ. Bradykinin triacetate pentahydrate (mol. wt 1325) were purchased from Bachem Inc., Marina Del Rey, CA, dextran (av. mol. wt 500,000) and sodium meta-periodate from Sigma Chemical Co., St. Louis, MO; sodium borohydride was purchased from Fisher Scientific Co., Houston, Tex.; cyanogen bromide from Aldrich Chemical Co., Milwaukee, WI; benzoylarginine ethyl ester (BAEe) from Cyclo Chemical, Los Angeles, CA and hippuryl-glycylglycine (Hip-Gly-Gly) from Pfaltz & Bauer, Inc., Stamford, CO.

Coupling to cyanogen bromide-activated dextran (CNBr dextran). Dextran was activated with cyanogen bromide according to the method of Axén *et al.* [10]. To 50 ml of an aqueous 2% solution of dextran (500,000 mol. wt) that was continuously stirred at room temperature was added 150 mg cyanogen bromide. The pH of the mixture was kept between 10.7 and 11.0 with 3 N NaOH. After the pH was stabilized at 11.0 (10–15 min.), it was lowered to 7.6 with a few crystals of $NaH_2PO_4 \cdot H_2O$.

Solutions of aprotinin (1 mg/ml), kallikrein (5 mg/ml) and bradykinin (2.5 mg/ml) were prepared in 0.1 M sodium phosphate buffer, pH 7.6. One ml

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of each of these solutions was allowed to react with 4 ml of the CNBr-dextran solution for 60 hr at 5°. The mixtures were agitated during this incubation period by attaching the reaction vessels to a rotating disk.

The separation of free aprotinin and kallikrein from that bound to dextran was achieved by filtration at 5° and 40 PSI N_2 through an Amicon XM100A membrane with 3 × 200 ml washes of 0.1 M sodium phosphate buffer, pH 7.6. Bradykinin was removed from that coupled to CNBr dextran by dialyzing it at 5° against 4 liters of 0.1 M sodium phosphate buffer, pH 7.6, first for 1 hr and then for 3 hr; subsequently overnight against 1 M NaCl, and finally against water for 1 and 3 hr.

Coupling to sodium meta-periodate-activated dextran (SMP dextran). Dextran was oxidized according to the following procedure. One g dextran (500,000 mol. wt) was dissolved in 15 ml H_2O at room temperature. Sodium meta-periodate (3.5 g) was dissolved in 10 ml of hot water and added to the dextran. The reaction vessel was covered with aluminum foil and incubated overnight (16–18 hr) at 4°. The oxidized dextran was dialyzed at room temperature against 4 liters water for 1 and then for 3 hr. The pH of the oxidized dextran was adjusted to 7.4 with 0.2 M sodium phosphate buffer, pH 7.6.

Solutions of aprotinin (0.4 mg/ml), kallikrein (2.0 mg/ml), bradykinin (1 mg/ml) and SQ 21541 (1 mg/ml) were prepared in 0.1 M sodium phosphate buffer, pH 7.6. Equal volumes of oxidized dextran and of compounds to be coupled were allowed to react for either 16 or 60 hr in the refrigerator. The reactive aldehyde groups remaining on the oxidized dextran at the end of the coupling reaction were reduced by adding 0.1 ml of a freshly prepared sodium borohydride solution (50 mg/ml of H_2O) for each ml of reaction mixture and incubating for 45 min at 4°. This step also converts to stable secondary amines the unstable Schiff bases that are formed when the peptides and proteins react with oxidized dextran.

The uncoupled compounds were separated from those bound as described above for CNBr dextran. The SQ 21541 SMP-dextran complex was separated from unbound SQ 21541 in the same way that bradykinin was separated from the bradykinin dextran complexes.

Analysis of the dextran complexes. The amount of protein or peptide coupled to dextran was determined by amino acid analysis according to the procedure of Spackman *et al.* [11] with a Beckman model 121 automatic amino acid analyzer. Aliquots of the complexes were acid hydrolyzed for 20 hr at 110° in 6 N HCl. After hydrolysis the samples were dried and the amino acids dissolved in 0.2 M sodium citrate buffer, pH 2.2. The samples were filtered through 0.45 μm Millipore filters to remove particulate matter. The amount bound was calculated from known amino acid composition excluding methionine and cysteine. When SMP-dextran was used, arginine was also excluded from the calculations.

Assays. Kallikrein activity was measured by following the hydrolysis of 1×10^{-3} M BAEe by the enzyme at room temperature in a Cary 118 recording spectrophotometer at $\lambda = 254$ nm [12]. The final incubation volume was 1.5 ml. One unit of enzyme activity equals 1 μmole substrate cleaved in 1 min. Aprotinin activity was measured by assessing its ability to inhibit the hydrolysis of BAEe by kallikrein. The inhibitor was pre-incubated with the enzyme for 10 min at room temperature. SQ 21541 activity was measured by assessing its inhibition of the hydrolysis of Hip-Gly-Gly (10^{-3} M) by purified swine kidney angiotensin I converting enzyme [13, 14]. The inhibitor was pre-incubated for 10 min at 37° with the enzyme and then Hip-Gly-Gly was added. The change in the absorption at $\lambda = 245$ nm of the reaction mixture was recorded during the next 5 min with a spectrophotometer. From the differences in the slopes of the lines obtained with or without SQ 21541 or its SMP dextran complex in the incubation medium, the per cent inhibition was established.

The biological potencies of bradykinin dextran complexes were determined on isolated guinea pig ileum and rat uterus preparations [12]. The immunological activities of the complexes were assessed in a bradykinin radioimmunoassay that employed [^{125}I]Tyr^5-bradykinin as the labeled antigen, rabbit anti-bradykinin serum, and dextran-coated charcoal to separate bound from free antigen [15]. The bradykinin-dextran complexes were compared to bradykinin as inhibitors of labeled antigen binding.

The hydrolysis of bradykinin or bradykinin dextran by purified swine kidney converting enzyme was determined in two ways. Bradykinin, 2×10^{-5} M, or an equivalent concentration of its dextran complex was incubated with the enzyme for varying lengths of time at 37° and the residual bradykinin measured by radioimmunoassay. Free and dextran bound-bradykinin were also tested as inhibitors of the hydrolysis of Hip-Gly-Gly by converting enzyme. Purified swine kidney converting enzyme was equilibrated at 37° for 10 min, then Hip-Gly-Gly (10^{-3} M) was added, followed immediately by bradykinin or bradykinin SMP-dextran. The volume of the final reaction mixture was 1 ml. The rate of hydrolysis of Hip-Gly-Gly at 37° was recorded as described above. The reactions were done in 0.1 M Tris 0.1 M NaCl buffer, pH 7.4. In all cases, the length of time required to hydrolyze 0.028 μmole Hip-Gly-Gly ($\Delta O.D._{254} = 0.01$) was measured and the rate, v_0, calculated. From these rates of hydrolysis, the inhibition of the enzyme by bradykinin or bradykinin bound to SMP dextran was determined.

RESULTS

Kallikrein. Kallikrein (36 μg) was bound per mg CNBr-dextran as determined by amino acid analysis. This calculation is based on the assumption that no dextran was lost during coupling and purification. From the amount of kallikrein allowed to react with CNBr-dextran, 56 per cent had been coupled. Bound kallikrein had 72 per cent of the esterase activity of the native enzyme (Fig. 1). The activity of uncoupled kallikrein was 121 units BAEe/mg of protein.

The SMP dextran kallikrein complex had only trace amounts of activity. The amount of kallikrein coupled to SMP dextran was too low to be detected when a 0.4-ml aliquot of the purified reaction mixture was acid hydrolyzed and subjected to amino acid analysis.

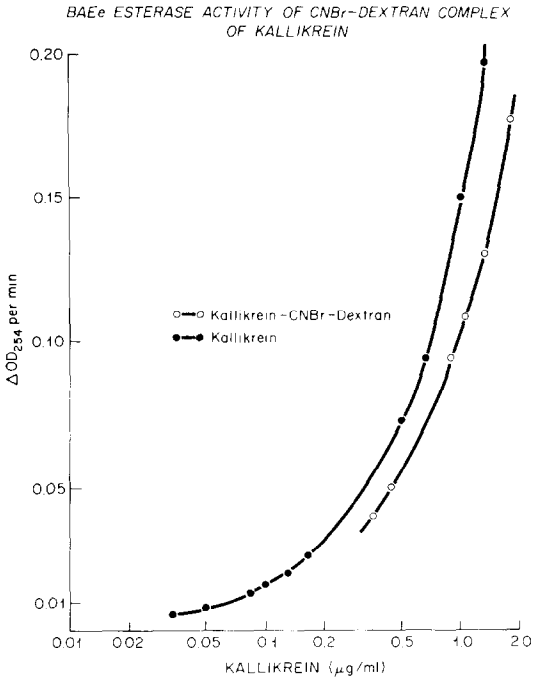

Fig. 1. Effect of increasing the concentration of free and bound kallikrein on the rate of BAEe hydrolysis. Key: ●——●, purified pig pancreatic kallikrein; O——O, kallikrein bound to CNBr–dextran. Abscissa: log concentration of kallikrein in μg/ml. Ordinate: rate of hydrolysis of BAEe in Δ O.D.$_{254}$/min.

Aprotinin (Trasylol). Thirty-five per cent of the aprotinin allowed to react with CNBr–dextran was coupled covalently (4.4 μg/mg). Figure 2 shows the inhibition of BAEe esterase activity of purified kallikrein by free and bound aprotinin. When the I_{50} values of the aprotinins were compared, the CNBr–dextran complex had 41 per cent of the activity of the uncoupled inhibitor. Aprotinin allowed to react with SMP–dextran did not inhibit the enzyme.

SQ 21541. The mean amount of SQ 21541 bound to SMP–dextran for four preparations was 9.1 μg/mg of dextran (S.D. 0.8). Based on the total amount of SQ 21541 allowed to react in these preparations, 38 per cent (S.D. 4.4) had been bound. SQ 21541 SMP–dextran was tested as an inhibitor of converting enzyme (Fig. 3). Calculated from the I_{50} values of the four preparations, SQ 21541 bound to SMP–dextran has 24 per cent (S.D. 4.0) the inhibitory activity of the uncoupled peptide.

Bradykinin. The amount of bradykinin bound to SMP–dextran in five preparations was 10.2 μg/mg of dextran (S.D. 2.7). Of the total amount of bradykinin allowed to react with dextran, 45 per cent (S.D. 15) had been bound. The corresponding figures for bradykinin coupled to CNBr dextran were 7 μg/mg of dextran and 23 per cent.

The relative biological potency (unbound bradykinin = 100 per cent) of bradykinin SMP–dextran was 29 per cent (S.D. 12) when assayed on the isolated rat uterus and 18 per cent (S.D. 8) when assayed on the isolated guinea pig ileum. The bradykinin CNBr–dextran complex had only 6 per cent (S.D. 6) of the biological potency of bradykinin on the rat uterus.

The relative immunological activities of the bradykinin–dextran complexes were determined by radioimmunoassay. Figure 4 shows typical inhibition curves obtained when bradykinin–dextrans compete with labeled bradykinin for antibody binding sites. Estimates of the relative immunological activities (bradykinin = 100 per cent) were made by comparing the concentrations that yielded 50 per cent inhibition. The relative immunological activity of the bradykinin SMP–dextran was 80 per cent (S.D. 17) while that of bradykinin CNBr–dextran was 92 per cent (S.D. 30).

The hydrolysis of bradykinin and bradykinin bound to SMP–dextran during 2 hr of incubation at 37° with purified swine kidney converting enzyme was followed by measuring the remaining immunoreactive bradykinin in a radioimmunoassay (Fig. 5).

Bradykinin coupled to SMP–dextran was less susceptible to hydrolysis by converting enzyme than uncoupled bradykinin. At the end of 1 hr of incubation, about 80 per cent of the kinin SMP–dextran conjugate was still immunologically active while native bradykinin was completely inactivated presumably by the removal of C-terminal Phe-Arg dipeptide [16, 17] (Fig. 5). To see whether this was due to inhibition of the enzyme by the dextran, oxidized–reduced dextran was incubated with uncoupled bradykinin and converting enzyme. The rate of bradykinin hydrolysis did not change significantly (Fig. 5). Therefore, the decreased rate of hydrolysis of the bradykinin SMP–dextran complex was not due to inhibition of the enzyme by dextran itself.

The effects of bradykinin and bradykinin SMP–dextran on the rate of hydrolysis of Hip-Gly-Gly by converting enzyme were also studied (Fig. 6). Bradykinin bound to SMP–dextran was less effective than native bradykinin in inhibiting the hydrolysis of Hip-Gly-Gly. For the same decrease in the rate of Hip-Gly-Gly hydrolysis, coupled bradykinin had to be present in higher concentration than free bradykinin. Oxidized–reduced dextran itself inhibited the hydrolysis of Hip-Gly-Gly, although this was only about 20 per cent of the inhibition obtained with comparable concentrations of the bradykinin SMP–dextran complex.

Non-covalent binding. To establish with certainty that the activity of the kallikrein, aprotinin, SQ 21541, and bradykinin dextran complexes was due to covalently bound material and not to material simply adsorbed on the dextrans, control preparations were made in which the proteins and the peptides were incubated with either non-activated dextran or oxidized–reduced dextran, and the mixtures were carried through the various purification steps. None of the control preparations had activity that could be attributed to adsorbed material. They were inactive relative to the covalently coupled preparations. We could not detect any amino acids in acid hydrolysates of control preparations.

DISCUSSION

These experiments have shown that components of the kallikrein–kinin system can be coupled covalently to soluble macromolecules and retain activity *in vitro*. The proteins and peptides linked to 500,000 mol. wt dextran include the kininogenase, kallikrein the kal-

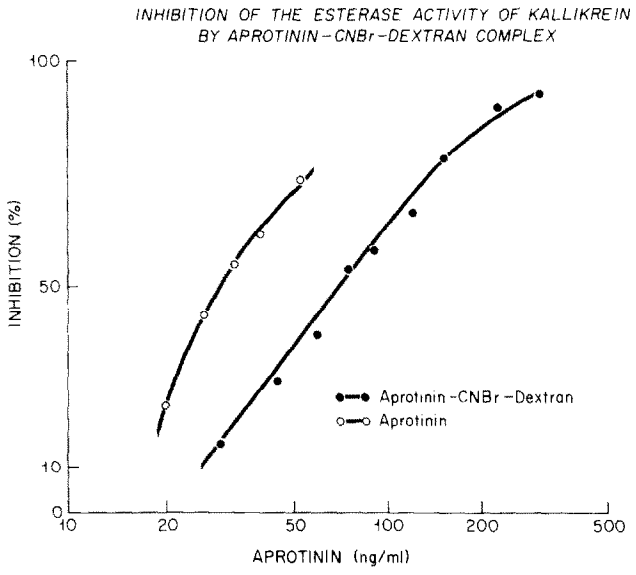

Fig. 2. Inhibition of the enzymatic hydrolysis of BAEe by free and bound aprotinin (Trasylol). Highly purified pig pancreatic kallikrein was the source of enzyme. Key: O—O, aprotinin; ●—●, aprotinin bound to CNBr-dextran. Abscissa: log concentration of aprotinin in ng/ml. Ordinate: per cent of inhibition.

likrein inhibitor aprotinin, the nonapeptide bradykinin, and the angiotensin I converting enzyme or kininase II inhibitor SQ 21541. These materials have biological importance and some are even used clinically. For example, aprotinin has been used in some instances to combat pancreatitis and other conditions [16]. SQ 20881, a nonapeptide converting enzyme inhibitor which is structurally related to the hexapeptide SQ 21541, has been used in clinical experiments to lower the elevated blood pressure of hypertensives. This compound can block the conversion of angiotensin I to II or the inactivation of bradykinin by inhibiting converting enzyme or kininase II [17]. It has been shown previously that components of the kinin system such as kallikrein [18], aprotinin [19, 20], converting enzyme [21], and Hageman factor [22] retained their activities when linked to insoluble matrices.

The coupling of kallikrein to CNBr-dextran did not appear to affect its esterase activity since plotting the activity against the enzyme concentration yielded parallel curves for free and bound enzyme (see Fig. 1). The kallikrein CNBr-dextran complex was found to retain approximately 75 per cent the esterase activity of uncoupled kallikrein. This compared favorably with kallikrein bound to the hydroxysuccinimide

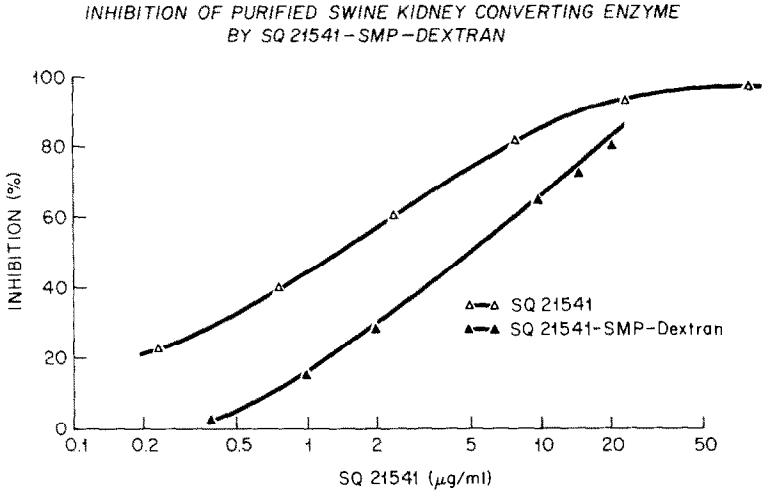

Fig. 3. Inhibition of the hydrolysis of Hip-Gly-Gly by free and bound angiotensin I converting enzyme inhibitor SQ 21541. Highly purified pig kidney angiotensin I converting enzyme (kininase II) was used. Key: Δ—Δ, SQ 21541; ▲—▲, SQ 21541 bound to SMP-dextran. Abscissa: log concentration of inhibitor in μg/ml. Ordinate: per cent of inhibition.

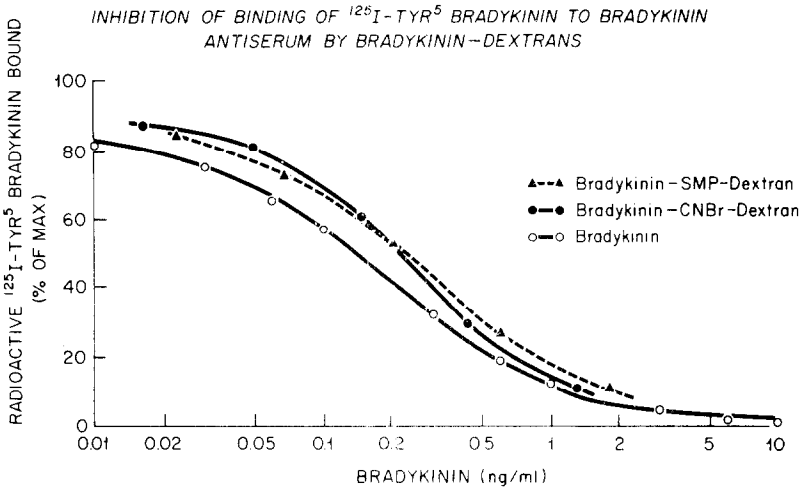

Fig. 4. Inhibition of the binding of [^{125}I]Tyr^5-bradykinin to a bradykinin antiserum by bradykinin and by bradykinin–dextran complexes. The amount of radioactivity specifically bound to antibody in the absence of unlabeled bradykinin is 100 (control). Key: O——O, bradykinin; ▲——▲, bradykinin–SMP–dextran; ●——●, bradykinin CNBr dextran. Abscissa: log concentration of bradykinin in ng/ml. Ordinate: per cent of labeled bradykinin bound.

ester of polyvinylpyrrolidone, which had about one-fourth the activity of free kallikrein on BAEe [6].

SQ 21541 (Arg-Pro-Gln-Ile-Pro-Pro) is a peptide inhibitor of angiotensin I converting enzyme and has an I_{50} of 32 μg/ml when assayed with the rabbit lung enzyme [9]. We obtained an I_{50} of 1.4 μg/ml or 1.8×10^{-6} M for this analogue with purified swine kidney converting enzyme. These differences in I_{50} might be due to the fact that a crude converting enzyme preparation was used when the higher I_{50} value was obtained.

Bradykinin coupled to SMP- or CNBr dextran was still biologically and immunologically active. The immunological activity of either complex was always greater than its biological activity. Possibly there is more steric hindrance to the accommodation of the macromolecular kinin complexes at the receptor sites than there is at the antibody binding sites. This difference in activities is not surprising since, as Haber [23] has reported, the immunological reactivities of bradykinin analogues do not necessarily reflect their biological activities.

The most frequent use of soluble bradykinin macromolecular complexes has been in the production of bradykinin antisera [24–26]. Bradykinin, when coupled to polylysine or rabbit serum albumin, was reported to have less than 0.1 per cent of activity of free bradykinin on the isolated rat uterus, and the antisera induced with them had low titers [25]. In light of this report, we were interested in seeing whether bradykinin attached to SMP–dextran and having about one-fourth the biological activity of free bradykinin might be a more suitable immunogen. In a pilot experiment, two rabbits were immunized with bradykinin SMP–dextran after a schedule which in the past has resulted in useful antisera [15]. No antibodies were produced. Thus, in these preliminary experiments, the bradykinin macromolecule was not antigenic. However, the immunogenicity and other properties of dextran may be altered as a result of the oxidation procedure. For example, SMP–dextran does not release histamine from rat mast cells (A. Goth, personal communication) although dextrans

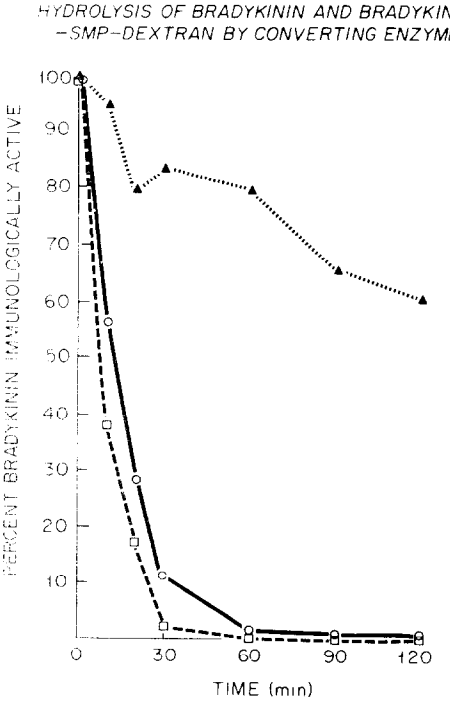

Fig. 5. Hydrolysis of bradykinin and a bradykinin–SMP–dextran complex by highly purified pig kidney angiotensin I converting enzyme (kininase II) determined by radioimmunoassay. Key: O——O, bradykinin; □——□, bradykinin plus oxidized reduced dextran incubated together, ▲——▲, bradykinin coupled to SMP–dextran. Abscissa: time in min. Ordinate: per cent of bradykinin concentration at '0' time.

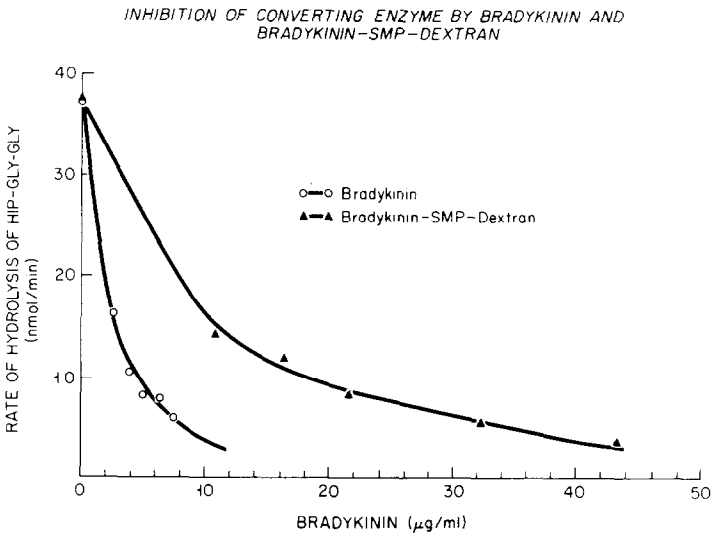

Fig. 6. Inhibition of the hydrolysis of Hip-Gly-Gly by bradykinin and bradykinin bound to dextran. Pig kidney converting enzyme was used. Key: O——O, bradykinin; ▲——▲, bradykinin coupled to SMP–dextran. Abscissa: concentration of bradykinin in μg/ml. Ordinate: rate of hydrolysis of Hip-Gly-Gly in nmoles/min as determined by Δ O.D.$_{254}$.

are active. That the peptide dextran complex was not antigenic may be a useful property if repeated administration of the materials *in vivo* is required.

Bradykinin SMP–dextran was less susceptible than native bradykinin to hydrolysis by purified swine kidney converting enzyme (see Fig. 5) or by the enzyme in human vascular endothelial cells grown in tissue culture [27]. The mechanism of this protection is not known but could be due to the increased size of the molecule, since longer analogues of bradykinin are inactivated more slowly by the enzyme than the nonapeptide [28]. The bradykinin SMP–dextran complex inhibited Hip-Gly-Gly hydrolysis by converting enzyme less than free bradykinin did. This also indicates that the dextran–bound peptide has a lower affinity for the enzyme than free bradykinin. The decreased inactivation *in vitro* of the bradykinin–dextran complex by converting enzyme suggests that this complex may be more active *in vivo*. However, structural constraints at the receptor level may negate any positive effects of decreased degradation. It should be noted in this regard that Suzuki *et al.* [29] have prepared an insulin CNBr–dextran complex that was more effective in lowering blood glucose levels *in vivo* than unbound insulin and was more resistant to degradation than the uncoupled polypeptide.

In summary, the per cent yield of the binding reactions and the activity *in vitro* of the soluble macromolecular dextran complexes formed warrant their further testing *in vivo*.

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