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Separation of Peptide Components of Urinary Kinin (Substance Z)* (33973)

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Werle and Erdös (1, 2) described the existence of a biologically active substance in normal human urine and named the partially purified peptide Substance Z. The substance was hypotensive and contracted isolated smooth muscle preparations of various laboratory animals. Some subtle differences such as stability in alkali and action on rat colon seemed to distinguish this material from kallidin. Gomes (3) indicated that the peptide might be identical with bradykinin, and afterwards it was frequently referred to as urinary kinin. (4) Subsequently, during chromatography of the peptide on a cellulose column, two different components were separated and named Z_1 and Z_2 (5, 6). It was also indicated that the major component was identical with bradykinin and the minor one with kallidin (7). Other investigators attempted to link changes in excretion of the urinary peptide or peptides to various pathological conditions (8). Because of the heterogeneity of the substance and the possible clinical importance of its presence in urine, we undertook to separate and identify its various components. A preliminary report on the subject was published recently (9).

Materials and Methods. The glassware used in the purification process was coated with silicone and treated with 0.1% hexadimethrine (10). Urine was collected in plastic containers from normal male volunteers and extracted as fresh as possible at 4°. The pH of the urine was between 4 and 5 after 1 ml of 0.002 *M* *p*-toluenesulfonic acid was added per 100 ml (11). Subsequently, 100 mg of Amberlite IRC-50 resin in the H^+ form was suspended in each 100 ml of urine. The pH was adjusted to 4 with 10 *M* formic acid and the suspension was stirred for 1 hr (12). The peptides adsorbed on the resin were eluted

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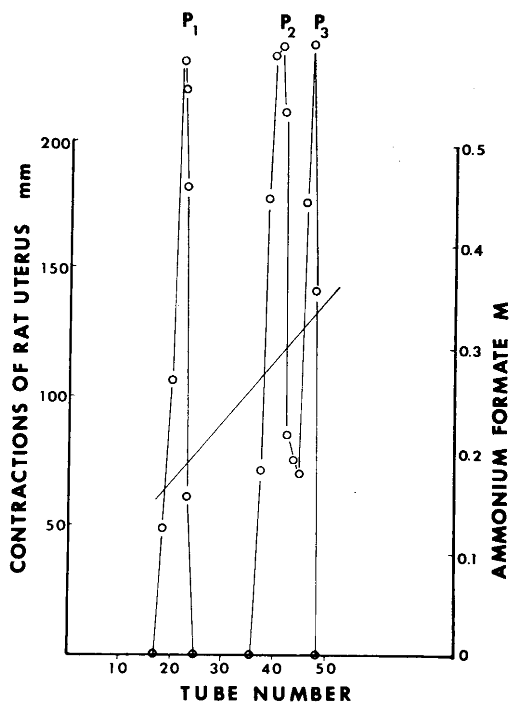

FIG. 1. Separation on the three urinary peptides on a CM-Sephadex C-25 microcolumn: abscissa: tube number; ordinates: contractions of rat uterus (mm) and concentrations of ammonium formate.

with 1 ml of 0.2 *M* ammonium formate/100 ml of urine and the pH was adjusted to 9, while stirring the mixture for 2 hr. After desorption, the resin was removed by filtration through a sintered glass funnel. The pH of the filtrate was adjusted to 3 with 1 *N* HCl and a resulting cloudy precipitate was removed by centrifugation. The supernatant was desalted on a Sephadex G-10 column using 0.002 *M* HCl as eluent. The biological activity of the filtrate was assayed using the isolated rat uterus. The active portion was lyophilized in 0.25% casein and stored as a dry powder.

The next step consisted of chromatographing Substance Z on a CM-Sephadex C-25 column. On the average 200 mg of extract were dissolved in about 5 ml of 0.05 *M* ammonium formate of pH 5 and adsorbed on a CM-Sephadex column (0.4 $\times$ 20 cm). The peptides were eluted and separated with a linear gradient of ammonium formate starting at 0.05 *M* concentration (pH 5) and increasing to 0.5 *M* (pH 7.5) (13, 14). The concentrations of ammonium formate that eluted the peptides were estimated from the gradient curve. The individual points on the curve were checked in control studies with a Radiometer conductivity meter with a CDC 114 flow type conductivity cell. One hundred 2-ml fractions were collected. In control studies synthetic bradykinin, kallidin, and Met-Lys-bradykinin were chromatographed under identical conditions. The percentage of recovery was established by adding 20 μg of synthetic bradykinin and 10 μg of synthetic kallidin to 1 liter of distilled water and carrying the peptides through the various purification procedures as described.

The radioactivity of [2, 3-L-proline-^{14}C] bradykinin chromatographed on a CM-Sephadex column was determined by mixing 1 ml of each fraction collected with 10 ml of Bruns and Christian's scintillation fluid (15) and counted in a Nuclear-Chicago scintillation counter.

The biological activities of the components of Substance Z were established by assays using the isolated rat uterus (6), the systemic arterial blood pressure of the rabbit (6) and the autoperfused hind limb of the dog (16).

The insoluble polymer of porcine pancreatic carboxypeptidase B was prepared in this laboratory (17) following the method of Levin et al (18), originally used for polymerizing trypsin. Labeled bradykinin was obtained through the support of the Radioactive Peptide Program of the National Heart Institute, National Institutes of Health, USPHS.

Results. CM-Sephadex column chromatography of the urinary peptide fractions yielded three active peaks as detected by bioassay with the isolated rat uterus. The three peaks, called P_1, P_2 and P_3 emerged from the CM-Sephadex column at the following molar concentrations of the eluting ammonium formate: P_1, 0.17–0.21; P_2, 0.25–0.32; and P_3, 0.29–0.35. A typical experiment is shown in Fig. 1. In control experiments the same concentrations of ammonium formate eluted the added synthetic kinins, bradykinin, Met-Lys-bradykinin, and kallidin. Bradykinin was

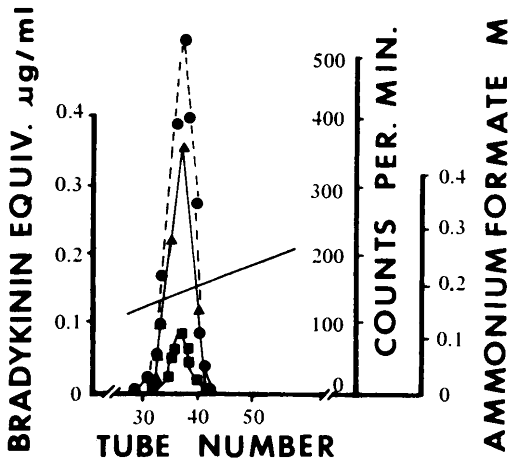

FIG. 2. Chromatography of a mixture of urinary kinin (P_1) and [2, 3-proline-^{14}C] bradykinin on a CM-Sephadex C-25 microcolumn: (▲), activity of peptides on isolated rat uterus expressed in bradykinin equiv.; (■), estimated biological activity of added labeled bradykinin ●, radioactivity, (cpm). Urinary kinin (P_1) and added labeled bradykinin were eluted identically.

eluted as P_1, Met-Lys-bradykinin as P_2, and kallidin as P_3. No. P_2 peak was found in urine samples stored frozen before extraction.

When 0.73 μg of labeled [2, 3-L-proline-^{14}C] bradykinin with an activity of 27.4 μCi/μmole was added to the urinary kinin fraction before CM-Sephadex chromatography, the radioactive material emerged with P_1 (Fig. 2).

The relative effects of various kinin derivatives depend on the bioassay employed and since the longer analogues are usually more active on the blood pressure than on the rat uterus, (6) P_1, P_2, and P_3 were compared to synthetic bradykinin in the three test systems. The results are summarized in Table I.

The relative activity of P_1 on the rat uterus, rabbit blood pressure, and dog hind limb perfusion pressure was about the same as bradykinin. As expected P_2 and P_3 were more potent on the circulation *in vivo* than on the isolated smooth muscle *in vitro* (Table I). The ratios of activities for P_2 and P_3 were very similar to those reported for Met-Lys-bradykinin and kallidin (6).

Of the 20 μg of synthetic bradykinin and 10 μg of kallidin added to 1 liter of water and purified by the same techniques as the urinary peptides, 36 and 10% were recovered. Because the synthetic Met-Lys-bradykinin preparation used was contaminated by another kinin component, its recovery could not be determined accurately. It was estimated to be similar to that of kallidin.

When P_1, P_2, and P_3 were incubated with purified pancreatic carboxypeptidase B (6) their biological activities were immediately destroyed. Adding the insoluble polymer of carboxypeptidase B(17) to P_1 or P_2 also inactivated the peptides.

Discussion. These experiments indicate that the Substance Z, the urinary kinin is a mixture of peptides. Bioassay, elution pattern during chromatography, isotope dilution study with labeled bradykinin, and sensitivity to pancreatic carboxypeptidase B proved that P_1 was identical with bradykinin. No labeled kallidin and Met-Lys-bradykinin (Met-kallidin) were available; however, the identity of P_2 with Met-Lys-bradykinin and P_3 with kallidin was suggested by other experiments. The P_2 was eluted from the CM-Sephadex column with the same concentration of ammonium formate as required for eluting added synthetic Met-Lys-bradykinin and P_3 as kallidin. The results of the bioassay were in agreement with reported effects of kallidin and Met-Lys-bradykinin (6). The sensitivity to carboxypeptidase B indicated that the peptides have a basic carboxyl terminal amino acid.

Carboxypeptidase B inactivated kinins even when the enzyme was coupled to insoluble polymers. The use of insoluble enzymes

TABLE I. Ratios of Biological Activities of Urinary Kinins.

	P_1	P_2	P_3
Rabbit blood pressure[a]: rat uterus[b]	1	9.8	4.1
Dog hind limb flow[c]: rat uterus[b]	1.2	1.8	2.7

[a] Systemic arterial blood pressure, iv injection.
[b] Isolated rat uterus *in vitro*.
[c] Perfusion pressure in autoperfused dog hind limb, ia inj. Synthetic bradykinin was the standard used.

has various advantages. For example, the polymerized enzyme was stable when stored in the refrigerator, while soluble carboxypeptidase B has to be kept frozen (17).

If we compensate for the losses that occurred during purification and for the relatively lower activity of kallidin and Met-Lys-bradykinin when compared to bradykinin on the isolated rat uterus (6) under optimal conditions, the kinin content of 1 liter of fresh, pooled normal urine can be estimated to be 10–36 μg of bradykinin, 6–7 μg of kallidin, and 13–25 μg of Met-Lys-bradykinin. In frozen urine the amount of kallidin decreases and Met-Lys-bradykinin can completely disappear.

The heterogeneity of the urinary hypotensive material was also observed previously by several investigators (5, 6, 19, 20). Between 1960 and 1963, Erdös, Renfrew, and Sloane purified Substance Z. The procedure consisted of adsorption the substance on zinc ferrocyanide (2), elution with alkaline ethanol, extraction with acidified butanol, and chromatography on aluminum oxide column. When the material was rechromatographed on CM-cellulose column two active peaks were found on isolated smooth muscles. Identification of the peptides was attempted by peptide mapping (20). One peak consistently behaved as synthetic bradykinin. The other one was more elusive, but accumulated evidence indicated that it was identical with kallidin (Quarterly Fundamental Research Reports of Mellon Institute, 1961, Pittsburgh, private communication). Two different kinins were also found in the urine later by Deshpande (19). In the meantime, however, Jensen and his associates (7) noticed during the purification of urinary kinin by paper chromatography, a second active material, that was not identical with bradykinin (22), but was similar to kallidin (6,23,24).

Until now Met-Lys-bradykinin was found after release from the precursor kininogenase substrate (kininogen) only when this plasma protein was denatured by acid treatments. Because kininogen is usually not present in human urine, the *in vitro* release of this peptide at pH 4 or 5 of the urine collected is unlikely. Thus the occurrence of a peptide, that behaved in various tests very similarly to Met-Lys-bradykinin, in addition to bradykinin and kallidin which are present in normal urine, is of interest.

Summary. During the purification of Substance Z (urinary kinin), three biologically active peptide components were separated. Isotope dilution studies, biological and chromatographic studies suggested the first peptide to be identical with bradykinin. The investigations also indicated that the two other peptides were kallidin and Met-Lys bradykinin.

Some of the urine samples used were collected by Drs. J. Colmore and H. Schweem with voluntary cooperation of the inmates of Oklahoma State Penitentiary. The synthetic peptides used were donated by Dr. E. Schröder, Schering A. G.; Dr. L. B. Achor, Sandoz, Inc.; and Dr. B. Merrifield of Rockefeller Univ.

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A Study Correlating Virus Particle Frequency with Disease Progression in a Transmitted Murine Leukemia* (33974)

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(Introduced by S. J. Wilson)

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In a detailed pathogenesis study, Potter and Richter (1) described and charted the development and spread of leukemia induced by cellular transmission in the C58 strain of mouse. Since this study most of the published works have involved virus-induced (2–4) and radiation-induced (5) pathogenesis studies of leukemia. The pathogenesis studies by Siegler and Rich (2, 3) reported the light microscopic findings with special emphasis on the preneoplastic changes in the thymus.

The presence of virus particles in organs of leukemic mice have been reported in detail (6, 7). Buffet *et al.* (4) reported the isolation of leukemic virus from organs of Ha/ICR Swiss mice as early as 1 week following inoculation with cell free filtrate of leukemic tissues, therefore indicating the presence of virus particles prior to the development of leukemia. Rich and Johns (8), in a recent publication, reported a similar level of virions in the thymus of their controls when compared with the virus-inoculated ICR/Ha Swiss mice. This implied that the increase in virions was not causally related to the development of the leukemia that followed.

It was our purpose to study relationships between the appearance of virus and the development of murine leukemia following the inoculation of leukemic tissue homogenates.

Materials and Methods. The mice used in this study were randomly inbred axenic CFW mice designated as CFW_w (9). The axenic CFW_w mice were used because of (a) their low indicence of spontaneous leukemic (<0.1%), and (b) the apparent low number of virus particles (9).

A CFW_w mouse (second passage) originally derived from a case of spontaneous lymphocytic leukemia in a conventional CFW_w mouse was selected as donor material for this study. The spleen, lymph node, and thymus were examined by electron microscopy and both intracytoplasmic type A and type C particles were present. A total of twenty-two 2–4-day-old mice were inoculated intraperitoneally with 0.1 ml of a 20% homogenate of spleen, thymus, lymph nodes, and liver from the donor animal. To eliminate the possibility of mice dying with leukemia before they could be examined, the moribund mice were sacrificed early. This selective process left some animals that did not develop leukemia until the end of the experiment. The inoculated mice were autopsied at approximately

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