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THE EXTRACTION OF HUMAN URINARY KININ (SUBSTANCE Z) AND ITS RELATION TO THE PLASMA KININS

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Human urinary kinin (substance Z) has been extracted by modifications of the methods previously described by Gomes (1955) and Jensen (1958). The separation of two oxytocic fractions from such extracts by paper pulp chromatography (Walaszek, 1957; Jensen, 1958) could not be confirmed. Substance Z could not be distinguished from kallidin, bradykinin or glass-activated kinin by parallel quantitative assays, thus confirming that these four substances are very closely related.

Diluted human urine has a marked stimulant effect on the superfused rat uterus (Gaddum, unpublished observations). This finding was followed up by Gomes (1955), who showed that the active principle was a polypeptide similar to bradykinin. More recent chemical evidence suggests that these substances may be different (Gomes, 1957). Werle and Erdös (1954) made similar observations, and called the active principle substance Z. Walaszek (1957) and Jensen (1958) using different chromatographic methods have separated two oxytocic fractions from extracts of human urine. If these fractions really represent different substances, it is important to compare them biologically with each other and with the kinins derived from plasma.

In this investigation, substance Z has been extracted from urine with butanol (Gomes, 1955), by adsorption on IRC-50 (Jensen, 1958) and by various modifications of these methods. Attempts have been made to confirm the presence of two active fractions in such extracts by paper pulp chromatography. Extracts of substance Z have been compared with three kinins prepared from human plasma, kallidin (Werle, 1937; Werle and Berek, 1950), bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949) and glass-activated kinin (Armstrong, Jepson, Keele and Stewart, 1954, 1955, 1957). From the results of parallel quantitative assays it is concluded that the active principle in extracts of substance Z is either identical with or closely related to that responsible for the activity of these three plasma kinins.

METHODS

Rat Uterus.—Virgin rats weighing 120 to 200 g. were injected with stilboestrol (10 μg./100 g.) 16 to 18 hr. before use. Uteri were suspended in either a 2 ml. bath or superfusion apparatus (Gaddum, 1953) at 30 to 31°; the de Jalon solution contained atropine sulphate (1 mg./l.).

Guinea-pig Ileum.—A 2 to 3 cm. segment of terminal ileum from animals weighing 120 to 250 g. was either suspended in a 2 ml. bath or superfused using Tyrode solution containing atropine sulphate (1 mg./l.) and mepyramine maleate (1 mg./l.), at 37°.

Rat Stomach.—The method described by Vane (1957) for the estimation of 5-hydroxytryptamine was followed. Male animals of 300 to 400 g. were used; the stomach was suspended in a 10 ml. bath containing oxygenated Tyrode solution at 37°. A dose cycle of 5 min. with 45 sec. contact was satisfactory.

Rat Duodenum.—The proximal 3 cm. of duodenum from rats weighing 150 to 250 g. was suspended in a 2, 10, or 15 ml. bath or superfused, using atropinized (1 mg./l.) de Jalon solution at 30 to 31°. The tissue was stored at 4 to 6° for 2 to 3 hr. before use. A dose cycle of 3 or 5 min. with 30 sec. contact was used.

Rat Ileum.—A 3 cm. segment of terminal ileum from starved rats (150 to 250 g.) was either superfused or suspended in a 2 ml. bath, using atropinized (1 mg./l.) de Jalon solution at 31°. A dose cycle of 5 to 10 min. was used depending upon the speed of response of the preparation, the drug being allowed to act for 40 to 90 sec.

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Rat Colon.—A 3 cm. segment of ascending colon from rats weighing 150 to 250 g. was either superfused or suspended in a 2 ml. bath. Spontaneous activity was very troublesome; it was reduced but by no means abolished by lowering the temperature to 25° and using de Jalon solution. A dose cycle of 3 or 4 min. with 40 to 60 sec. contact was usually satisfactory.

Rat Blood Pressure.—The technique described by Crawford and Outschoorn (1951) was followed, except that the anaesthetic was a mixture of urethane (60 mg./100 g.) and sodium barbitone (50 mg./100 g.) as recommended for the assay of depressor substances by Amin (1953).

Rabbit Blood Pressure.—Animals weighing 1.0 to 2.5 kg. were anaesthetized with sodium barbitone (180 mg./kg.) injected intravenously. The trachea was cannulated and artificial respiration applied when necessary. Blood pressure was recorded from a carotid artery with a mercury manometer and injections were made into a femoral vein. Heparin (30 mg./kg.) was injected intravenously when the dissection was complete.

Adsorption on IRC-50.—Amberlite resin IRC-50 (chromatographic grade and 100 to 200 mesh) was pre-treated by the method of Hirs, Moore, and Stein (1953). Columns 25 to 40 cm. × 2 to 3 cm. were prepared and equilibrated at *p*H 6 with a phosphate buffer. Fresh human urine, filtered and adjusted to *p*H 6, was passed through the column at 10 ml./min. The column was washed with water (about 4 l.) until the washings were clear. The active principle was eluted with either N hydrochloric acid as described by Jensen (1958) or N sodium hydroxide followed by phosphate buffer at *p*H 9. The rate of elution varied in different experiments from 0.5 to 10 ml./min. A 300 cm. head of pressure was necessary in alkaline elutions to achieve a rate of 10 ml./min., the only resistance being that of the column.

Adsorption on Paper Pulp.—Paper pulp columns were prepared by suspending Whatman No. 1 cellulose powder in the solvent (either 7% phenol or water) and allowing the paper to settle by gravity. The dimensions of the column were 12.0×1.7 cm. The extract of substance Z, dissolved in 2 ml. of solvent, was applied to the top of the column, which was then washed with 50 ml. solvent. Elution was attempted with 0.4% v/v acetic acid and then with 0.1N-hydrochloric acid. Washing and elution were maintained at a constant rate (0.5 ml./min.), and 2.5 or 5 ml. samples were collected on a fraction collector.

Butanol Extraction.—The method described by Gomes (1955) for extracting substance Z from urine was followed with minor modifications. Fresh urine was adjusted to *p*H 1.5 with hydrochloric acid, saturated with sodium chloride and extracted with an equal volume of *n*-butanol. The mixture was centrifuged at 2,000 rev./min. for 15 min. and the organic phase transferred to a precipitation jar, from which the air had been displaced with nitrogen. Five vol. cooled diethyl ether (peroxide-free) were added and the precipitate allowed to settle for 3 to 4 hr. at 4 to 6°. The supernatant was then removed by suction and the precipitate dried *in vacuo*.

Standard Substance Z.—An extract of substance Z prepared by the butanol method above was used as the master standard, 1 mg. being called 1 unit. Substandards were prepared by butanol extraction, IRC-50 adsorption or by a combination of these methods and were standardized against the master standard by 2 + 2 dose assay on the rat uterus.

Preparation of Kallidin. — Freeze-dried human plasma (citrated) was re-constituted with water, and incubated for 10 min. at 37° with N hydrochloric acid (1 in 10) to destroy the kininase and kallikrein inactivator (Horton, 1958). The plasma was then adjusted to *p*H 7.3 with sodium bicarbonate, and incubated at 37° for 2 hr. with human urinary kallikrein (30 mu./ml.). The kallidin was extracted by pouring the plasma into 2 vol. boiling absolute alcohol and boiling for 5 min. The mixture was centrifuged and the supernatant decanted off. The residue was re-extracted with 66% alcohol and the combined supernatants were evaporated to dryness *in vacuo*.

Preparation of Glass-activated Kinin.—Polythene containers and pipettes were used throughout the initial stages. Freshly collected heparinized (10 units/ml.) human blood was centrifuged at 3,000 rev./min. for 1 hr. at 4°. The plasma was transferred to another tube and re-centrifuged. The upper ¾ was pipetted into an unsiliconed glass tube and stirred with a glass rod. The development of activity was followed on the rat uterus. After 20 min. contact, the kinin was extracted with alcohol as described for kallidin. The final residue was shaken with ether to remove lipids, and re-dried *in vacuo*.

Bradykinin.—This was prepared by the action of trypsin on ox globulins. It had an activity of 7.1 units/mg. and was kindly supplied by Dr. M. Schachter.

Preparation of Human Urinary Kallikrein.—Pooled male human urine (5 l.) was concentrated to a fifth of its volume in a climbing film evaporator. The concentrate was dialysed through cellophane against running tap water for 48 hr. The kallikrein was then precipitated by the addition of 4 vol. acetone at 4°. The precipitate was separated and dried *in vacuo*. The amorphous material was powdered and standardized against a sample of Padutin (Bayer) kindly supplied by Dr. E. Werle.

RESULTS

Butanol Extraction of Substance Z from Human Urine

Substance Z was extracted from urine with butanol by the method of Gomes (1955). The recovery was estimated by comparing the activity of the extract with that of the crude urine. In different experiments yields varied from 0.5 to 10% in contrast to the 100% reported by Gomes. The reasons for this discrepancy were investigated.

It was possible that substance Z is unstable at the *p*H (1.5) of extraction. However, since urine adjusted to *p*H 1 could be boiled for 1 hr. without detectable loss of activity, that possibility was excluded. Extraction with 1 vol. *n*-butanol might be incomplete. When the separated butanol layer (after extraction) was shaken with water, 80 to 100% of the original substance Z was found in the aqueous phase. This suggested that the loss of activity must occur during the ether precipitation.

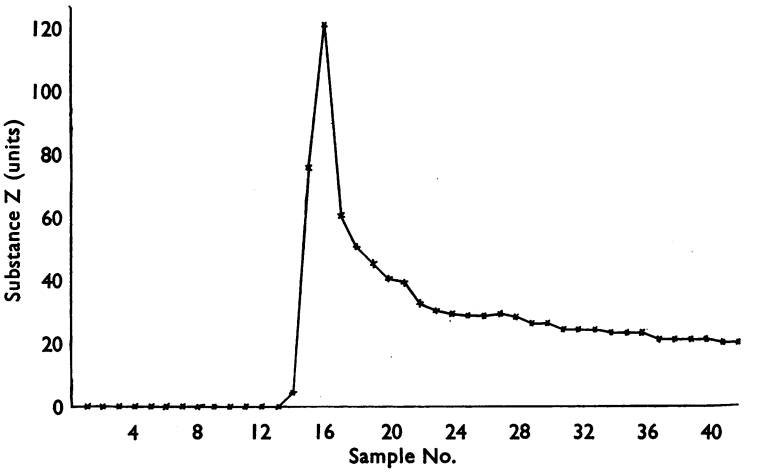

FIG. 1.—IRC-50 column 37·0 × 2·5 cm. 3 l. of human urine (10 units substance Z/ml.) applied at *p*H 6. Elution (1 ml./min.) with N hydrochloric acid. Recovery (samples 14 to 42)=32%. Each sample was 25 ml. in volume.

If the ether was allowed to stand exposed to the air either before or during the precipitation, recoveries were always poor (0.5 to 2%). If fresh ether was used and the precipitation carried out in an atmosphere of nitrogen, recoveries of 10% were invariably obtained. It seemed probable that ether precipitation was an inefficient method of obtaining substance Z from the butanol phase.

It was found that recoveries of 80 to 90% could be obtained by evaporating the butanol phase to dryness *in vacuo* at 50° using a capillary air inlet. Although more of the active material was recovered than with ether precipitation, there was a corresponding increase in the amount of impurities, and so the final dried powder was only slightly more active on a weight basis than that obtained by ether precipitation.

Adsorption of Substance Z on IRC-50

Jensen (1958) has shown that the Amberlite resin IRC-50 is suitable for extracting substance Z from large volumes of urine. He eluted the active principle with hydrochloric acid and obtained recoveries up to 60%.

Several experiments were performed using this method. Recoveries varied from 10 to 50%. These low figures were partly the result of considerable tailing, which persisted even when the elution rate was slow (0.5 ml./min.) (Fig. 1). In this way large volumes of eluate containing a relatively low concentration of substance Z were obtained. Since one of the purposes of using the column was to concentrate the activity into smaller volumes, it was important to find a way of overcoming this difficulty.

Elution of substance Z, a polypeptide, from IRC-50 with acid depends upon depressing the ionization of the column. If the *p*H is raised the capacity of the column for cations is increased, but the peptide is converted into an anionic form and will not be retained. This proved to be a good way of eluting substance Z from IRC-50.

Batchwise experiments showed that substance Z could be eluted from IRC-50 at *p*H 9. Sodium hydroxide was added in an amount calculated from previous titrations to raise the *p*H of the column from 6 to 9. In a batchwise experiment, the resin would rapidly equilibrate at this *p*H, but when carried out on a column a *p*H gradient was established, the upper parts being more alkaline. M sodium phosphate buffer (*p*H 9) was added after the sodium hydroxide. As the buffer passed through the *p*H was readjusted to 9, alkali being displaced to raise the *p*H lower down. Ultimately, the whole column reached *p*H 9, and at that point a small number of highly active fractions were collected with recoveries of 80 to 100% (Fig. 2). Since substance Z is rather unstable in alkali, elution was carried out rapidly (10 ml./min.), some protection from the alkaline solvent front being provided by the buffering action of the column.

Elution could also be effected with phosphate buffer alone, but large quantities of buffer were required, elution was much slower and the peak was not so sharp.

Eluate samples from IRC-50 columns were extracted by the butanol method described above, and the butanol phase was evaporated to dryness. Good recoveries were obtained. The final dried powder was stable and contained 10 to 15 units/mg.

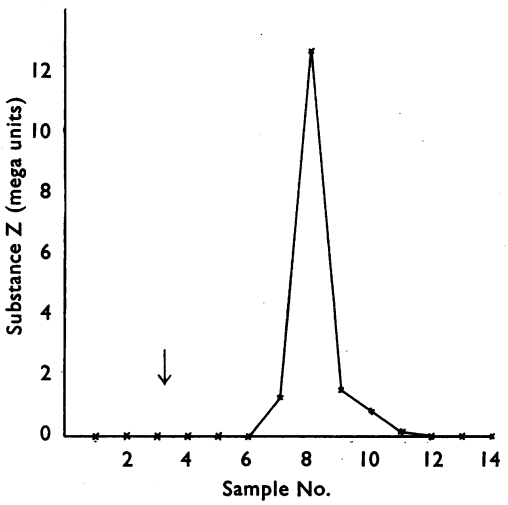

FIG. 2.—IRC-50 column 27·5 × 2·0 cm. 2 l. of human urine (10 units substance Z/ml.) applied at *p*H 6. Elution (10 ml./min.) with 80 milliequivalents sodium hydroxide followed by sodium phosphate buffer (*p*H 9) at arrow. Recovery=82%. Sample volume, 25 ml.

Paper Pulp Chromatography

Numerous experiments were performed in an attempt to repeat the work of Walaszek (1957) and Jensen (1958) in which two fractions were separated from substance Z extracts by paper pulp chromatography.

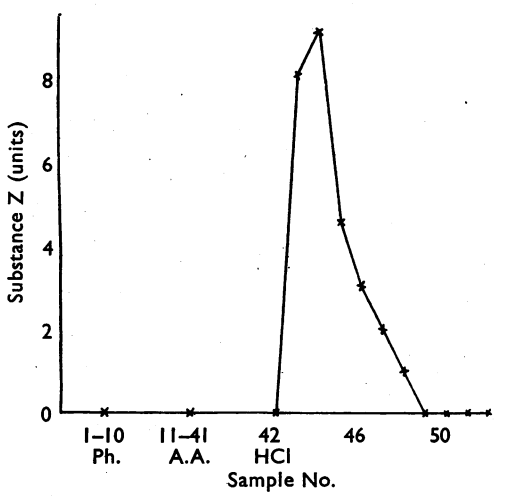

FIG. 3.—Paper pulp column 12·5 × 1·7 cm. prepared by suspending cellulose powder in 7% phenol. 55 units of substance Z extract, dissolved in 2 ml. 7% phenol, applied. Column washed with 50 ml. 7% phenol (**Ph.**). Elution with 150 ml. 0·4% v/v acetic acid (**A.A.**) followed by 50 ml. 0·1 N hydrochloric acid (**HCl**) at 0·5 ml./min. Recovery=50%. Sample volume, 5 ml.

A typical result obtained when using 7% phenol as the solvent is shown in Fig. 3. An active fraction corresponding to Walaszek's Z_1 could not be eluted with 0.4% v/v acetic acid, using up to 250 ml., whereas a single peak of activity appeared on elution with 0.1 N hydrochloric acid, corresponding to Walaszek's Z_2 fraction.

When water was used instead of 7% phenol, results similar to those reported by Jensen (1958) were obtained. A peak of activity could be washed through the column with water, and a second peak occurred on elution with 0.1 N hydrochloric acid (Fig. 4). It seemed probable that the first peak represented excess substance Z from overloading the column ; this was confirmed in two ways.

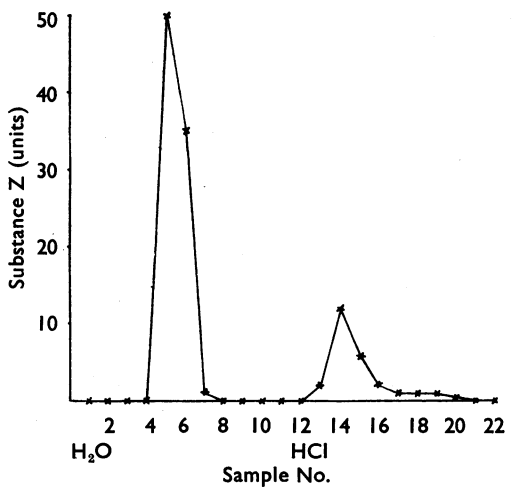

FIG. 4.—Paper pulp column 12·5 × 1·7 cm. prepared by suspending cellulose powder in distilled water. 131 units of substance Z extract, dissolved in 2 ml. distilled water, applied. Column washed with distilled water (H_2O). Elution with 0·1 N hydrochloric acid (**HCl**) at 0·5 ml./min. Total recovery (washings plus eluates)=87%. Sample volume, 5 ml.

The experiment illustrated in Fig. 4 was repeated. Columns of the same size were used, but the amount of substance Z extract applied was reduced from 131 units to 88 and 38 units respectively in the two experiments. The results are shown in Fig. 5 (cf. Fig. 4). Approximately the same amount of activity, about 30 to 35 units, was eluted with hydrochloric acid from each of the three columns. On the other hand the size of the first peak fell from 86 to 42 to 0 units. Clearly, the capacity of a paper pulp column of these dimensions was about 35 units, and if more than this was applied the excess passed through in the aqueous washings.

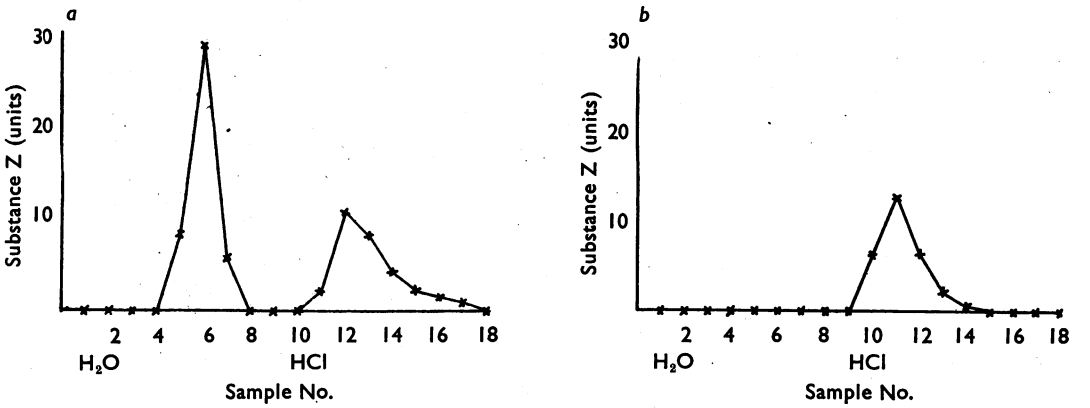

FIG. 5.—Columns prepared as in Fig. 4. In (*a*) 88 units and (*b*) 38 units of substance Z extract applied. Columns washed with distilled water (H_2O). Elution with 0·1 N hydrochloric acid (HCl) at 0·5 ml./min. Total recoveries (*a*) 90%, (*b*) 72%.

Active material (30 units) which passed unadsorbed through one column was applied to a second. All the substance Z was adsorbed, and most of it could be recovered by hydrochloric acid elution.

Thus the separation of two active fractions on paper pulp using 7% phenol could not be confirmed, and the appearance of two peaks when phenol was omitted was shown to be due to overloading of the column.

Substance Z and Kallidin

Kallidin is the plasma kinin formed by the action of kallikrein on plasma (Werle, 1937). Since it is said to differ from substance Z (Werle and Erdös, 1954), parallel quantitative assays were performed (Fig. 6). The results on four tissues are shown in Table I. Each figure represents the mean of three assays with the standard error. From these parallel assays it is clear that kallidin and substance Z must be closely related.

TABLE I
UNITS OF SUBSTANCE Z EQUIACTIVE WITH 1 MG. KALLIDIN

	Units (±s.e.)
Guinea-pig ileum	12·0±3·5
Rat duodenum	9·2±1·2
,, ileum	8·7±0·3
,, uterus	8·1±0·6

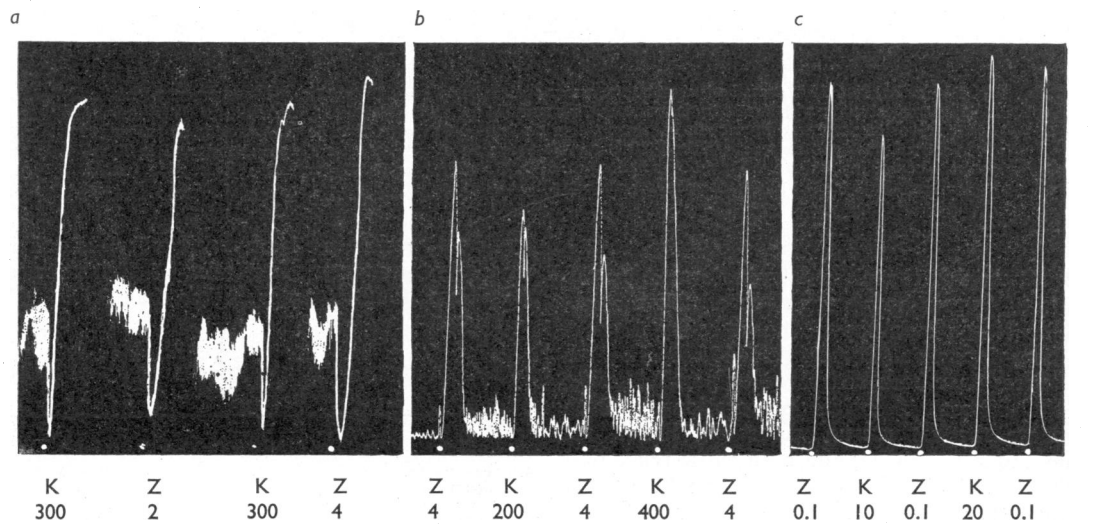

FIG. 6.—(*a*) Rat ileum suspended in a 2 ml. bath containing atropinized (10^{-6}) de Jalon solution at 31°. (*b*) Guinea-pig ileum suspended in a 2 ml. bath containing atropine (10^{-6}) and mepyramine (10^{-6}) in Tyrode solution at 37°. (*c*) Rat uterus suspended in a 2 ml. bath containing atropinized (10^{-6}) de Jalon solution at 30°. K=human kallidin extract (μg.). Z=substance Z extract (units).

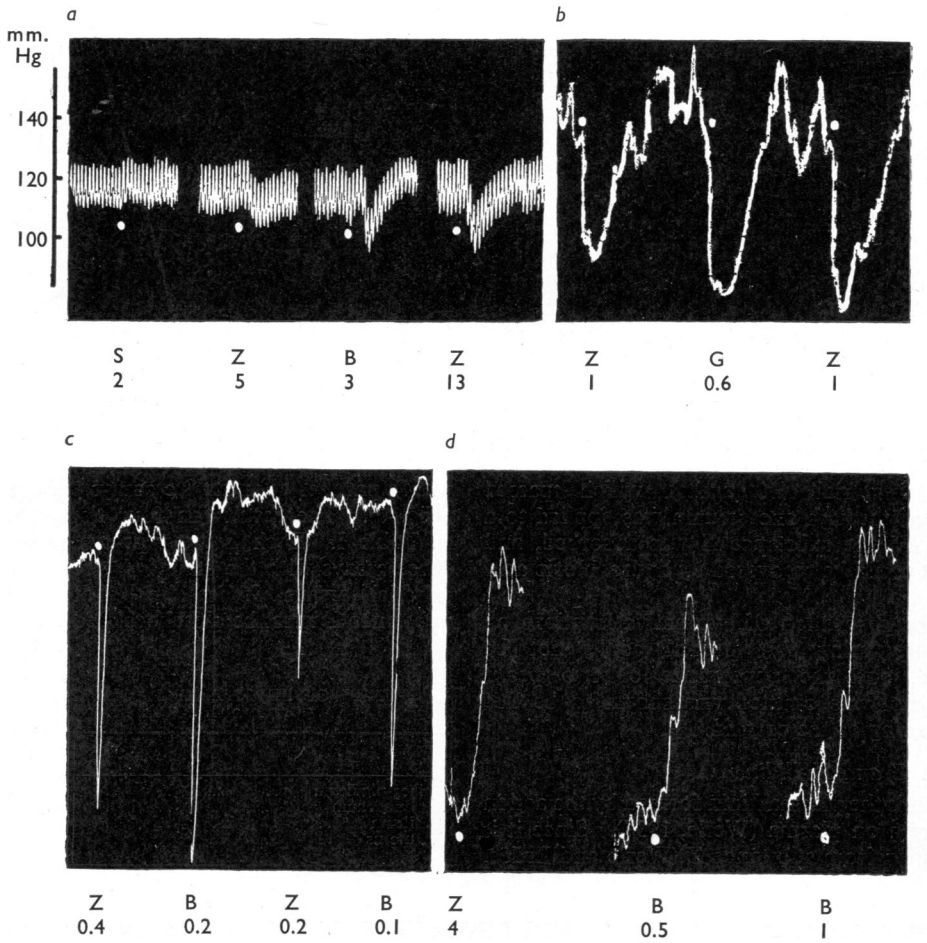

FIG. 7.—(*a*) Rabbit 1·5 kg. anaesthetized with intravenous sodium barbitone (180 mg./kg.). Blood pressure recorded from carotid artery with a mercury manometer. Heparin (45 mg.) was injected intravenously as anticoagulant. (*b*) Rat colon suspended in a 2 ml. bath containing de Jalon solution at 25°. (*c*) Rat duodenum suspended in a 2 ml. bath containing atropinized (10^{-6}) de Jalon solution at 31°. (*d*) Rat stomach suspended in 10 ml. bath containing oxygenated Tyrode solution at 37°. S=saline (ml.). Z=substance Z extract (units). B=bradykinin (units). G=glass-activated kinin (mg.).

Substance Z and Bradykinin

Bradykinin is the plasma kinin formed by the action of trypsin on plasma (Rocha e Silva *et al.*, 1949). It has been shown that substance Z is a similar polypeptide (Gomes, 1955, 1957). The two substances have been compared by parallel assays on six different biological preparations. Both stimulate the rat uterus, guinea-pig ileum, rat ileum and rat stomach, and both cause relaxation of the rat duodenum and a fall of the rabbit blood pressure (Fig. 7).

The values in Table II differ among themselves, but are not inconsistent with the view that the same active principle is responsible for the activity of the two extracts. The index of discrimination (Gaddum, 1955) is never higher than 2.5.

TABLE II

UNITS OF SUBSTANCE Z EQUIACTIVE WITH 1 UNIT BRADYKININ

	Units (±s.e.)
Guinea-pig ileum	10·0±3·0
Rat ileum	7·0±0·8
,, uterus	6·2±0·5
,, stomach	4·5±1·2
,, duodenum	4·4±1·0
Rabbit blood pressure	4·0±1·1

Substance Z and Glass-activated Kinin

When human plasma is exposed to a glass surface, a kinin is formed which causes pain and stimulates the rat uterus (Armstrong *et al.*, 1954, 1955, 1957). This substance is very similar to bradykinin (Jepson, Armstrong, Keele and Stewart, 1956). In view of the similarity of substance Z, it was of interest to compare it also with glass-activated kinin. The relative potencies obtained by parallel assays on six tissues are shown in Table III. In this series only one assay was performed on each tissue and the error represents the limits of a bracketing assay. In general the values agree well enough; 0.6 would fall within the error range of them all. It would seem that substance Z and the glass-activated kinin are also closely related.

TABLE III

UNITS OF SUBSTANCE Z EQUIACTIVE WITH 1 MG. GLASS-ACTIVATED KININ

	Units
Guinea-pig ileum	1·2±0·6
Rat duodenum	1·0±0·5
,, colon	1·0±0·4
,, ileum	0·8±0·3
,, uterus	0·5±0·2
,, blood pressure	>0·3

DISCUSSION

Extraction of Substance Z

Extraction of substance Z from human urine using the method described by Gomes (1955) gave poor yields. Precipitation with ether appears to be an inefficient way of recovering the active material from the butanol phase; much better recoveries have been obtained by evaporating the butanol to dryness *in vacuo*, and the latter procedure has been adopted in the final method.

Elution of substance Z from IRC-50 with alkali was found to be more satisfactory than the acid elution used by Jensen (1958). Recoveries were good and the active material was concentrated in very few fractions. As pointed out by Jensen (1958), columns of IRC-50 have the advantage that substance Z can be adsorbed very conveniently from large volumes of urine. If elution is carried out with alkali, little activity is lost and the dried samples contain inorganic phosphate as the main impurity. If the eluate samples are then extracted with butanol, little inorganic material passes into the organic phase and thus the main impurity is left behind. The extracts prepared by this combined method are less active but more stable than those prepared by Jensen (1958) using IRC-50 and paper pulp columns.

Paper Pulp Chromatography

Walaszek (1957) applied substance Z extracts to paper pulp columns in 7% phenol and was able to elute two peaks of activity with 0.4% acetic acid. He subsequently found that the second peak could be eluted more readily with 0.1 N hydrochloric acid, with a total recovery of 60 to 90%. In the present investigation no activity could ever be eluted with acetic acid and this accounted for the failure to separate two fractions. The active material could be eluted readily by 0.1 N hydrochloric acid in a single peak with yields of 50 to 70%. The difference of these findings from those of Walaszek (1957) may have been due to slight differences in technique or materials; it is interesting, however, that Jensen (1958) was also unable to recover a Z_1 fraction by this method.

Similar results were obtained if this type of experiment was performed using water instead of 7% phenol as the solvent. An apparent separation of two fractions (see Jensen, 1958) was shown to be due to overloading of the column. It provided no evidence for the presence of two different substances. These negative results do not, however, exclude such a possibility. Jensen (1958) produced some evidence to show that two fractions could also be separated by chromatography on an alumina column. That type of separation was not attempted in the present investigation.

Substance Z and the Plasma Kinins

Werle and Erdös (1954) concluded that substance Z and kallidin were different substances. Their evidence was that substance Z dialysed more slowly and that it was rather more stable to alkali than kallidin. Both these differences might be accounted for by differences in purity of the two extracts. In addition, they found that substance Z had no effect on the rat intestine unlike kallidin and bradykinin which stimulate that preparation. In the present investigation extracts of substance Z caused relaxation of the rat duodenum and rat colon, and contraction of the rat ileum, similar effects being observed with kallidin and bradykinin. It is possible that Werle and Erdös (1954) used too little substance Z in their experiments with the intestine.

Substance Z and kallidin could not be distinguished by parallel assays on four tissues suggesting that they may contain the same active principle. This is supported by the similar results with bradykinin, which is possibly identical with kallidin (Werle, 1953; Holdstock, Mathias, and Schachter, 1957; Mathias and Schachter, 1958).

The relative potencies of substance Z to bradykinin on the rabbit blood pressure, rat uterus and guinea-pig ileum (Table II) show certain similarities to those obtained by Gomes (1957). In both investigations substance Z appeared to be relatively more active than bradykinin on the blood pressure and relatively less active on the ileum, the uterus being intermediate. These differences are small, but they may be significant if, as Gomes (1957) suggests from counter-current distribution experiments, the active principles are different.

The kinin prepared by glass-activation of human plasma was not tested for its pain-producing activity and so is referred to here as glass-activated kinin and not as pain-producing substance (Armstrong *et al.*, 1954). This kinin is known to be very similar to bradykinin (Jepson *et al.*, 1956; Armstrong *et al.*, 1957), and its similarity to substance Z on parallel assays provided more evidence of the close relationship of all these peptides.

From these biological comparisons it is concluded that the same active principle may be responsible for the activity of the four kinins studied, but the question must be settled ultimately by chemical analysis of the isolated peptides.

In the meantime there seems little justification for the continued use of the term "substance Z," and it is suggested that it should be replaced by "urinary kinin," analogous to the terms plasma kinin (Lewis, 1958) and wasp kinin (Schachter and Thain, 1954), thus emphasizing their similarity without implying identity.

We are grateful to Dr. R. A. Cumming, Regional Director of the Blood Transfusion Service, the Royal Infirmary, Edinburgh, for supplies of blood and plasma.

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