Tri-*m*-tolyl phosphate is not neurotoxic.[1-3] It was administered orally to rabbit and *m*-(α-hydroxy)-cresol was found in urine. Hook *et al.*[1,2] found that a low toxic insecticide Sumithion, *O,O*-dimethyl *O*-3-methyl-4-nitrophenyl phosphorothioate, was metabolized to 2-nitro-5-hydroxybenzoic acid in several species of mammals. It appears that the hydroxylation of meta alkyl group may not relate with the activation of the esters, but with degradation.

This investigation was supported in part by a grant from Japan Ministry of Education.

Laboratory of Pesticide Chemistry,
Department of Agricultural Chemistry,
Kyushu University,
Fukuoka, Japan

MORIFUSA ETO
MISAKO ABE

REFERENCES

1. D. HENSCHLER, *Archs exp. Path. Pharmak.* **237,** 459 (1959).
2. H. F. BONDY, E. J. FIELD, A. N. WORDEN and J. P. W. HUGHES, *Br. J. Indust. Med.* **17,** 190 (1960).
3. W. N. ALDRIDGE and J. M. BARNES, *Biochem. Pharmac.* **6,** 177 (1961).
4. M. ETO, J. E. CASIDA and T. ETO, *Biochem. Pharmac.* **11,** 337 (1962).
5. M. ETO, S. MATSUO and Y. OSHIMA, *Agr. biol. Chem.* **27,** 870 (1963).
6. M. ETO, Y. OSHIMO and J. E. CASIDA, *Biochem. Pharmac.* **16,** 295 (1967).
7. R. L. BARON, D. R. BENNETT and J. E. CASIDA, *Br. J. Pharmac.* **18,** 465 (1962).
8. W. N. ALDRIDGE and J. M. BARNES, *Biochem. Pharmac.* **15,** 541 (1966).
9. J. D. TAYLOR, *Toxic. appl. Pharmac.* **11,** 538 (1967).
10. J. E. CASIDA, R. L. BARON, M. ETO and J. L. ENGEL, *Biochem. Pharmac.* **12,** 73 (1963).
11. J. E. CASIDA, *Biochem. Pharmac.* **5,** 332 (1961).
12. G. E. R. HOOK, T. W. JORDAN and J. N. SMITH, *Enzymatic Oxidation of Toxicants* (Ed. E. HODGSON), p. 27, North Carolina State University, Raleigh (1968).

Biochemical Pharmacology, Vol. 20, pp. 969–973. Pergamon Press, 1971. Printed in Great Britain

Determination of human urinary kinin levels by radioimmunoassay using a tyrosine analogue of bradykinin*

(*Received* 17 *August* 1970; *accepted* 30 *October* 1970)

BRADYKININ (BK), kallidin, and methionyl-lysyl-bradykinin, are a group of closely related vasodilator peptides. It has been shown that the flushing attacks experienced by patients with the carcinoid syndrome[1,2] are frequently associated with elevated blood kinin levels and it is widely believed that they are important mediators of inflammation. The existence of these peptides in the blood,[3] urine,[4] and other body fluids of normal individuals, suggests that they may also have a physiological function but difficulties in quantitating the kinin levels in various situations have limited progress in defining any such role.

Radioimmunoassay has been applied to a number of the peptide and protein hormones present in biological fluids in low concentration and already a number of groups have reported the development of antibodies to bradykinin.[6–8] So far one report[9] has appeared describing application of these methods to measure kinin levels in blood and synovial fluid. The present report describes the use of the radioimmunoassay to measure kinin levels in the urine of normal human subjects and compares the figures obtained with those from a bioassay procedure. The relative ease with which large numbers of samples can be processed simultaneously in the former method confers great practical advantages even when as in urine the concentration present permits a biological assay to be performed quite readily.

Methods

Development of antibodies. BK was coupled to either ovalbumin or human gamma globulin by means of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodi-imide HCl.[10] The conjugated BK dissolved in water was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into rabbits. Each rabbit received BK complexed with only one of the carrier proteins and was injected with 1 mg of coupled BK each month. Antibodies were developed within 6 months using either carrier protein.

Labelling the antigen. It is not possible to label BK with radioiodine since it does not contain a tyrosyl residue. An analogue of BK in which the phenylalanyl residue in the 8 position was replaced by a tyrosyl residue ([Tyr^8]-BK) was synthesized in this laboratory by the solid phase technique.[11] Aliquots of this peptide were iodinated using a modification of the chloramine-T method.[12]

Radioimmunoassay. All dilutions were made with 0·1 M tris-HCl pH 7·4 containing 0·2% gelatin, 0·1% neomycin and 0·01 M EDTA. The incubation mixture consisted of:

0·10 ml immune serum at the chosen suitable dilution.
0·02 ml 10^{-1} M phenanthroline HCl.
0·05 ml [^{125}I-Tyr^8]-BK containing 8000 counts/min.
0·50 ml diluent buffer containing either standard peptide or the unknown.

This mixture was incubated at 5° for 18 hr following which the [^{125}I-Tyr^8]-BK bound to antibody was separated from the unbound by absorbing the latter on dextran coated charcoal.[13]

The specificity of the antibodies was investigated by constructing displacement curves with BK,† kallidin,‡ met-lys-BK‡ and angiotensin II. These displacement curves were obtained in the usual manner by plotting the percentage of labelled peptide bound by antibody against the concentration of standard peptide added.

For all estimations of urinary kinins, BK† standards were used.

Bioassay. Estimates of urinary kinin were made by conventional four point assay using the isolated perfused rabbit ear vein.[14]

Urine collections. Sixteen urine samples from eight normal males were collected into glass vessels treated with hexadimethrine bromide to prevent non-specific adsorption of kinins.[15] Aliquots were transferred into hexadimethrine-treated polythene tubes and immersed in boiling water for 1 hr to inactivate kininases. Each boiled sample was assayed by both methods on the day of collection and was kept at 4° while awaiting processing. Duplicate immunoassays were performed on the urine at two dilutions giving four such estimates for each sample.

Recovery of internal standards by immunoassay. BK standards of 50, 100, 200 and 400 picogram were added to four urines diluted 1 in 100 with diluent buffer and the recovery of these internal standards was estimated by radioimmunoassay according to the technique described.

Results

Sera from rabbits immunized with BK-protein complex as described were capable of binding the labelled tyrosyl analogue and this combination could be inhibited by the addition of picogram amounts of kinins to the incubate (Fig. 1). Addition of up to 1 μg angiotensin II, had no effect on the displacement of the labelled peptide. The dilution of the different antisera needed to bind 50 per cent of the [^{125}I-Tyr^8]-BK used (8000 counts/min) varied from 1/5 to 1/1000. Serum from control rabbits showed no capacity to bind the iodine-labelled peptide.

The displacement curves shown in Fig. 1 were obtained by adding increasing amounts of BK, kallidin or met-lys-BK to the antibody and [^{125}I-Tyr^8]-BK incubation mixture. With BK the degree

* Supported in part by a Grant-in-Aid from the National Heart Foundation of Australia.

† Provided by courtesy of Dr. E. Stürmer and Dr. I. S. Collins of Sandoz Ltd.

‡ Obtained through the support of the radioactive peptide synthesis programme of the National Heart Institute, National Institutes of Health, Bethesda, Maryland, and by courtesy of Dr. J. J. Pisano.

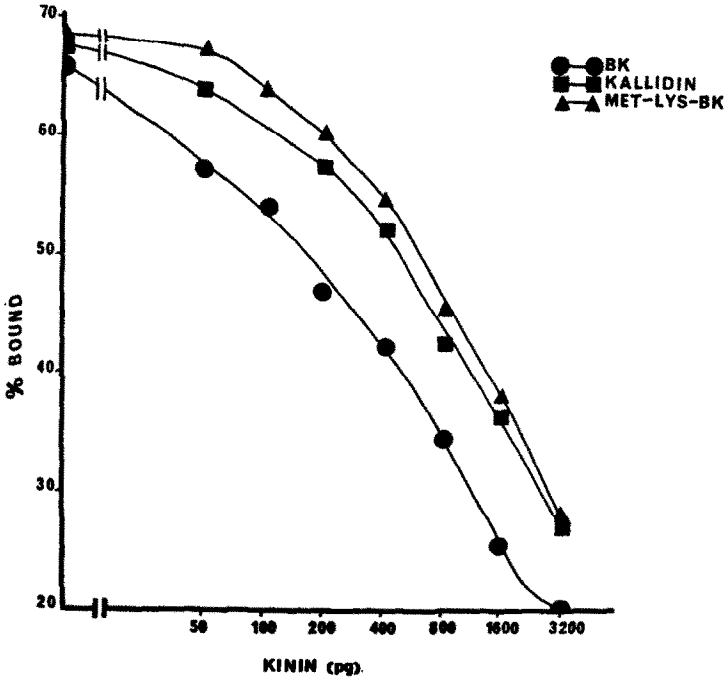

FIG. 1. Displacement of ^{125}I-Tyr^{8}-BK by increasing amounts of BK, kallidin and met-lys-BK.

of displacement increases rapidly with the amount added over the range of 50–1600 picogram. Kallidin and met-lys-BK have similar ability to displace the labelled peptide but have less than one-half the efficiency of BK.

When urine diluted with diluent buffer replaced the standard peptide in the incubation mixture, displacement of the labelled peptide was obtained. Dilutions of 1/50 and 1/100 usually resulted in displacement on the sensitive part of the curve permitting assay of urine levels. The mean percent difference between duplicate estimations was 16·9 per cent (S.E. 3·3%). When tested by analysis of variance the difference between estimates at the two dilution levels was not significantly different to that between duplicates.

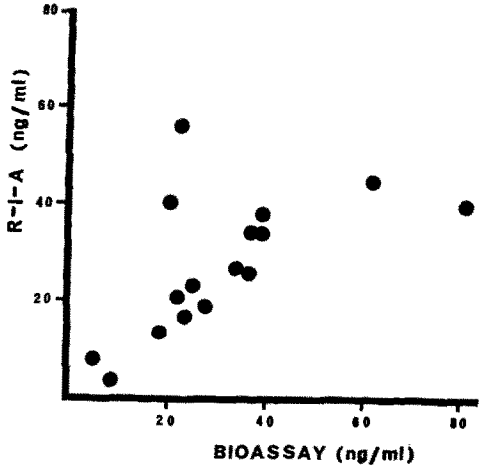

FIG. 2. Plot of urinary kinin levels by radioimmunoassay against those by bioassay for 16 urine samples.

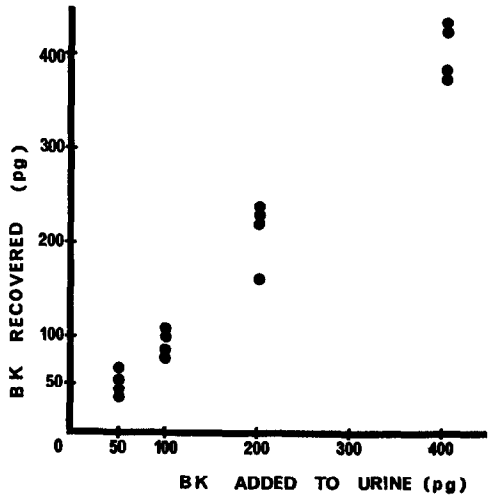

FIG. 3. Recovery of BK added to urine as internal standard determined by radioimmunoassay.

The levels of urinary kinin obtained by the bioassay and immunoassay are compared in Fig. 2 and there is a significant correlation between estimates obtained by the two assays ($P < 0.05$). Most of the points fall below the line of identity indicating that the results of radioimmunoassay are on the whole lower than bioassay.

To determine whether components of urine were interfering with detection of kinins by radioimmunoassay, recovery of internal standards by immunoassay was measured. These results are shown in Fig. 3 in which the estimated values are plotted against the actual values of peptide added. The resulting calculated regression line had a slope of 1·03 and a negative intercept of 3·8 pg. The 90 per cent confidence limits on the slope of this line are 1·1–0·96, while the 90 per cent confidence limits for the intercept are −74·6–67·0 pg showing that the calculated regression line does not significantly differ from the line of identity. Thus the lower levels for urinary kinin obtained by radioimmunoassay are not accounted for by reduction of recovery due to some interfering constituent of urine.

Discussion

Moderately good agreement was obtained between levels estimated by radioimmunoassay and those found by bioassay although the former were in most cases lower. This does not suggest that kinin metabolites or other substances in urine are interfering with the assay by binding to the antibody. If this were the case the levels would be above those from bioassay since the peptide fragments would have little or no biological activity. Furthermore dilution of urine gave the same slopes for displacement as the standard curve showing that the displacing material has the same immunological reactivity as authentic BK.

The disparity between the two sets of results could be accounted for in part by the experimental variability of the methods. A more likely reason for the fairly consistent divergence is that the relative activities of the different kinins may differ in the two systems. Miwa *et al.*[16] have shown that there are considerable amounts of both kallidin and met-lys-BK as well as BK in human urine. In the perfused rabbit ear vein kallidin and met-lys-BK have 53 and 48 per cent respectively of the activity of BK on a weight basis. A comparison of the relative potencies of the 3 kinins in the radioimmunoassay (Fig. 1) shows that kallidin and met-lys-BK are less effective when compared to BK in this assay than they are in the bioassay.

The immunoassay described will facilitate studies on urine kinins since the method is capable of handling many more estimations in a given time than the bioassay. However the differences between the performance of closely similar kinins in immunoassay compared with their biological activity must be remembered in the interpretation of results obtained.

University of Melbourne,
Department of Medicine,
Austin Hospital,
Heidelberg, Victoria 3084,
Australia

M. L. MASHFORD
M. L. ROBERTS

REFERENCES

1. D. T. MASON and K. L. MELMON, *J. clin. Invest.* **45**, 1685 (1966).
2. M. L. MASHFORD and R. ZACEST, *Aust. Ann. Med.* **16**, 326 (1967).
3. R. ZACEST and M. L. MASHFORD, *Aust. J. exp. Biol. med. Sci.* **45**, 89 (1967).
4. J. H. GADDUM and E. W. HORTON, *Br. J. Pharmac.* **14**, 117 (1959).
5. M. L. MASHFORD and R. ZACEST, *Aust. J. exp. Biol. med. Sci.* **45**, 661 (1967).
6. T. L. GOODFRIEND and D. L. BALL, *J. Lab. clin. Med.* **73**, 501 (1969).
7. J. SPRAGG, K. F. AUSTEN and E. HABER, *J. Immunol.* **96**, 865 (1966).
8. H. RINDERKNECHT, B. J. HAVERBACK and F. ALADJEM, *Nature, Lond.* **213**, 1130 (1967).
9. T. C. TALAMO, E. HABER and K. F. AUSTEN, *J. Lab. clin. Med.* **74**, 816 (1969).
10. T. L. GOODFRIEND, L. LEVINE and G. D. FASMAN, *Science* **144**, 1344 (1964).
11. J. M. STEWART and J. D. YOUNG, *Solid State Peptide Synthesis*. W. H. Freeman, San Francisco (1969).
12. H. G. KWA and F. VERHOFSTAD, *Biochim. biophys. Acta* **133**, 186 (1967).
13. V. HERBERT, K. S. LAU, C. W. GOTTLIEB and S. J. BLEICHER, *J. clin. Endocrinol.* **25**, 1375 (1965).
14. J. D. HOROWITZ and M. L. MASHFORD, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **263**, 332 (1969).
15. V. EISEN, *Br. J. Pharmac. Chemother.* **22**, 87 (1964).
16. I. MIWA, E. G. ERDÖS and T. SEKI, *Life Sci.* **7**, 1339 (1968).

Biochemical Pharmacology, Vol. 20, pp. 973–975. Pergamon Press, 1971, Printed in Great Britain

Inhibition of tryptophan oxygenase *in vitro* by steroid compounds

(*Received* 10 *August* 1970; *accepted* 30 *October* 1970)

LIVER tryptophan oxygenase (TPO) is subject to induction both by its substrate and by adrenal glucocorticoids.[1] Although TPO activity has been shown to be elevated in the livers of pregnant rats[2,3] the administration of oestrone and progesterone in combination to non-pregnant females failed to produce an increase in enzyme activity.[3] Further, it was subsequently found that the subcutaneous injection of progesterone alone, in a dose of 4 or 8 mg daily for 28 days, caused a significant reduction of TPO levels in rat liver.[4]

In an attempt to investigate this problem further we have studied the effect of *in vitro* additions of progesterone and various other steroids on TPO.

Methods

Livers were obtained from adult female Sprague–Dawley rats killed by decapitation. A particle-free supernatant was prepared by centrifugation of a 10% (w/v) homogenate at 105,000 *g* for 60 min at 4°, and the TPO activity determined by the method of Leklem *et al.*[5]

The steroids were dissolved in 100% ethanol such that the required amount for addition to the enzyme assay medium was present in 0·1 ml. Control assays were carried out with each experiment, both in the presence and absence of ethanol, so that every inhibition study had its own control, prepared from the same liver as was used in flasks containing the steroid. The ethanol did not affect the activity of TPO. The mean control value for TPO obtained in 22 assays was 3·4 ± 0·8 μmoles kynurenine produced/g wet liver tissue/hr.