THE KALLIKREIN-KININ SYSTEM IN PLASMA OF POIKILOTHERM VERTEBRATES

by

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ABSTRACT

The little studied plasma kallikrein-kinin system of poikilotherm vertebrates was investigated in several species of fish, amphibians, and reptiles, and compared to the well-known mammalian enzyme system. It was found that the plasmas of all fish and amphibians tested differed from reptilian and mammalian plasma in their inability to release a kinin-like factor when reacted with trypsin or glass, and no evidence was obtained to suggest that these plasmas contain enzymic machinery which can produce a kinin. However, it was shown that heat-denatured plasma from these animals did develop biological activity when treated with hog pancreas kallikrein, an enzyme specific for releasing kinins. Thus, the equivalent of a kininogen might exist in these plasmas.

Since turtle plasma produced a kinin by endogenous enzymes, detailed studies of this system were conducted. By a variety of criteria, enzymic mechanisms for kinin production in this plasma were closely similar to those of mammalian plasma. However, purification of the turtle kinin released by endogenous enzymes, followed by pharmacological and chemical tests showed that this kinin was chemically different from bradykinin, its mammalian counterpart. Data obtained from amino acid analysis of the peptide, and from certain

pharmacological tests, strongly suggested that the structure of turtle kinin is 6-thr-bradykinin; i.e., that a threonine residue has been substituted for a serine. The possible significance of this finding is discussed.

Preliminary studies of the pharmacological effects of bradykinin on aspects of blood pressure and flow in the turtle itself are described. Intra-arterial injections of bradykinin over a wide range of doses always produced a pressor response which could be greatly reduced by adrenergic blockade. This is in contrast with the effect of the peptide in mammals, where there is typically a hypotensive response which cannot be reduced by adrenergic blockade. The significance of this difference is discussed, and approaches to future investigations are suggested.

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DEFINITIONS AND ABBREVIATIONS

Definitions

Due to the complexity of the factors involved in the kallikrein-kinin system, the nomenclature of its various components has often been ambiguous and redundant. Recently, M.E. Webster (1966) chaired a committee which recommended certain terms and definitions in an effort to clarify the situation. These terms, which have been adopted by most workers, will be used in this thesis as defined below:

Kinins: A general term indicating a polypeptide which resembles bradykinin in its structure and pharmacological activity.

Bradykinin: Refers to the nonapeptide, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, as isolated by Elliott et al (1961).

Lys-bradykinin: Refers to the decapeptide Lys-bradykinin, formed by the action of glandular kallikreins on kininogen (Werle and Trautschold, 1963).

Kallidin: A commonly used term for lys-bradykinin.

Kininogen: Refers to all biologically inactive proteins that release a kinin when reacted with appropriate proteolytic enzymes.

Kininogenase: Refers to any enzyme that liberates a kinin from an inactive protein substrate; e.g. kallikreins, trypsin,

pepsin, snake venom.

Kallikrein: A type of naturally occurring kininogenase which rapidly and specifically liberates a kinin from kininogen. These are identified by species and source; e.g., hog pancreas kallikrein.

Prekallikrein: Refers to the inactive precursor of a kallikrein.

Kininase: A general term referring to enzymes that inactivate kinins.

Abbreviations Used

BK	-	bradykinin	Gly	-	glycine
Lys-BK	-	lys-bradykinin	His	-	histidine
K	-	kallidin	Ile	-	isoleucine
HPK	-	hog pancreas kallikrein	Leu	-	leucine
T	-	trypsin	Lys	-	lysine
BK-eq	-	bradykinin equivalent	Met	-	methionine
Ala	-	alanine	Pro	-	proline
Arg	-	arginine	Phe	-	phenylalanine
Asp	-	aspartic acid	Ser	-	serine
Cys	-	cystine	Tyr	-	tyrosine
Cyt	-	cysteic acid	Thr	-	threonine
Glu	-	glutamic acid	Val	-	valine

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CHAPTER 1. INTRODUCTION

In 1949 Rocha e Silva described a potent hypotensive polypeptide, which was released from mammalian plasma by snake venom and by trypsin. The factor was named bradykinin. Since this time, plasma kinins and the enzymes which release them from their alpha-globulin precursor in the blood have been the subject of numerous studies, and the history and current state of the art have been presented in detail in many reviews and symposia (see Schachter, 1969).

The structure of purified mammalian bradykinin was determined by synthesis (Boissonas, Guttmann, and Jaquenod, 1960) and by amino acid analysis of trypsin released kinin (Elliott, Horton, and Lewis, 1961). Later work has shown that certain glandular kallikreins and/or acid treatment also release lys-bradykinin (Webster and Pierce, 1963) and met-lys-bradykinin (Elliott, 1963; Schröder, 1964) from plasma globulins. Both of these peptides have also been identified in human urine (Miwa, Erdos, and Seki, 1969). All three kinins are biologically active and it is now recognized that the hypotensive, thermolabile and large molecular weight glandular kallikreins described by Frey and his coworkers in the 1930's (reviewed by Schachter, 1969) exert their pharmacological effect by releasing these kinins from a specific substrate in blood plasma.

A variety of other treatments of mammalian plasma kininogen result in the production of yet other bradykinin-containing fragments in addition to varying proportions of the plasma kinins already described. The complex interrelationships between the various treatments and the peptides released have been reviewed by Pierce (1968) and Hamberg (1969), and are summarized in Figure 1. While these studies have contributed to an understanding of structural features of plasma kinins and kininogens, they are of little value in understanding a physiological role for kinins, since most of these active peptides are converted to bradykinin in native plasma, and the endogenous plasma kallikreins produce mostly bradykinin to begin with (Pierce, 1968).

Throughout the study presented here, emphasis has been placed on kinin production by activation of the natural plasma kallikreins present in plasma, since this process may imitate physiological release. Studies with enzymes other than those present in the blood, such as trypsin or glandular kallikreins, were included only for comparison. While there is disagreement on details of the interaction of many kininogenases with different types of kininogen, most workers would agree that the diagram in Figure 2 expresses the major events which occur when kinin is released from intact plasma by a glass surface, trypsin or glandular kallikrein.

Figure 1

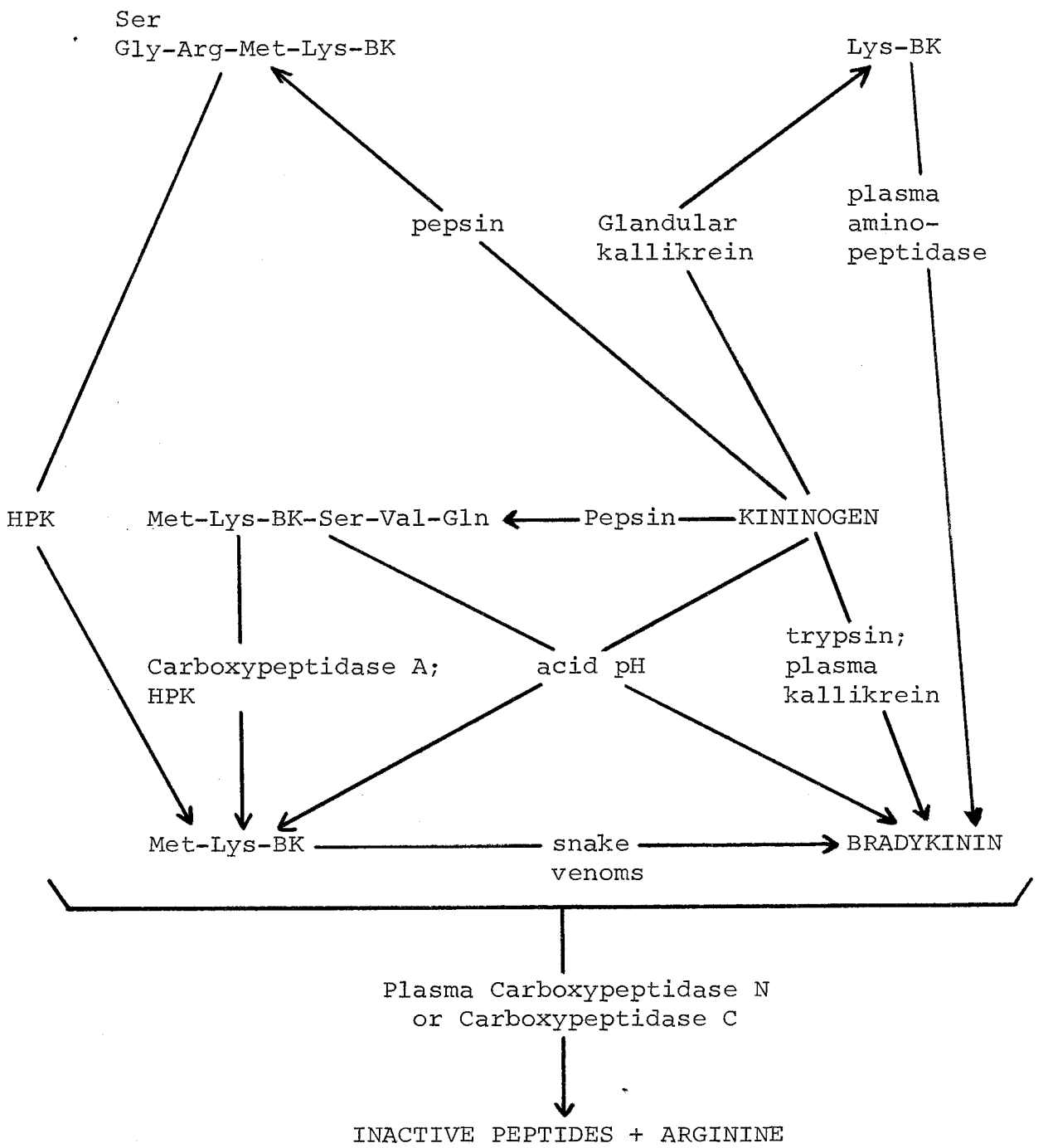
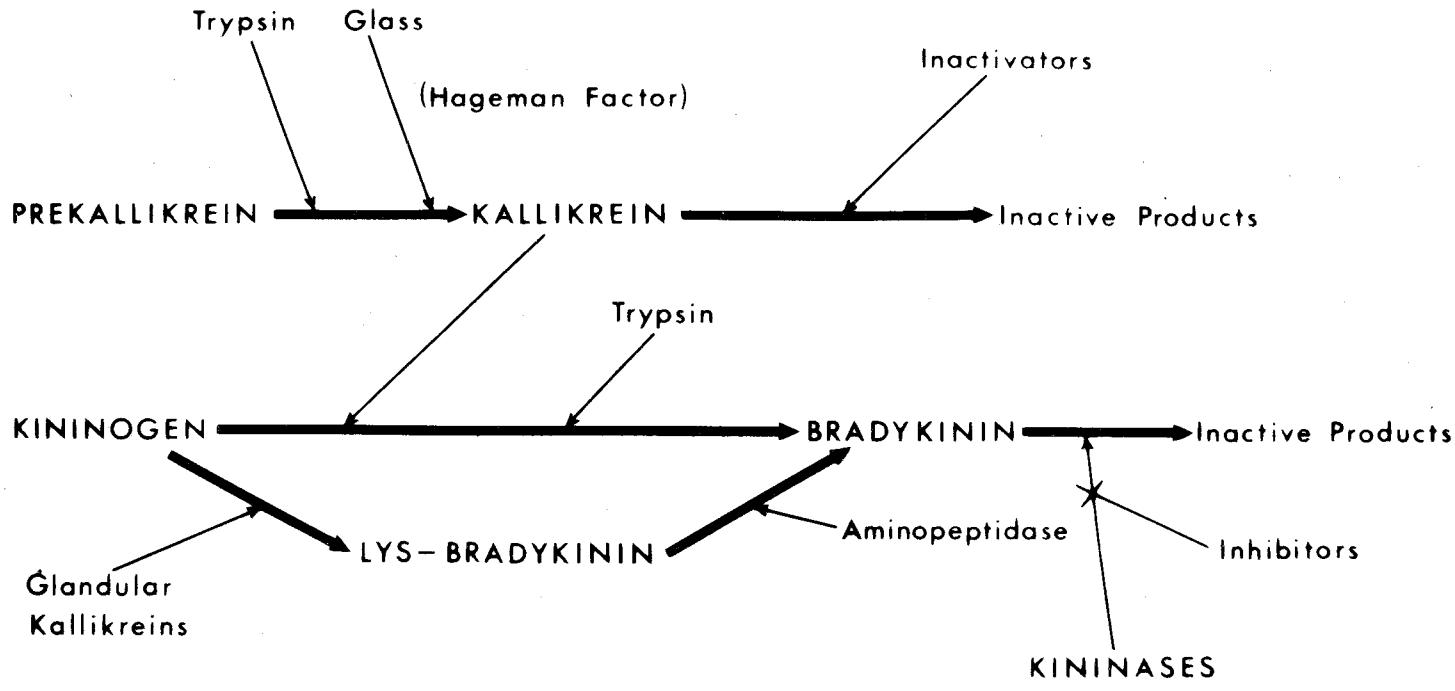

Ser
Gly-Arg-Met-Lys-BK
Lys-BK
pepsin
Glandular kallikrein
plasma amino-peptidase
HPK
Met-Lys-BK-Ser-Val-Gln
Pepsin
KININOGEN
Carboxypeptidase A; HPK
acid pH
trypsin; plasma kallikrein
Met-Lys-BK
snake venoms
BRADYKININ
Plasma Carboxypeptidase N or Carboxypeptidase C
INACTIVE PEPTIDES + ARGININE

Figure 1. Diagrammatic summary of the interrelationships of kininogen, kinins, and kinin-containing fragments. From Pierce, 1968.

Figure 2. Schematic representation of the effect of glass surface, trypsin, and glandular kallikrein on major enzymatic events involved in kinin release in mammalian plasma.

Figure 2



Mammalian Plasma Kallikrein-Kinin System

It is generally agreed that trypsin, a powerful kininogenase, will release maximum amounts of kinin from all kininogens known to occur in mammalian tissues (Schachter, 1969). On the other hand, plasma kallikrein degrades only a portion of the total kininogen. For convenience, this fraction of the kininogen will be referred to as substrate 1 (Jacobsen 1966 a).

There is increasing evidence for the existence of numerous molecular species of kininogens, kallikreins, and kininases (reviewed by Schachter, 1969). This kallikrein-kinin system is therefore a complex "damped cascade" pathway similar to the blood clotting reactions (as described by Hemker and Hemker, 1969), and sharing with them the initial step of Hageman Factor activation (Margolis, 1958; Temme, et al, 1969).

Non-Mammalian Kinins

Kinin-like polypeptides have now been isolated from a number of non-mammalian sources. These have been reviewed in detail by Pisano (1968) and Schachter (1968, 1969). Briefly, wasp venoms (Vespa sp. and Polistes sp.) contain at least three kinins, one identified as gly-bradykinin (Prado et al, 1966). Bradykinin itself has been isolated from frog (Rana temporaria) skin in concentrations as high as 200-250 ug/g fresh tissue (Anastasi, Erspamer, and Bertaccini, 1965). Three pharmacologically active peptides have recently been found in the skin of a small Brazilian amphibian, P. rohdei, one of

which has the structure; bradykinin-Ile-Tyr-HSO_4. It is remarkable that the fundamental bradykinin chain is an integral part of all non-mammalian kinins whose structures have been established. The active research presently being carried out on a wide variety of insect and reptilian venoms is likely to identify an increasing number of vasoactive, bradykinin-containing peptides.

However, little is known of plasma kinins or endogenous enzymes that release them in non-mammalian vertebrates. It has been shown that ornithokallikrein from the bird pancreas releases from bird plasma a polypeptide which is hypotensive on bird blood pressure, but is inactive in mammals (Schachter, 1969). The structure of this factor differs significantly from known kinins (Werle, et al, 1967). It is not clear whether bird plasma can release enzymes which produce this ornithokinin. However, Erdos, Miwa and Graham (1967) have shown that if Hageman Factor was supplied, glass beads released from duck plasma a kinin indistinguishable from bradykinin. Bradykinin itself is, however, inactive on fowl blood pressure (Erdos, 1966).

Data on the occurrence of kinins and kallikreins in poikilotherms is very scarce. Diniz and Carvalho (1963) reported a single relatively low value for trypsin-released kinin from denatured toad plasma. While the present study was in progress

three very brief, though pertinient, papers have appeared: Erdos et al (1967) reported the liberation of plasma kinins by glass activation in alligator and turtle plasmas; Vogel et al (1969) studied the response of fish blood pressure to various kinins and kallikreins and concluded that there were no hints of a kinin-liberating system comparable to that of mammals; A.C. Alba Lavras et al (1969) came to a similar conclusion for snake plasma, but did report that hog pancreas kallikrein released from this plasma a hypertensive smooth muscle stimulating agent different from bradykinin. These important studies will be discussed in later chapters.

CHAPTER 2. GENERAL METHODS

Several general techniques and procedures were followed throughout this work, and these are detailed below:

A. Siliconing Procedure

Kinins in solution lose activity when in contact with glass (Margolis and Bishop, 1963), presumably through adsorption to negative sites on its surface. This loss, as well as unwanted kallikrein activation, can be greatly reduced by pretreatment of the glass surface with silicone. All glassware to come in contact with kinins was therefore washed, exposed for 10 seconds to a 1% solution of Siliclad (Clay Adams, N.Y.), rinsed with distilled water, and dried for 20 minutes at 100°C, before use in any experiment.

B. Rat Uterus Bioassay

There are many bioassays which might be used to measure kinin production (Erdos, 1966), and sensitive techniques for radioimmunoassay are being developed (Spragg, Haber, and Austen, 1966; Talmo, Haber, and Austen, 1969). However, the rat uterus bioassay is still the most accurate and sensitive method for routine estimation of plasma kinin activity. The assay is simple to carry out, will measure a concentration of bradykinin as low as 50 pg/ml, and allows computation of potency for an unknown with 95% confidence limits between

± 10 and 20%.

The rat uterus assay was therefore used to study kinetics of kinin release from denatured and intact plasma. It was also a basis for pharmacological tests, and was used to follow kinin activity through purification, and several chemical tests.

Female virgin rats (Wistar strain) 180-220 g in weight were used. Only rats in full oestrus (indicated by vaginal smear) were selected. The animal was killed by a sharp blow to the back of the head. The uterus was exposed by a sagittal incision in the abdominal wall, rapidly dissected free of mesentery and fatty tissue, and placed in warm (30°C) buffered saline, which was Munsick's modification of Van Dyke-Hastings Solution (Munsick, 1960). The composition of this fluid, which supported the tissue throughout the bioassay, is given below:

Chemical	Amount/L	Chemical	Amount/L
NaCl	6.704 g	glucose	0.5 g
KCl	0.459 g	*phosphate buffer	10 ml
$NaHCO_3$	2.590 g	Na phenol-sulfonephth-alein	0.054 g
$CaCl_2$	0.5 ml, 1 M solution		

*...Phosphate buffer consisted of solutions of Na_2HPO_4 (22.714 g/L) and Na H_2PO_4 (6.349 g of hydrated form/L) combined so that the final pH was 7.40.

One horn of the uterus was then placed in a 5 ml organ bath containing Van Dyke-Hastings solution. The organ bath and

a connecting 2 L reservoir were individually gassed with a mixture of 5% CO_2 and 95% O_2, which kept the pH at 7.4, and both were in a water bath regulated to ± 0.1°C, and the assay temperature set between 30 and 33°C. The posterior end of the uterus was secured to a glass muscle hook at the bottom of the organ bath, and the ovary was connected to a writing lever by surgical thread under a tension of 1 - 2 g. The solutions to be assayed were added to the organ bath by means of glass pipettes or Hamilton glass syringes, in a volume never exceeding 0.2 ml, and the resulting contractions were recorded on smoked paper on a kymograph. After each response had reached maximum contraction, the bath was flushed with an excess of buffer from the reservoir, and the tissue was allowed to recover for at least 5 minutes. The smoked paper with recorded traces was dipped in shellac and the responses later measured to the nearest mm.

Estimation of activity for a large number of samples (e.g., eluates from chromatography columns) was based on log dose-response curves, or on single four point assay groups. Where maximum accuracy was desired, five four point groups consisting of matched responses to high and low doses of standard and unknown, were obtained according to the method of Holton (1948). All assays used synthetic bradykinin (Sandoz, 0.1 mg/ml) as standard, and results throughout were expressed as ug (or ng) bradykinin-equivalent (BK-eq) per ml fluid.

C. Source of Animals

a) Fish. The following animals were obtained by otter trawl near Friday Harbour, U.S.: starry flounder (Platichthys stellatus), butter sole (Isopsetta isolepis), lemon sole (Parophrys vetulus), cod (Gadus macrocephalus), skates (Raja binoculata and Raja rhina), dogfish (Squalus suckleyi) and ratfish (Hydrolagus colliei).

Trout (Salmo gairdnerii) were obtained from a commercial supplier in Port Coquitlam, B.C.

b) Amphibians. Frogs (Rana catesbiena) were obtained from College Biological Supply, Seattle, and maintained at 10-15°C in the laboratory. Amphiuma tridactylum was ordered from North Carolina Biological Supply Co., Durham, North Carolina, and kept in large aquaria at room temperature until use.

c) Reptiles. Turtles (Pseudemys scripta elegans) were obtained from College Biological Supply, and kept in moist tanks at 10-15°C until use.

d) Mammals. Seal blood (Phoca vitulina richardi) was obtained from captive animals at the University of British Columbia. Killer whale blood (Orcinus orca) was taken from Skana, at the Vancouver Public Aquarium. Dog blood was donated by a golden retriever kept in the Physiology Department, at the University of British Columbia, and white rats (Wistar strain), rabbits and guinea pigs were obtained from the vivarium

also at U.B.C.

D. Blood Sampling

Blood from all animals was withdrawn into sterile, chilled plastic syringes (Tomac) previously rinsed in a 10 mg% heparin-saline solution. Basic techniques used for each species are given below in Table 1. All animals listed in the Table were destroyed after removal of blood, except for the seal, dog, and killer whale.

E. Sampling of Kinin-Producing Reactions

Although kinin releasing methods varied between experiments, certain basic techniques were used to sample all kinin producing reactions in the various plasmas. Plasma, whether heated or intact, was always diluted with 2 volumes of the appropriate saline (Appendix B). At zero time, the kininogenase or activator was added to the mixture, which had a total volume of 5-10 ml. At the desired time intervals small samples (usually 0.5 or 1.0 ml) were removed from the incubate in a 1 ml sterile plastic syringe and rapidly injected into 2 volumes of distilled water or the appropriate saline preheated to 100°C in a siliconed glass test tube. The final dilution of the plasma was 1/9. These samples were kept in a boiling water bath for a further 10 minutes, then removed and stored at 4°C until assay on the isolated rat uterus.

This technique had certain advantages for this study:

Table 1. Summary of methods used in withdrawing blood.

Species	Source of blood	Volume/animal (ml)	Anaesthetic
Starry flounder	Dorsal aorta, near anal fin	2-6	MS-222
Butter sole	"	"	none
Lemon sole	"	"	"
Ratfish	"	"	"
Trout	"	"	MS-222
Cod	Cardiac puncture	5-10	none
Dogfish	"	"	"
Skates	"	"	"
Amphiuma	*Systemic artery near heart	10-30	MS-222
Frog	Cardiac puncture	2-5	Ether
Turtle	*Left aorta	10-70	Nembutal
Seal	Dorsal aorta	20	none
Killer whale	Caudal artery	20	"
Dog	Leg vein	20	"
Rat	*Dorsal aorta	7-15	Ether

*...These blood vessels were exposed by mid-ventral incisions, and subsequent dissection. A square about 6 x 6 cm was cut in the turtle plastron, using a Desoutter Autopsy saw. Underlying muscle was carefully trimmmed away using a scalpel, and the square discarded. All of these vessels were clamped, then cannulated with the syringe distal to the clamp. The artery clamp was then removed, and the blood withdrawn.

1. The rapid inactivation allowed serial samples to be taken at time intervals short enough (to 30 second intervals) to allow the kinetics of kinin production to be studied.
2. The treatment achieved a partial purification, since much protein was precipitated, while the kinin remained in the aqueous supernatant saved for assay.
3. The samples obtained can be tested directly on the rat uterus, whereas other methods of halting such reactions, by the use of acetone or ethyl alcohol, for example, require that these interfering substances be eliminated before assay. The latter time consuming steps could be avoided in the present study, thereby allowing the author to deal with the numerous small samples obtained.

F. Preparation of Sephadex G-25 for Gel Filtration Chromatography

96 g (dry weight) of Sephadex G-25 beads (fine grade, lot # 9053) were stirred slowly with 3 volumes of distilled water, and allowed to swell for three hours. The magnetic stirrer was then shut off and the gel allowed to settle. The supernatant and fines were decanted and replaced with 3 volumes of 0.02 M ammonium acetate buffer, pH 5.0 (Appendix B). This mixture was then stirred, allowed to settle, and was decanted and re-washed with the buffer at least 3 more times over a 5 hour period.

A gel slurry in the same buffer was then applied with

constant stirring to a 100 x 2.5 cm siliconed glass column filled with 0.02 M ammonium acetate buffer. A small wad of glass wool weighed down with a few glass beads prevented the gel from running out of the column. A very slow flow through the column, less than 5 ml/hour, was maintained throughout most of the building process. After all of the gel had settled the column was 90-96 cm in height.

After building was complete, a small disc of filter paper was placed on top of the gel and buffer was allowed to flow through the column at about 12 ml/hour, for at least 24 hours. The eluate was periodically monitored for conductivity and pH, and compared to the original buffer.

Before any experimental material was applied, the column was tested for homogeneity and resolution by applying a solution of Blue Dextran 2000 (Pharmacia) and 1.0 g NaCl in 20 ml of the same buffer. These two substances are ideal for calibration, since Blue Dextran is totally excluded from the gel, while NaCl penetrates freely. This material was washed through with 0.02 M buffer, and collected in 100 drop fractions with a fraction collector (LKB). The Blue Dextran intensity in the eluate was estimated by eye, or measured on a Unicam spectrophotometer (#17914) at λ = 625 nM. Salt concentration was measured as conductivity at 20^{o}C on a CDM-2d conductivity meter

(Radiometer, Copenhagen).

After calibration, the column was washed with several volumes of 0.02 M buffer, prior to application of the experimental sample.

G. Preparation of CM-Sephadex for Ion Exchange Chromatography

4 g (dry weight) of CM-Sephadex gel (medium grade, lot #6071) were placed in 100 ml distilled water and stirred for 3 hours. The slurry was then filtered on a Buchner funnel with Whatman #1 filter paper, and washed through with at least 500 ml of a number of solutions, in the following order: 0.5 N NaOH, distilled water, 0.5 N HCl, distilled water, 0.5 N NH_4OH, excess distilled water, followed by 1.5 L 0.02 M ammonium acetate buffer.

A slurry of the gel in 50 ml of buffer was applied with constant stirring to a 25 ml siliconed burette previously filled with buffer. Glass wool placed just above the stopcock prevented the gel from running out of the column. After the column had built to a height of 15-20 cm, the remaining slurry was discarded, and 0.02 M buffer was allowed to flow through the gel for at least 24 hours. The ionic strength and pH of the eluate was periodically checked against the original buffer to ensure that equilibrium had occurred.

Kinin-containing samples were applied slowly, in volumes ranging from 2 to 27 ml, and were washed on with at least 30

ml of 0.02 M buffer. The column was then subjected to an ionic strength gradient, from 0.02 up to 1.0 M ammonium acetate, pH = 5.0. A 50 ml mixing chamber was connected to the column, both equilibrated in 0.02 M buffer. The gradient was initiated by connecting to the mixing chamber a reservoir containing the high strength buffer, which gradually replaced the 0.02 M buffer in the mixing chamber as buffer was eluted from the column.

H. Estimation of "Lowry" Peptide

The estimation of total peptide concentration was hased on the colorimetric method of Lowry, Rosebrough, Farr and Randall (1951).

1. Reagents.

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 0.5% Cu $SO_4 \cdot 5H_2O$ in 1% potassium tartarate

C. 1 N Folin-Ciocalteau Phenol reagent

2. Standards. A series of protein solutions containing 10, 25, 50, 150, 200 and 300 ug/ml, were prepared by dilution from a 50 mg% Bovine Serum Albumin Stock (fraction v, Armour Pharmaceuticals).

3. Measurement of peptide content. 0.2 ml of the unknown and standards were pipetted into a series of 2 ml test tubes. To each sample 1 ml of reagent C was added, shaken, and allowed to stand for 15-30 minutes. 0.1 ml of Folin Ciocalteau

reagent was then added very rapidly and mixed immediately. After a period of 40-60 minutes the colour was fully developed, and the samples were read against a blank on a Unicam SP 600 spectrophotometer at λ = 750 nM, using the red filter photocell. The concentration of protein ("Lowry" peptide) in the experimental samples could then be read in ug/ml from the standard curve.

CHAPTER 3. COMPARATIVE STUDIES ON FISH, AMPHIBIAN, REPTILIAN AND MAMMALIAN PLASMAS

A. Denatured Plasma

The first logical step in characterizing a kallikrein-kinin system is to determine whether a kininogen is present; i.e., whether smooth muscle stimulating activity can be developed in the test plasma by treatment with strong kininogenases. This is most easily done using heat-denatured plasma, since this treatment eliminates natural kininase activity which would destroy any kinin produced. The entire kallikrein-kinin system, if it exists, is reduced to a suspension of kininogen, which may be treated with kininogenases such as trypsin, or with the more specific kallikreins, such as hog pancreas kallikrein. The method therefore bypasses the pre-enzyme to enzyme conversions normally required to release kinin in native plasma.

These initial studies on denatured plasma were carried out in two stages: 1) the effect of trypsin on whole blood of fish and mammals, and 2) the effect of trypsin and hog pancreas kallikrein on fish, amphibian, reptilian and mammalian plasmas.

1. Effect of trypsin on whole blood from fish and mammals

A survey of the kininogen content of several fish plasmas was carried out using the method of Brocklehurst and Zeitlin (1967), which allows separate determination of kininogen and free kinin. Blood was collected from fish and from mammalian

controls (seal and killer whale) as described previously (p 13) and immediately injected into 3 volumes of chilled 95% ethanol. These samples could be stored in polypropylene tubes at 0°C for at least a week, with no loss of kininogen. In the laboratory these samples were centrifuged, washed with 80% ethanol, homogenized, resuspended in phosphate buffer, pH = 7.4 (Appendix B), and incubated with trypsin at 37°C for 30 minutes. These incubates were then placed in a boiling water bath for 10 minutes to ensure inactivation of all trypsin. Biological activity of the samples was assayed on the isolated rat uterus using synthetic bradykinin as standard. Controls to which no trypsin had been added were always included, and tested for activity.

An attempt was made to estimate levels of free kinin present in the blood at the time of sampling. The ethanol extract and the washings obtained from the procedure described above were pooled, and placed in a boiling water bath for 10 minutes. The sample was then reduced to dryness in a flash evaporator, and taken up in warm (60°C) saturated NaCl solution acidified to pH 1.5 with HCl. This solution was twice extracted in a separating funnel with 2 volumes of redistilled 1-N-butanol. The butanol was pooled and evaporated to dryness under reduced pressure in a water bath at 50°C. These samples were stored dry at - 20°C until assay on the rat uterus

in the presence of atropine (1 ug/ml) and bromolysergic acid diethylamide (0.5 ug/ml). Full details of the method are given in Appendix C.

Results. Table 2 summarizes results for trypsin treated blood. No values for free kinin are given, since no activity was detected in any sample, mammalian or fish. It is evident that trypsin released no kinin-like activity from fish blood. One sample of starry flounder blood did appear to show a low oxytocic activity, but this was the result of an early experiment and could not be repeated in several trials. Therefore the significance of this positive result was dubious, and it can be concluded, tentatively, that fish blood treated as described above does not act as a substrate for the kininogenase trypsin, as it does in mammals.

Values obtained for seal and killer whale blood are the first reported for these species, and are in the range typical of mammals (Diniz and Carvahlo, 1963). These positive results were obtained in experiments conducted in parallel with those on fish blood, thereby underlining the validity of the negative data obtained for fish.

Control samples free of trypsin never contained oxytocic activity.

The absence of detectable amounts of free kinin in mammalian samples is not surprising, since normal levels are expected to

Table 2. Kininogen in fish and mammalian blood, as determined by incubation with trypsin.

Species*	Kininogen (ug/ml plasma¢, BK-eq)	Mean
Seal	0.94, 0.70, 1.56	1.07
Killer whale	3.75, 2.50, 3.00	3.08
Trout	0.06, 0.01, 0.01, 0.01, 0.01, 0.01	none
Starry flounder	0.18, 0.01, 0.01, 0.01, 0.01	doubtful
Butter sole	0.01, 0.01, 0.01, 0.01	none
Lemon sole	0.01, 0.01	none
Cod	0.01, 0.01	none
Skate (*R. rhina*)	0.01, 0.01	none
Skate (*R. binoculata*)	0.01, 0.01	none
Ratfish	0.01, 0.01	none
Dogfish	0.01, 0.01	none

*...scientific names are given on page 11.

¢...where biological activity was observed, values were converted from ug/ml blood, to ug/ml plasma, BK-eq, using hematocrit readings.

be extremely low (e.g., about 2.8 ng/ml blood in man, Brocklehurst and Zeitlin, 1967).

2. Effect of trypsin and hog pancreas kallikrein on vertebrate plasmas.

While the method of Brocklehurst and Zeitlin described previously has distinct advantages for field work and is useful in separating free kinin from that held in the kininogen precursor, it has serious disadvantages. The method involves reacting trypsin with a homogenized sample of whole blood proteins which include ruptured blood cells, and all particulate matter in the original blood. It is difficult to relate results obtained to events which might occur physiologically, particularly when examining previously untested blood. In addition, the kininogen has been denatured, precipitated, and resuspended. While this procedure apparently does not affect the reproducibility of results in mammalian plasmas, it may artificially increase the amount of substrate available to kininogenases (Hamberg and Rocha e Silva, 1957).

Further experiments with trypsin and kininogen were redesigned to approach physiological conditions more closely. Blood plasma was used as a source of kininogen. Denaturation of the plasma was accomplished by heating only to 65°C, rather than by boiling with ethanol. Finally, the more specific hog pancreas kallikrein (HPK) was used, along with trypsin, to release possible kinins.

Experiment 1

Method. Blood was obtained from the test animals as described previously (Chapter 2) and centrifuged at 2000 g for 40 minutes, at 4°C. The plasma was decanted, heated to 65°C for 30 minutes, diluted with the appropriate saline (Appendix B) to which HPK or trypsin had been added, as follows:

plasma	-	2 ml
buffered saline, pH = 7.4	-	4 ml
*HPK or trypsin	-	2 mg
Total volume	-	6 ml

*...Hog pancreas kallikrein had 10 K.U./mg

All incubations with these mammalian enzymes were carried out in siliconed glass test tubes in a water bath at 37°C ± 0.1°C. The pH of the buffered salines was 7.4, and that of intact plasma, about 7.8. The pH of the incubate was about 7.6. Both trypsin and HPK function efficiently over this range of pH.

The following controls were always included:

a)

plasma (heated)	-	1 ml
buffered saline	-	2 ml
Total volume	-	3 ml

b)

plasma (heated)	-	1 ml
buffered saline	-	2 ml
HPK or trypsin, previously boiled for 10 minutes	-	1 mg
Total volume	-	3 ml

At the desired time intervals the incubates described

above were sampled as described in Chapter 2, cooled, and assayed on the rat uterus bioassay.

Results. Samples from the controls listed above never exhibited a production of kinin activity. However, a few mammalian controls contained a low level of activity, but this was unlikely to be due to kinin, since it was independent of enzyme treatment, and was reduced or abolished by BOL (0.5 ug/ml) and atropine (1.0 ug/ml).

All other plasmas never had activity in the controls, thus ensuring, a) that activity did not arise spontaneously, but was the result of the enzyme's action on the heated plasma, and b) that the enzymes (trypsin and HPK) were totally inactivated by 10 minutes boiling, and were in themselves totally inactive on the isolated rat uterus.

With respect to the relative efficiency of trypsin and HPK in releasing kinin activity, the vertebrate plasmas tested were clearly divided into two groups, as shown in Figure 3a & b:

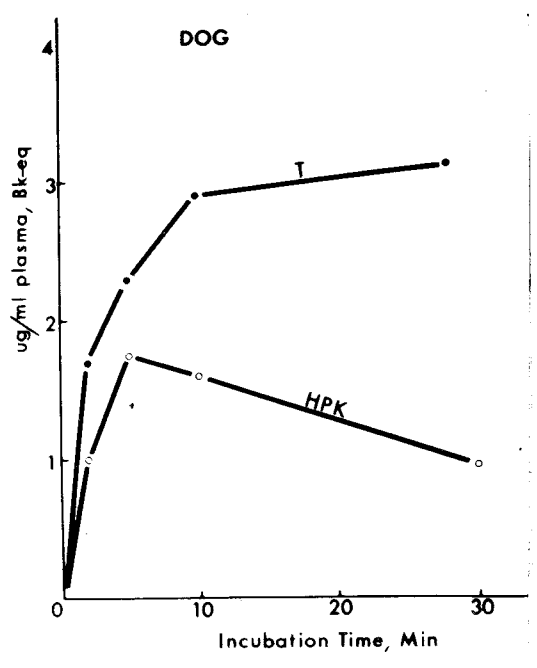
Group 1 - Mammalian and turtle plasmas, which released large amounts of activity to both enzymes, but always most to trypsin, and,

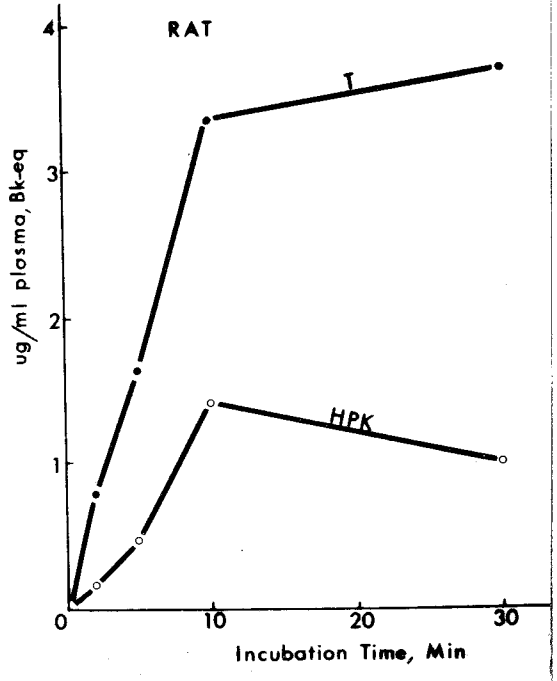
Group 2 - Amphibian and fish plasmas, which released little, if any, activity to trypsin, but which always developed significant biological activity in the presence of HPK.

In considering these results it may be important to be

Figure 3 a. Effect of trypsin (T) and hog pancreas kallikrein (HPK) in releasing kinin from dog, rat and turtle plasmas. Plasma was denatured by heating to 65°C for 30 minutes, after dilution 1:2 with saline. Trypsin was used at 1 mg/ml undiluted plasma, and hog pancreas kallikrein at 10 k.u./ml undiluted plasma. Biological activity released is expressed as ug/ml undiluted plasma, BK-eq, as determined on the rat uterus bioassay.

Figure 3 a.


DOG
ug/ml plasma, Bk-eq
4
3
2
1
0
10
20
30
T
HPK
Incubation Time, Min


RAT
ug/ml plasma, Bk-eq
4
3
2
1
0
10
20
30
T
HPK
Incubation Time, Min

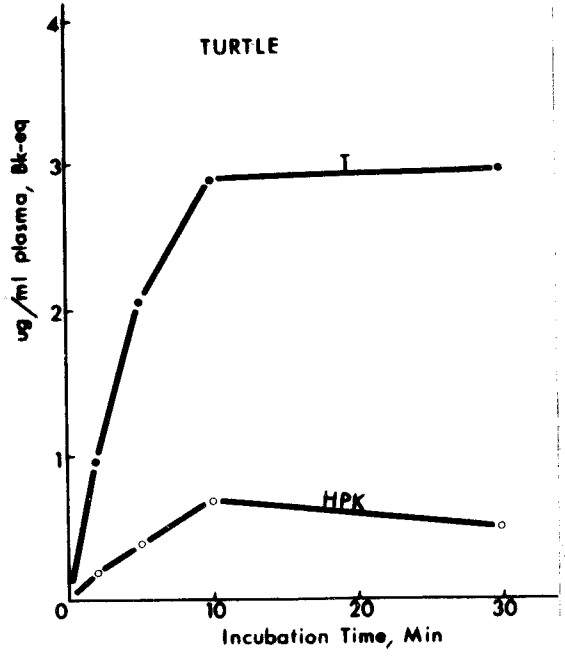
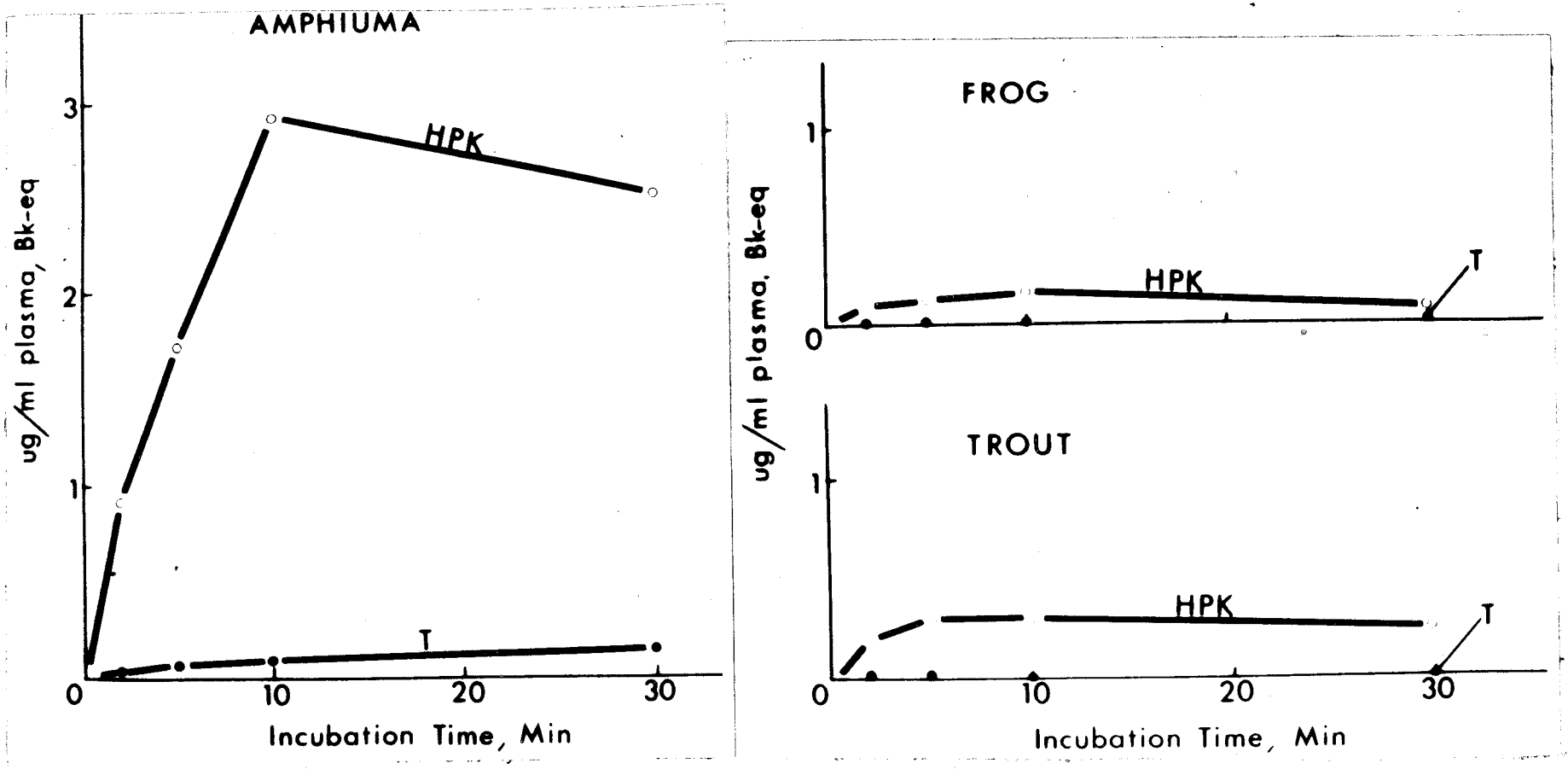

TURTLE
ug/ml plasma, Bk-eq
4
3
2
1
0
10
20
30
T
HPK
Incubation Time, Min

Figure 3 b. Effect of trypsin (T) and hog pancreas kallikrein (HPK) in releasing kinin from Amphiuma, frog and trout plasmas. Conditions of incubation as described in Figure 3 a.

Figure 3 b.


AMPHIUMA
ug/ml plasma, Bk-eq
3
2
1
0
HPK
T
10
20
30
Incubation Time, Min
FROG
1
0
HPK
T
TROUT
1
0
HPK
T
10
20
30
Incubation Time, Min

be aware of differences in the action of trypsin and HPK on a given substrate. Trypsin has been shown to release kinin from all types of kininogen, while HPK may act on only one (e.g., Habermann, 1966). Also, pancreatic kallikrein releases lys-bradykinin from denatured mammalian kininogen, while trypsin releases exclusively bradykinin (Schachter, 1969). Since lys-bradykinin is about 2/3 as active as bradykinin on the rat uterus (Stürmer and Berde, 1963), values given for activity released might be considerably higher in terms of molar amounts of kinin liberated.

The release of kinin from heated rat plasma by HPK (Figure 3 a) was an unexpected result, since several workers (Fasciolo and Halvorsen, 1946; Vogt, 1966; Jacobsen, 1966b) have stated that this kallikrein releases no kinin from rat plasma. However, Jacobsen (1966 b) and Gautvik and Rugstad (1967) have shown that rat plasma releases lys-bradykinin on incubation with rat saliva, and conclude that a kininogen is present (termed substrate 2) which will yield kinin to at least some glandular kallikrein. Since HPK concentrations used in the present study were greater than those used by most workers, it seems possible that this higher concentration of enzyme might degrade the "substrate 2". This would explain the apparent contradiction.

It is also possible that the HPK preparation (Nutritional

Biochemicals Corp.) used in these experiments differed slightly from that used by other workers (usually Padutin, Bayer), or that there are intra-specific variations in kininogen linkage among strains of laboratory rats.

With regard to the activity released by HPK in group #2 plasmas, it is possible that enzymes other than those which produce lys-bradykinin in mammalian plasma might be responsible for the appearance of this activity, since there are at least 5 active kallikreins and two prekallikreins in hog pancreas extracts (reviewed by Schachter, 1969). It is a further possibility that a protease impurity could contribute to results in plasmas from both groups.

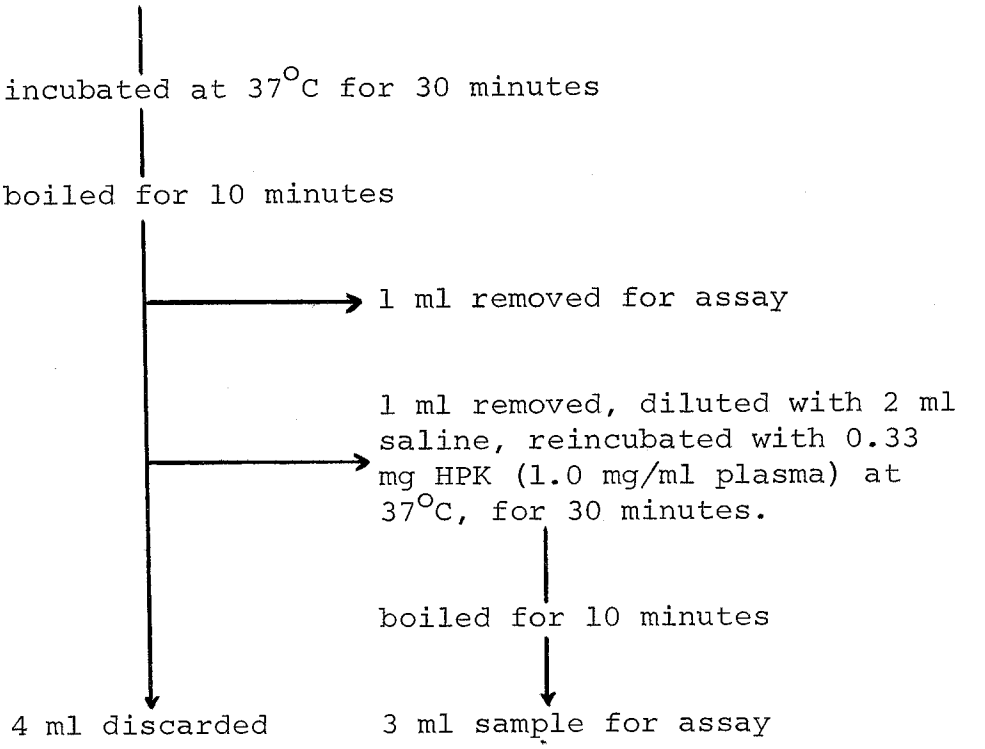
In any case, the major conclusion drawn from the experiment (i.e., the separation of the plasmas into two groups) is unaltered.

Experiment 2

Although the previous experiment suggested that trypsin and HPK act on different substrates in amphibian and fish plasmas, the possibility remained that trypsin itself might inactivate the oxytocic factor it released in these plasmas. Therefore, further experiments were carried out to test whether HPK could release this active principle from plasma previously treated with trypsin.

Method. 2 ml samples of plasma were heated, diluted with saline and incubated at 37°C with either trypsin or HPK, as described in Experiment 1 (p 24). Plasma from Amphiuma (group #2) and from rats (group #1) were used in these experiments. After 30 minutes, 2 samples were taken from these tubes, one for immediate assay, the other for reincubation with the alternative enzyme, either HPK or trypsin, as follows:

heated plasma	-	2 ml
buffered saline, pH = 7.4	-	4 ml
trypsin (or HPK)	-	2 mg
Total volume	-	6 ml



The final dilution of the plasma was 1/9, for all samples.

Results. Figure 4 is a histogram summarizing the results of

these reciprocal experiments. As expected from Experiment 1, HPK released more activity from Amphiuma plasma than did trypsin, and trypsin released the most from rat plasma. However, the reincubation with HPK released considerably more activity than did the initial incubation with trypsin in the same plasma. This observation supports the conclusion that HPK utilizes a substrate in Amphiuma plasma which is unavailable to trypsin.

B. Intact Plasma

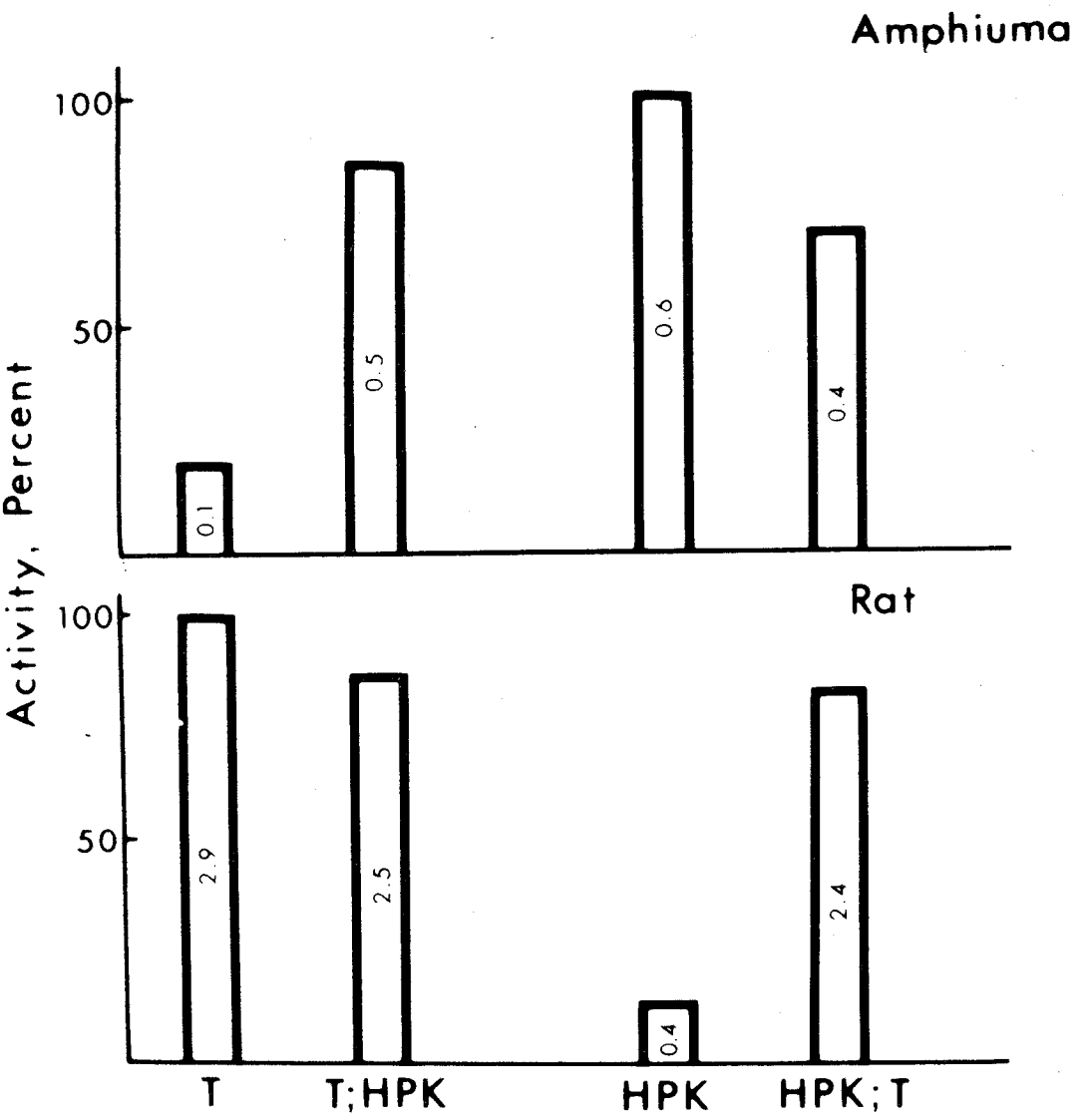
In order to determine whether other components of a plasma kallikrein-kinin system besides kininogen might be present in poikilotherm vertebrates, experiments were also carried out using undenatured, or intact plasma.

1. Kininases in fish, amphibian, reptilian and mammalian plasmas

Compared to other pharmacologically active peptides, bradykinin and lys-bradykinin are the most quickly inactivated in mammalian blood plasma (Erdos, 1966), and this homeostatic mechanism is probably a major factor in determining the duration of their action. In mammals, at least two kininases are responsible for this inactivation; plasma kininase I (Carboxypeptidase N), which splits the Phe^{8}-Arg^{9} bond of bradykinin (Erdos and Sloane, 1962), and plasma kininase II, which splits the Pro^{7}-Phe^{8} bond (Yang and Erdos, 1967). These enzymes can

Figure 4. Relative amounts of kinin released from heated Amphiuma and rat plasma in 30 minutes of incubation at 37°C with trypsin (T; 1 mg/ml plasma); with trypsin followed by hog pancreas kallikrein (HPK; 10 K.U./ml plasma); with hog pancreas kallikrein; and with hog pancreas kallikrein followed by trypsin. Values are based on two experiments, and the numbers in each bar are actual means, in ug BK-eq/ml plasma.

Figure 4


Amphiuma
100
50
0.1
0.5
0.6
0.4
Rat
100
50
2.9
2.5
0.4
2.4
Activity, Percent
T
T;HPK
HPK
HPK;T

be totally inhibited by a variety of chelating agents (Erdos, 1966).

Kininase activity in the test plasmas was compared to characterize the nature of this important component of the kallikrein-kinin system in these animals. The relative effect of 8-hydroxyquinoline sulfate (8-$HQSO_4$) on kininase activity was also compared in the different plasmas, since a), mammalian kininases are totally inhibited by low doses of this compound, thus providing another criterion for comparison, and b), further study of kinin release in intact plasma would depend upon the identification and use of an effective kininase inhibitor.

Method. Kininase activity was studied directly in the undiluted plasmas by following the disappearance of the oxytocic activity of artificially added synthetic bradykinin, 1 ug/ml plasma. At zero time 1 ug of bradykinin in 0.01 ml was added by means of a 25 ul Hamilton syringe to 1 ml of plasma in a siliconed glass test tube. At the desired times, 0.2 ml of the incubate was removed in a sterile 1 ml plastic syringe and rapidly injected into 1 ml of distilled water in a boiling water bath. After 10 minutes in the bath these tubes were removed, cooled, and assayed on the isolated rat uterus, as described in Chapter 2.

The effect of different concentrations of the kininase inhibitor 8-$HQSO_4$ on the kinetics of bradykinin inactivation was also studied in each plasma. Experimental tubes were set up

and sampled exactly as described above, except that the required amount of 8-$HQSO_4$ in 0.2 ml of saline was mixed with the plasma prior to the addition of synthetic bradykinin.

Control incubates containing bradykinin and saline (but no plasma) were included in each experiment. This precaution ensured that the disappearance of bradykinin in those incubates which contained plasma was not due to adsorption on the glass surface, nor to spontaneous breakdown in the saline.

Results. The graphs in Figure 5 a & b show the amount of bradykinin remaining in the plasmas at the different sampling times. In all plasmas bradykinin was rapidly inactivated in the absence of 8-$HQSO_4$. However, in rat and turtle plasma (Figure 5 a) 0.5 mg 8-$HQSO_4$/ml undiluted plasma was sufficient to completely inhibit kininase activity, whereas up to 8 times this amount of the inhibitor (4 mg/ml plasma) only partially inhibited kininases in Amphiuma, frog and trout plasmas (Figure 5 b).

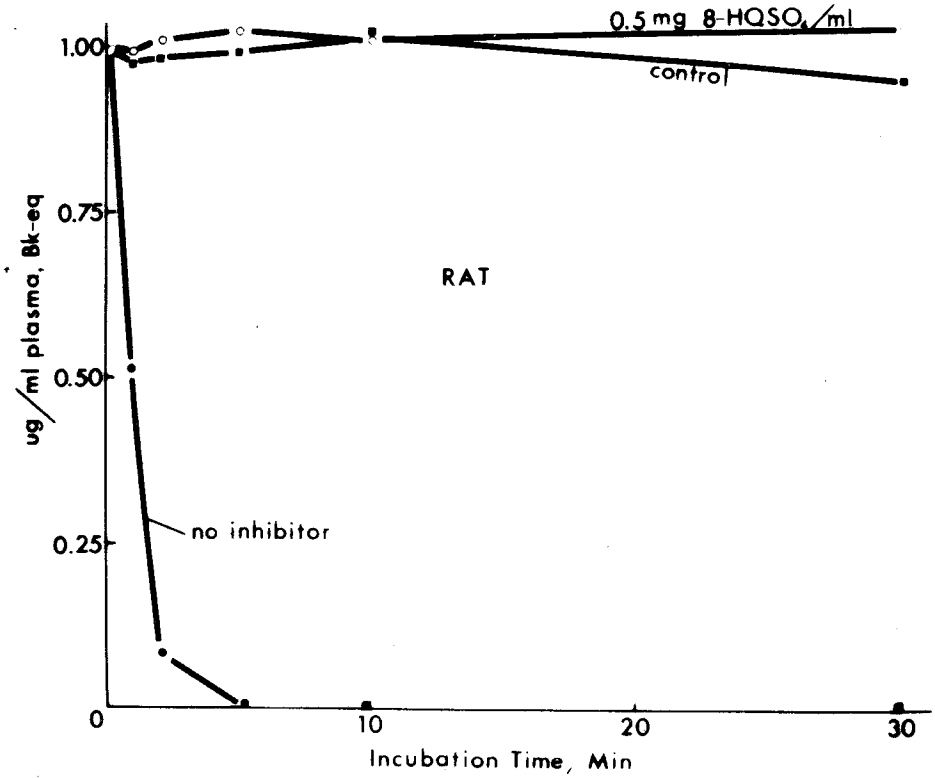
Saline controls never showed any loss of kinin activity over the sampling period.

All plasmas tested had considerable kininase activity. However, with respect to the efficiency of 8-$HQSO_4$ as an inhibitor these plasmas were once more clearly divided into two groups:

Group 1. - Rat and turtle plasmas, where kininase activity was completely inhibited by low levels of 8-$HQSO_4$, and

Figure 5a. Inactivation of synthetic bradykinin (1 ug/ml) by intact, undiluted rat and turtle plasma is indicated by the curves with solid dots. The effect of 0.5 mg/ml 8-$HQSO_4$ on bradykinin inactivation is given by curves with hollow dots, and activity in control incubates (1 ug bradykinin in saline) is indicated by solid squares. All incubates were at 20°C. Values are means from two experiments.

Figure 5 a.


0.5 mg 8-HQSO4/ml
control
1.00
0.75
0.50
0.25
0
ug/ml plasma, Bk-eq
RAT
no inhibitor
10
20
30
Incubation Time, Min

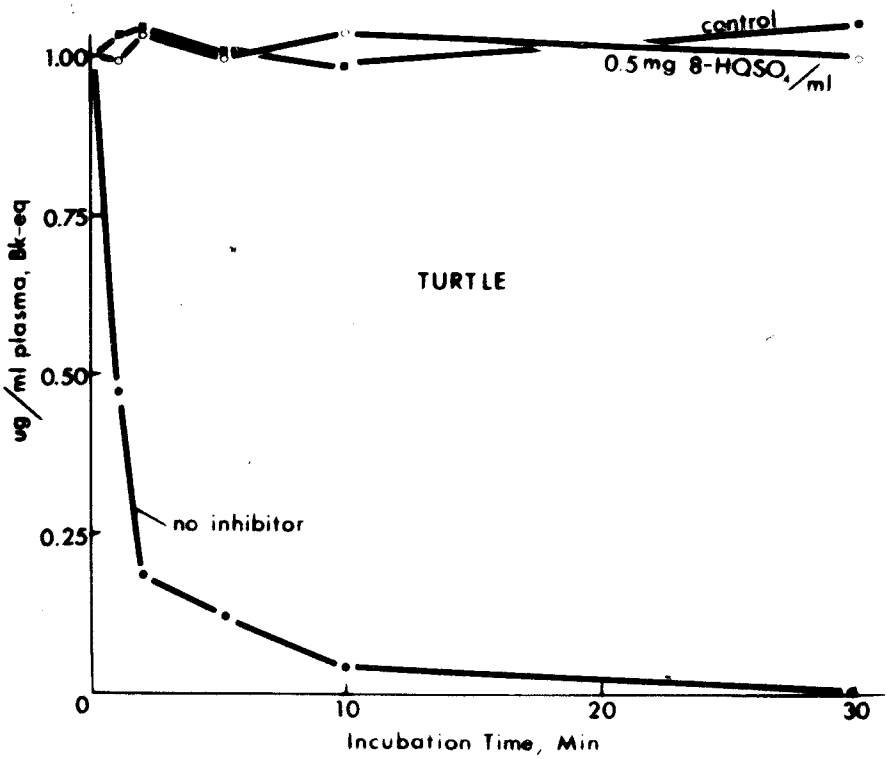
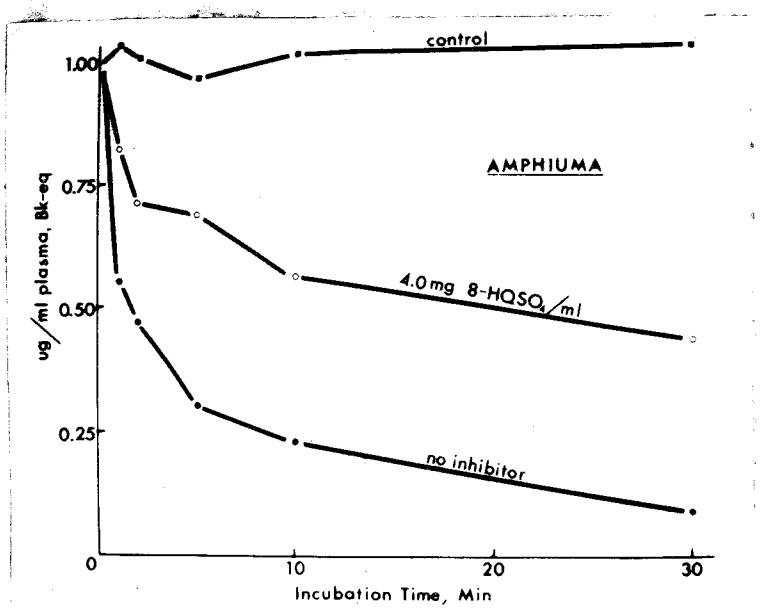
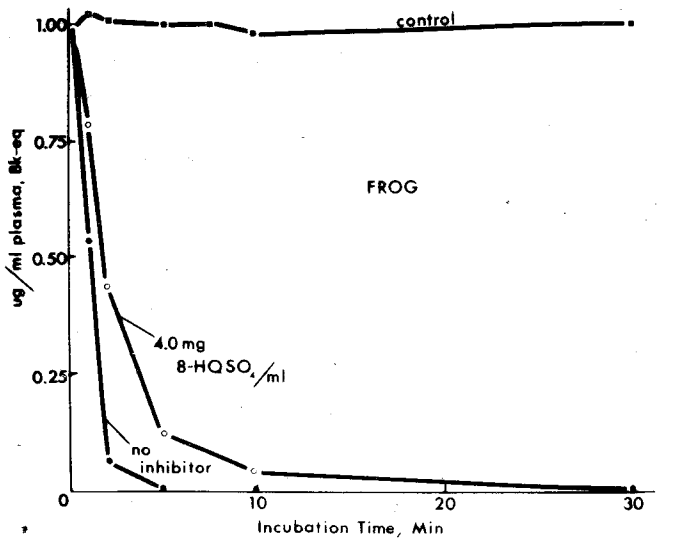
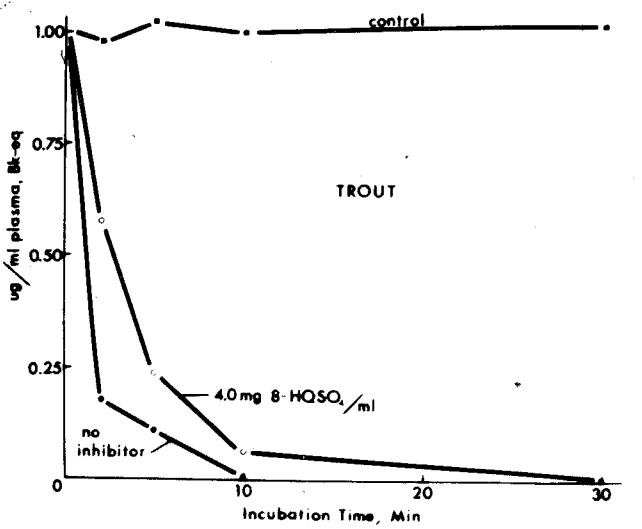

control
0.5 mg 8-HQSO4/ml
1.00
0.75
0.50
0.25
0
ug/ml plasma, Bk-eq
TURTLE
no inhibitor
10
20
30
Incubation Time, Min

Figure 5 b. Inactivation of synthetic bradykinin (1 ug/ml plasma) in Amphiuma, frog and trout plasmas. Legend for curves, and conditions of incubation are described in Figure 5 a.

Figure 5 b.


control
AMPHIUMA
4.0 mg 8-HQSO4/ml
no inhibitor
ug/ml plasma, Bk-eq
1.00
0.75
0.50
0.25
0
10
20
30
Incubation Time, Min


control
FROG
4.0 mg
8-HQSO4/ml
no
inhibitor
ug/ml plasma, Bk-eq
1.00
0.75
0.50
0.25
0
10
20
30
Incubation Time, Min


control
TROUT
4.0 mg 8-HQSO4/ml
no
inhibitor
ug/ml plasma, Bk-eq
1.00
0.75
0.50
0.25
0
10
20
30
Incubation Time, Min

Group 2. - Frog, Amphiuma and trout plasmas, where kininase activity was only partially reduced by much higher concentrations of the compound.

The result suggests at least two explanations: Firstly, the enzymes which contribute to bradykininase activity in Group 2 plasmas may differ qualitatively from those in Group 1; Secondly, differences could be due to the different chemical environments in these plasmas. Most of the kininase inhibitors described to date are chelating agents which inhibit metalloenzymes in general (Erdos and Wohler, 1963). It has been suggested that the action of these structurally different inhibitors may rest on the binding of a metal cofactor (Erdos and Wohler). In this case, the ionic environment in the plasma would be a very important factor in determining the efficiency of inhibiton. It is therefore a possibility that differences in concentrations of certain ions (perhaps divalent cations) in trout, Amphiuma and frog plasmas might account for the ineffectiveness of 8-$HQSO_4$ as a kininase inhibitor in these animals.

2. Relative effectiveness of glass surface, trypsin, and hog pancreas kallikrein in releasing kinin from undenatured vertebrate plasmas.

As has been mentioned previously, an effective kininase inhibitor is essential to any study of kinin production in intact plasma. Studies described in the previous experiment

indicated that 8-$HQSO_4$ was an effective inhibitor in some plasmas, and at least partially effective in all. Therefore activation of endogenous kallikrein-kinin systems by glass surface (as in Figure 2) could at least be attempted.

The object of these preliminary experiments was simply to determine whether glass activation would result in the appearance of kinin-like activity via the endogenous enzymes in lower vertebrate plasmas, as it does in mammals. The magnitude of these responses would be compared to kinin production from the same plasma by trypsin and HPK.

Method. Margolis (1963) indicated that 50 cm^2 of glass surface per ml of undiluted plasma rapidly released kinin from human plasma at room temperature, and this amount of acid-washed glass ballotini (Jencon's No. 8, 0.6 g = 50 cm^2) was used in this experiment.

Plasma was diluted 1:2 with the appropriate saline (Appendix B) containing 8-$HQSO_4$ in a 50 ml polypropylene test tube. The tube was clamped into an Eberbach water bath at 20°C, and shaken continuously. At zero time, the glass beads were added from a 5 ml glass beaker. At 0, 2, 5, 10, 30, and 60 minutes of incubation 0.5 ml samples were removed, injected into 1 ml of boiling water, and stored for assay.

Exact amounts used in the preparations were as follows:

	Group 1 (rat, turtle)	Group 2 (Amphiuma, frog and trout)
plasma	- 2 ml	- 2 ml
buffered saline	- 4 ml	- 4 ml
8-$HQSO_4$	- 2 mg	- 8 mg
glass beads	- 1.2 g	- 1.2 g
Total fluid volume	- 6.0 ml	- 6.0 ml

Incubations with HPK and trypsin were conducted in a water bath at 37°C, as follows:

	Group 1	Group 2
plasma	- 2 ml	- 2 ml
buffered saline	- 4 ml	- 4 ml
8-$HQSO_4$	- 2 mg	- 8 mg
Trypsin or HPK	- 2 mg	- 2 mg
Total fluid volume	- 6.0 ml	- 6.0 ml

Treatment of groups 1 and 2 differed only in the amount of kininase inhibitor. Two types of control incubates were included with experiments on each plasma. These were prepared exactly as described above, except that, a) 8-$HQSO_4$ was omitted, or b) glass or enzyme was omitted.

Results. HPK released no detectable kinin-like activity from any of these intact plasmas, which was in contrast to previous results with denatured plasma. Earlier experiments indicated that the HPK preparation had some intrinsic kininase activity

(Figures 3 a & b) which was not inhibited by 8-$HQSO_4$, but not enough to explain the complete lack of kinin activity in incubates from all sampling times. There are at least two possible explanations for this failure to develop free kinin;

a) that 8-$HQSO_4$ might have interfered with the action of HPK, and

b) that there are natural inhibitors of HPK in these plasmas which were destroyed by heating in previous experiments.

All other results are summarized in Table 3. Values are expressed as percents, based on maximum activities obtained in a given experiment on one plasma sample, usually at 10 or 30 minutes of incubation.

3. Effect of glass + Hageman Factor in releasing kinin from undenatured trout, frog and Amphiuma plasmas

Margolis (1960) has presented good evidence that glass beads activate and adsorb Hageman Factor in mammalian plasma, thereby initiating the kallikrein-kinin reactions. Bird plasma does not release a kinin when activated with glass, but Erdos et al (1967) found that duck plasma would release a kinin indistinguishable from bradykinin when activated with glass which had previously been rotated in mammalian plasma. These workers concluded that duck plasma lacks Hageman Factor, but possesses all other components of a kallikrein-kinin system. The trout, frog and Amphiuma plasmas tested in the present

Table 4. Mean percent of kinin-like activity released from intact and denatured plasma of several species by glass surface, trypsin, and hog pancreas kallikrein.

	Intact Plasma		Denatured Plasma		*Mean maximal activity/ml plasma
Species	Glass	Trypsin	Trypsin	HPK	
Dog	52(2)¢	100(2)	100(2)	56(2)	3.1(4)
Rat	83(6)	100(6)	100(4)	38(4)	3.0(10)
Turtle	68(7)	100(7)	100(3)	19(3)	1.9(10)
Amphiuma	-	-	6(3)	100(3)	2.9(3)
Frog	-	-	2(3)	100(3)	0.2(3)
Trout	-	-	-	100(3)	0.4(3)

*...values in this column are expressed as ug/ml plasma, BK-eq.

¢...values in parentheses indicate the number of experiments upon which each mean is based.

study released no kinin-like activity when treated with glass. To determine whether this failure could be due to a lack of Hageman Factor, as it is in birds, experiments similar to those described by Erdos et al were carried out with these plasmas.

Method. The method used was exactly as described for glass beads (p 38) except that the acid-washed ballotini were first rotated in 10 ml of fresh rat plasma for 10 minutes, which was ample time for adsorption of Hageman Factor (Margolis, 1960). The rat plasma was then poured off, and the beads were washed 5 times with saline prior to their addition to the test plasmas.

Results. None of the test plasmas developed kinin-like activity in response to the treatment described above.

C. Discussion and Conclusions

The fish, amphibian, reptilian and mammalian plasmas subjected to the basic qualitative tests described in this chapter had only two characteristics of the mammalian kallikrein-kinin system (Figure 2) in common; all had kininase activity, and all released biological activity when heated plasma was incubated with hog pancreas kallikrein. In all other tests, responses of the different plasmas clearly separated them into two groups, as summarized below:

TEST	RESPONSE	
	Group 1 (Turtle and mammalian plasmas)	Group 2 (Fish and amphibian plasmas
1. a. Trypsin on heated plasma	Maximal activity	Low levels of activity

TEST	RESPONSE	
	Group 1	Group 2
b. HPK on heated plasma	Sub-maximal activity	Maximal activity
2. 8-$HQSO_4$ on kininase in intact plasma	Total inhibition with 0.5 mg/ml plasma	Only partial inhibition with 4.0 mg/ml plasma
3. a. Trypsin on intact plasma	Maximal activity	No activity
b. Glass beads on intact plasma	Sub-maximal activity	No activity

Two conclusions may be drawn from the results of these preliminary studies:

a) No evidence was obtained to support the hypothesis that a kallikrein-kinin system analagous to that of the mammal exists in the plasma of those fish and amphibians tested:

b) Turtle plasma contains a kallikrein-kinin system comparable in those characteristics examined to that of the mammal.

With regard to the reptiles, evidence obtained in this study is supported by the work of Erdos, Miwa and Graham (1967) which was published during the course of these investigations. These workers studied glass released kinin-like activity in plasma of the common snapping (Chelydra serpentina ?) and elegant slider (Pseudemys scripta elegans ?) turtles, and of the alligator, using 1, 10 phenanthroline as kininase inhibitor. Basic chemical and pharmacological properties of the active principle led the authors to suggest that the reptilian kinin

was bradykinin and/or lys-bradykinin. In any case, these plasmas clearly belong in Group 1, as described above.

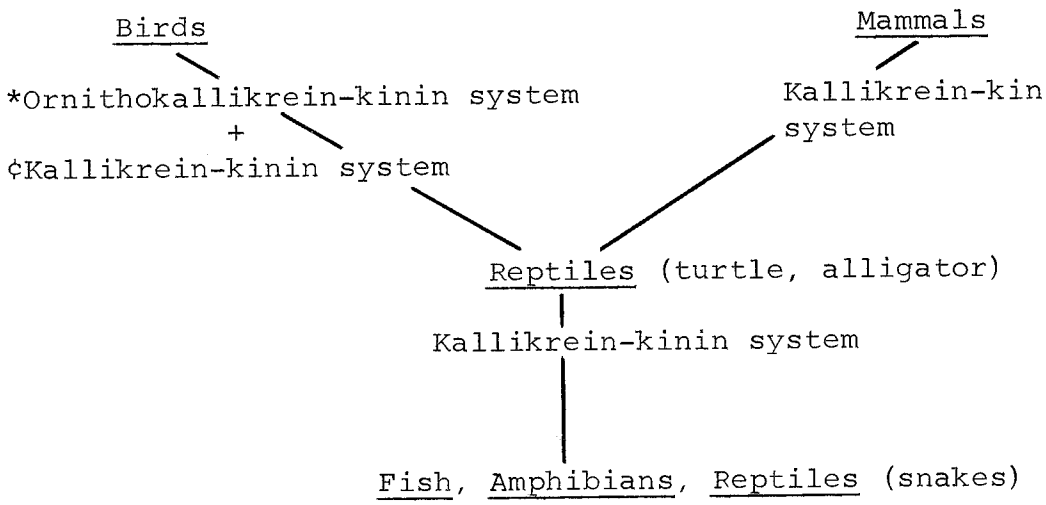
Recent observations of Lavras, et al (1969) on plasma of the snake Bothrops jararaca indicate that not all reptilian plasma fit into this category. These workers found no evidence for an endogenous kallikrein-kinin system capable of glass activation, and no kinin was generated by the action of trypsin. It was also demonstrated that HPK did release a factor which was oxytocic on the isolated rat uterus, but that this substance was different from mammalian kinins, since it was hypertensive (rather than hypotensive) on mammalian blood pressure. Plasma of this reptile therefore has characteristics ascribed to Group 2 (p 43).

The role of the kallikrein-kinin system in blood pressure regulation in a shark (Scyliorhinus stellaris) and a teleost (Silurus glanis) was examined by Vogel et al (1969). Bradykinin, Lys-bradykinin, hog pancreas kallikrein, fish pancreas kallikrein, and trypsin had no effect on fish blood pressure, and the authors have suggested that a kallikrein-kinin system does not exist in the animals. Data obtained in the present study are not in conflict with these results, or this conclusion, since Vogel et al did not investigate smooth muscle stimulating agents released by HPK from fish plasma.

Although the HPK released factor may not be a kinin, the observation that the enzyme can liberate a biologically active

principle in fish, amphibian, and snake plasmas encourages speculation on the possibility that there could be a physiological release and role for the factor. However, further research on the chemical nature of the agent, its pharmacological effects, and possible mechanisms of *in vivo* release (e.g., the effect of glandular extracts on plasma of each species) must be carried out in all species before any such possibility can be meaningfully discussed.

Any detailed consideration of evolution of plasma kallikrein-kinin systems would be premature at this time, since so few vertebrates have been examined However, considering the evidence presently available, the following picture emerges when major classes are ranked according to the system each possesses:



*...Werle et al, 1966.

¢...This kallikrein-kinin system lacks Hageman Factor.

It should be emphasized that the lines between groups in the above diagram do not imply evolution of one enzyme system to another by any direct means.

CHAPTER 4. THE KALLIKREIN-KININ SYSTEM IN TURTLE PLASMA

Experiments on fish, amphibian and reptilian plasmas described in Chapter 3 established turtle plasma as one of the few which might have an endogenous kallikrein-kinin system comparable to the well-studied mammalian system. Further investigations were therefore extended to the turtle Pseudemys scripta elegans, to characterize more precisely the mechanisms of the release of this kinin, and to compare this process with the release of bradykinin in mammalian plasma.

A wide variety of treatments will result in kinin production from plasma (Erdos, 1966), but few could be regarded as conceivable physiological events. Since glass surface initiates kinin production by activation of the animal's own enzymes, while causing a minimum of side effects, it may imitate physiological release of kinin much more closely than would at first appear. For this reason the method of glass activation was used to study kinin production. Details of current theories on glass activation of Hageman Factor and human prekallikrein have been reviewed by Webster (1968).

There are obvious difficulties in kinetic studies of a reaction whose final product must be measured by bioassay. Although the rat uterus assay is remarkably sensitive, and indispensible in measuring low concentrations of kinins, data

are less accurate and more difficult to obtain than in equivalent studies on reactions whose progress can be measured spectrophotometrically.

Interpretation of results is complicated by the presence of inactivators of enzymes and products, and by the plurality of enzyme-substrate interactions which contribute to a given concentration of kinin. More precise information on particular points in the system, such as kallikrein-kininogen interactions, can be obtained using purified preparations of kininogens (e.g., Kato and Suzuki, 1968), plasma kallikreins (e.g., Colman, Mattler, and Sherry, 1969), prekallikreins (e.g., McConnell and Mason, 1970) or glandular kallikreins (e.g., Takami, 1969). However, it was the purpose of this study to examine the different aspects of kinin production involving the entire kallikrein-kinin system, under conditions which might obtain *in vivo*.

There may be seasonal changes in hematocrit (Hutton, 1960) and blood coagulation (Jacques, 1963) in *P. scripta elegans*, which could result in seasonal changes in concentrations of components in the plasma kallikrein-kinin system. Since this study was primarily directed towards characterizing the qualitative mechanisms of the system, no seasonal or experimentally induced quantitative changes of this nature were explored. Turtles were kept at natural photoperiod and

constant temperature (15°C) in the laboratory; the animals were sampled throughout the year, and no consistent differences in kinin production were noted.

A. Preliminary Studies.

These studies were conducted to determine the effect on turtle kinin production of varying important conditions of incubations, such as the amount and type of kininase inhibitor, the dilution of plasma, or the amount of glass surface. Data from these experiments would allow selection of standard conditions for release, enabling the direct comparison of the characteristics of release with mammalian plasma. It would also allow the design of an experiment suitable for the production of the large amount of kinin required for future purification of the peptide.

1. Production of turtle kinin in the absence of a kininase inhibitor. Although detailed study of turtle kinin release would involve the use of a kininase inhibitor, and adoption of convenient sampling techniques with diluted plasma, it is nonetheless important to examine the release of turtle kinin in unmodified plasma, particularly since this is the closest approximation to in vivo activation of the enzyme system.

Method. Undiluted plasma obtained in the usual manner (Chapter 2) was placed in 5 ml lots in 10 ml polypropylene test tubes,

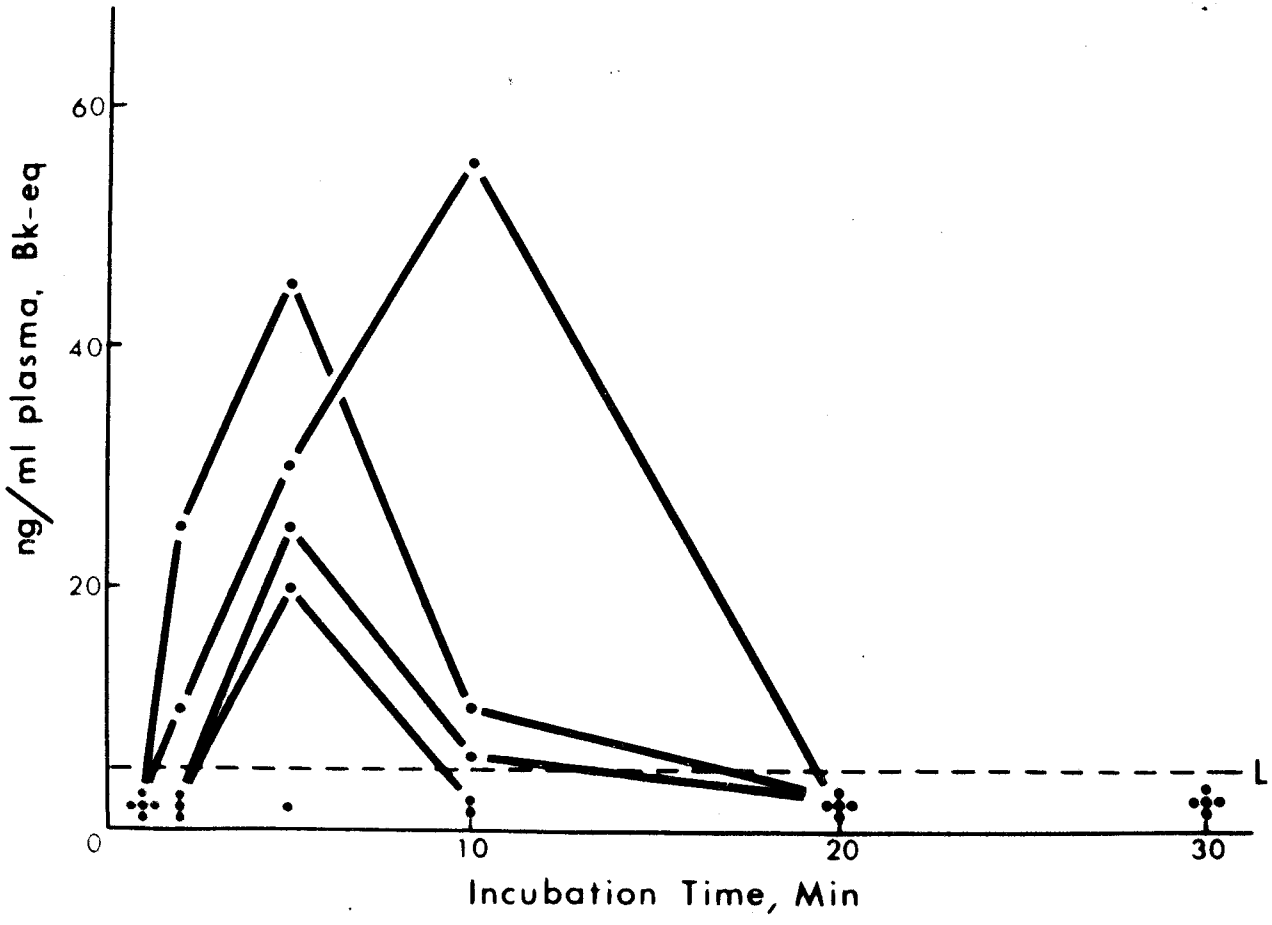
in an Eberbach water bath at 20°C, and activated with 6.0 g acid washed glass beads per tube (50 cm^2/ml plasma). At 1, 2, 5, 10, 20, and 30 minutes samples were removed and heated as described in Chapter 2 (p 12). Control lots containing no glass were incubated and sampled in the same way.

Results. Individual values for five experiments on plasmas of different turtles are presented in Figure 6. Samples taken before 2 minutes, and after 10 minutes of incubation never showed kinin activity. Some plasmas activated with glass did not contain measurable turtle kinin at any sampling time, while others did develop up to 55 ng/ml, bradykinin equivalent. Controls with no glass never developed activity over the sampling period

Unmodified turtle plasma can develop low levels of kinin activity, and it is clear that the plasma contains an active kininase, as suggested by earlier experiments with bradykinin (Figure 5 a). In mammals, the threshold dose for a depressor response is usually not less than 250 ng/kg (Erdos, 1966). If turtles are similar, then at this pH (about 7.8) and temperature (20°C) it should therefore require a very strong stimulus for the concentration of kinin to build up to a point where a major cardiovascular effect could be observed. It should be emphasized that there is at present no evidence on the physiology or pharmacology of kinins in reptiles.

Figure 6. Effect of glass surface (50 cm^2/ml plasma) in releasing kinin from turtle plasma containing no kininase inhibitor. Results of 5 separate experiments at 20°C are shown. Dotted line indicates the limit of sensitivity for the rat uterus bioassay. Activity is expressed as ng/ml plasma, BK-eq.

Figure 6


60
40
20
0
ng/ml plasma, Bk-eq
L
10
20
30
Incubation Time, Min

2. Effect of mammalian kininase inhibitors on turtle kininase. This experiment was designed to find a compound which could effectively inhibit kininases in turtle plasma, and therefore would allow the release of turtle kinin. The compounds 2-mercaptoethanol and 3-mercaptoproprionic acid (Sigma) have been shown to be potentiators of the in vivo effects of bradykinin in mammals (Erdos and Wohler, 1963), presumably through kininase inhibition. These compounds were tested in this study in the hope that the same inhibitor might be used in future in vivo and in vitro experiments. Derivatives of 8-hydroxyquinoline are effective inhibitors in vitro (Erdos, 1966; Rocha e Silva, 1963), as are metal salts of EDTA (Margolis, 1963; Erdos, 1966), although both are ineffective potentiators of in vivo bradykinin effects (Erdos and Wohler, 1963); these compounds were also tested.

All compounds were tested in two ways; a) in experiments to determine the effect of different concentrations of inhibitor on the rate of inactivation of bradykinin in intact plasma; and b) in experiments to determine the effect of different concentrations of inhibitor on the kinetics of turtle kinin release by glass activation.

Method. a) The method for tracing the rate of bradykinin des truction was exactly as described in Chapter 3, p 33. Inhibitors were added to the plasma in volumes never exceeding

0.2 ml of saline carrier.

b) Plasma diluted 1:2 with turtle saline was activated with 50 cm^2 glass surface/ml plasma, and sampled as described in Chapter 2. Inhibitors were added to the turtle saline prior to dilution of the plasma.

Results. Figure 7 indicates that 3-mercaptoproprionic acid and Na-EDTA in doses up to 4 mg/ml were ineffective in decreasing the rate of bradykinin inactivation, while 2-mercaptoethanol was partially effective. However, 8-$HQSO_4$ in doses above 0.5 mg/ml plasma was normally sufficient to give total protection to bradykinin (as discussed in Chapter 3, p 34).

These results were mirrored in the effects of these same compounds on turtle kinin production, in Figure 8. In these experiments, 8-$HQSO_4$ (1 mg/ml plasma) gave the highest values for turtle kinin at any incubation time, and after 1 hour (in one case 2 hours) of incubation with this inhibitor, the concentration of turtle kinin showed no decrease. 2-mercaptoethanol (2 mg/ml plasma) allowed considerable kinin activity to build up, but this was always destroyed in prolonged incubations. It is possible that very high concentrations of this substance might give complete protection to bradykinin and turtle kinin, but might also interfere with other aspects of kinin release.

Activation of turtle plasma in the presence of Na-EDTA or 3-mercaptoproprionic acid produced only traces of turtle

Figure 7. Effect of mammalian kininase inhibitors on the rate of inactivation of bradykinin (lug/ml plasma) in undiluted turtle plasma, at 20°C. Values are means for two experiments. The legend for the curves is as follows:

▲ .....no kininase inhibitor added

▽ .....up to 4 mg/ml 3-mercaptoproprionic acid

□ .....up to 4 mg/ml Na-EDTA

■ .....4 mg/ml 2-mercaptoethanol

● .....0.5 mg/ml 8-$HQSO_4$

X.....controls, containing only saline and synthetic bradykinin, lug/ml.

Figure 7

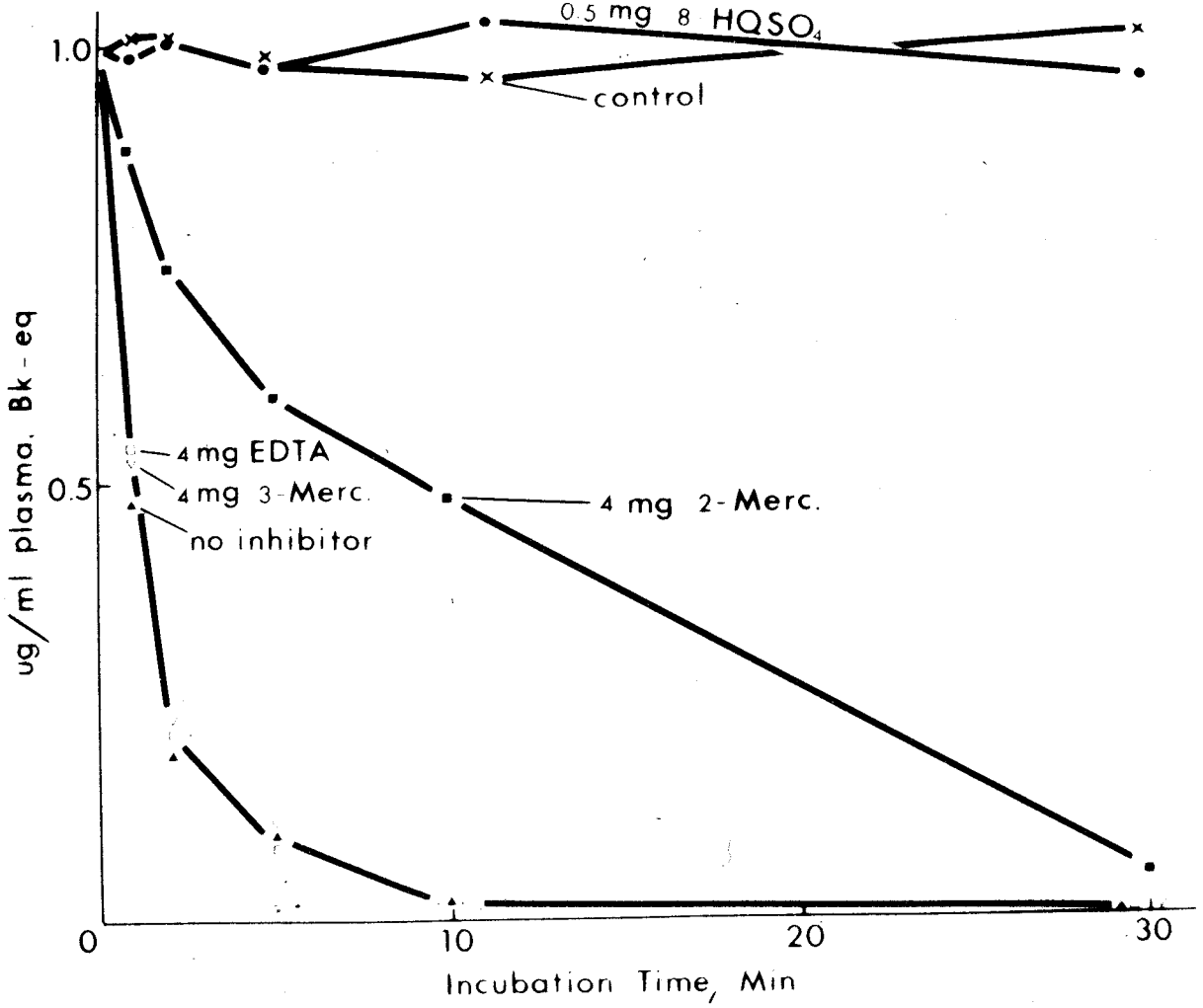

0.5 mg 8 HQSO4
control
1.0
0.5
0
4 mg EDTA
4 mg 3-Merc.
no inhibitor
4 mg 2-Merc.
ug/ml plasma, Bk-eq
10
20
30
Incubation Time, Min

Figure 8. Production of kinin from turtle plasma by glass surface (50 cm^2/ml plasma) at 20°C, in the presence of different kininase inhibitors. Activity present in the samples is expressed as ug/ml undiluted plasma, BK-eq. The legend for the curves is as follows:

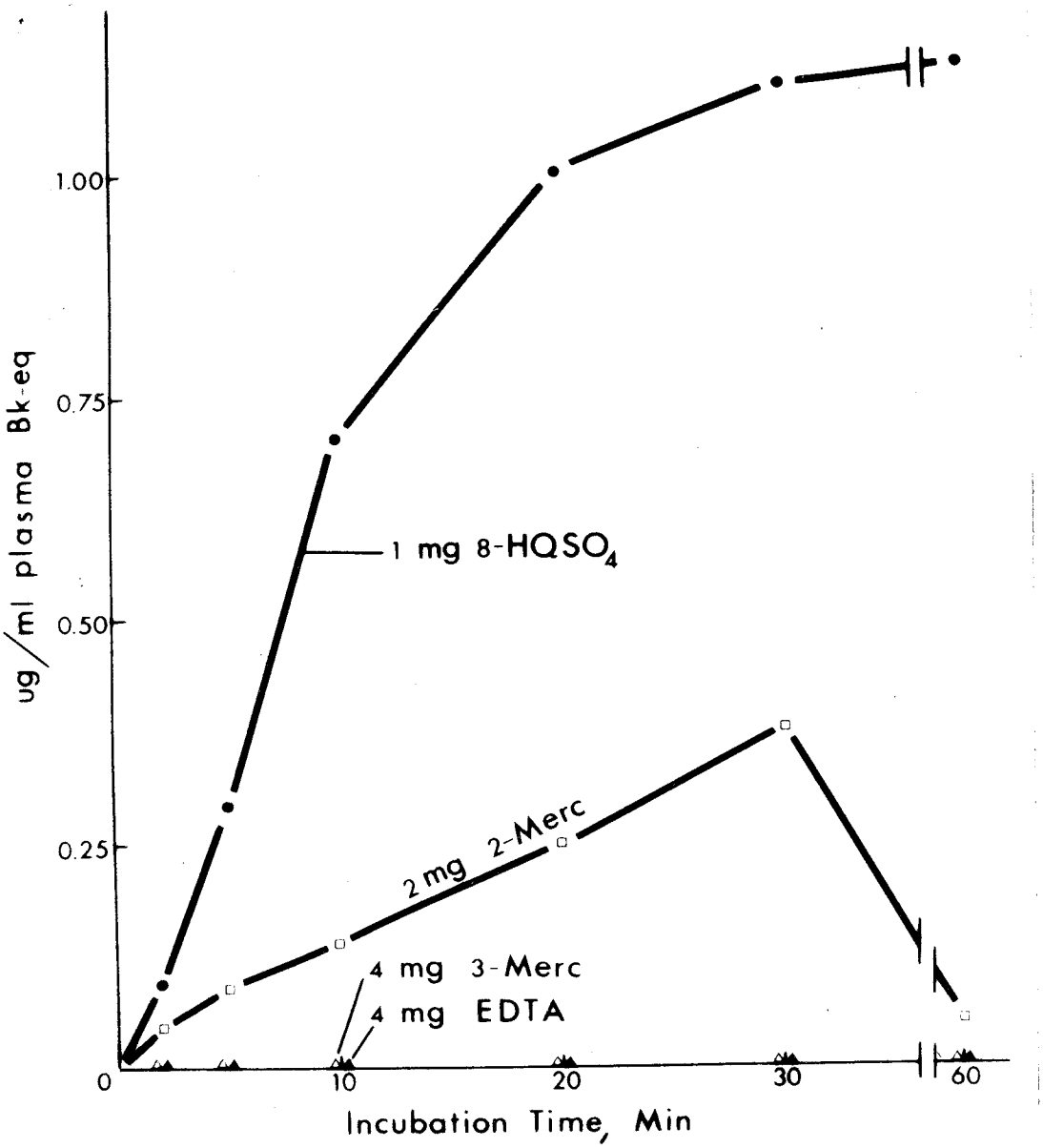
△ .....4 mg/ml 3-mercaptoproprionic acid

▲ .....4 mg/ml Na-EDTA

□ .....2 mg/ml 2-mercaptoethanol

● .....1 mg/ml 8-$HQSO_4$

Figure 8


1.00
0.75
0.50
0.25
0
ug/ml plasma Bk-eq
1 mg 8-HQSO4
2 mg 2-Merc
4 mg 3-Merc
4 mg EDTA
10
20
30
60
Incubation Time, Min

kinin activity no different from plasma activated in the absence of any inhibitor (Figure 6). Turtle plasma is therefore similar to rat and guinea pig plasma in that Na-EDTA is ineffective, and different from human (Margolis, 1963) and sheep (Campbell et al, 1968) plasmas in which EDTA is partially effective.

It was concluded from the experiments given here that the mercapto compounds, and Na-EDTA are unsatisfactory in vitro kininase inhibitors in turtle plasma. However, this does not exclude the possibility that these chemicals may potentiate in vivo effects in the turtle. In rats, for example, Ryan, Roblero and Stewart (1968) have shown that 2-mercaptoethanol may potentiate in vivo effects of bradykinin by inhibiting powerful kininases situated not in the plasma, but in the vascular endothelium in the lung.

3. Effect of plasma dilution on turtle kinin production. For most incubations it was found to be convenient to dilute plasma 1:2 with turtle saline (Appendix B). This procedure allowed the heated samples used in some experiments to remain in a liquid form, whereas undiluted plasma formed a gel when heated above 65°C. Since it is possible that the kinetics of kinin production could be significantly altered by dilution of the components from physiological concentrations, experiments were carried out to test the effect of varying dilution over

the range of dilutions to be used in future experiments.

Method. Using 1 mg 8-$HQSO_4$/ ml undiluted plasma, at 20°C, samples of plasma from one animal were incubated with 50 cm^2 glass surface, as follows:

	Plasma	Saline	8-$HQSO_4$	Glass Beads
a.	2 ml	none	2 mg	2.4 g
b.	2 ml	4 ml	2 mg	2.4 g
c.	1 ml	9 ml	1 mg	1.2 g

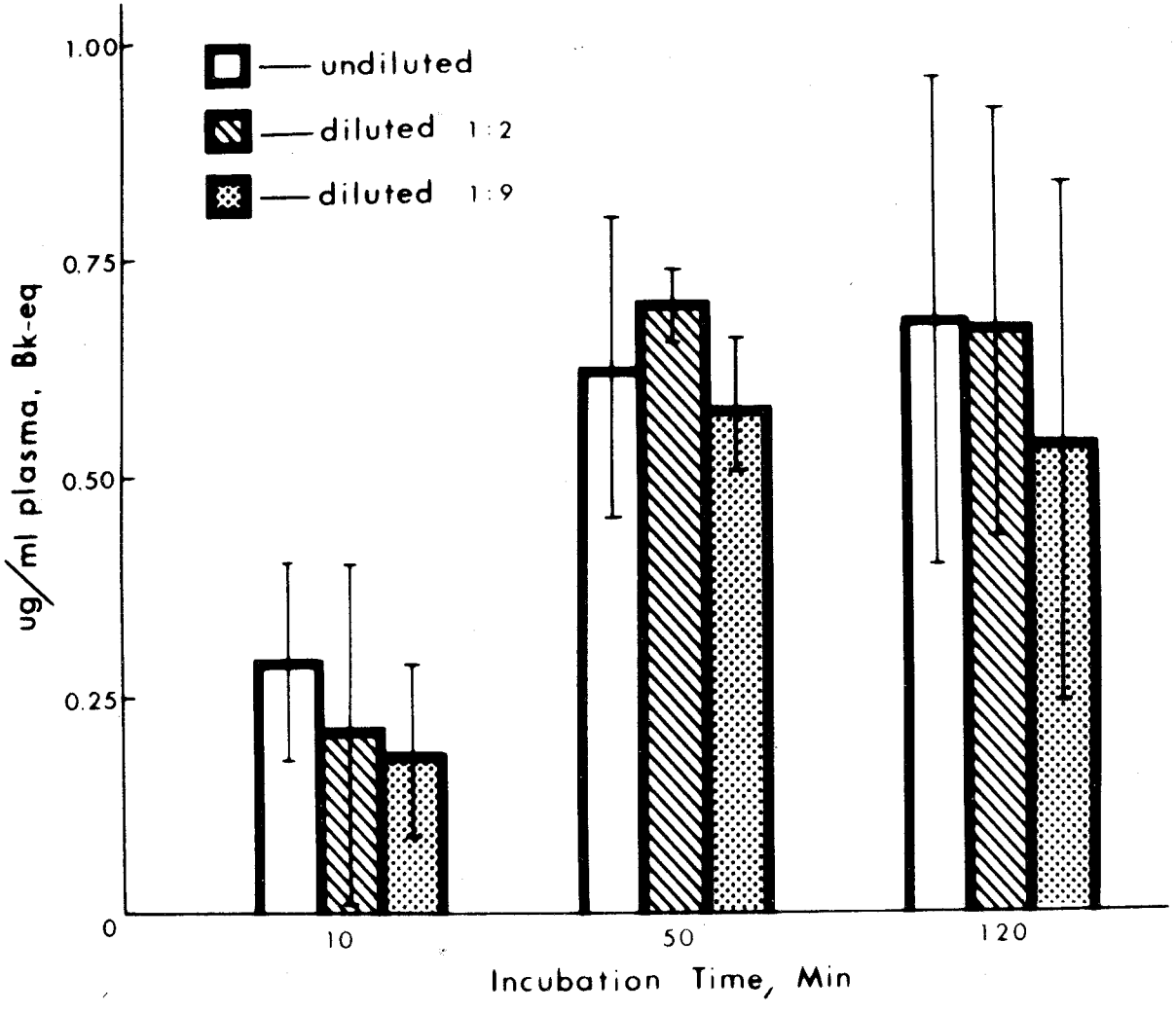
At T = 0, 10, 50, and 120 minutes samples were removed, inactivated by boiling, and stored for assay.

Results. Results for each dilution are summarized in Figure 9, in terms of bradykinin-equivalent/ml undiluted plasma present at each sampling time. Each bar represents the mean of three values, and 95% confidence limits are given. There was no significant difference between treatments, and it is doubtful whether a larger sample size would indicate that significant differences exist in activities present at 10 minutes (a measure of reaction rate) or at 50 and 120 minutes (a measure of maximum yield). In similar experiments on human plasma, Margolis (1963) found that dilution had little effect, although at very high dilutions, up to 1:100, both rate and yield of kinin production were reduced.

Throughout remaining experiments it was assumed that dilution of plasma to 1:2 did not cause major changes in the

Figure 9. Effect of dilution of plasma on rate and yield of turtle kinin production. Plasma was diluted with buffered saline as indicated in the Figure, and activated with 50 cm^2 glass surface/ml undiluted plasma, at 20°C. Activity at each of the sampling times is given as ug/ml undiluted plasma, BK-eq. Each bar represents the mean of 3 experiments, and 95% confidence limits are indicated.

Figure 9


1.00
0.75
0.50
0.25
0
undiluted
diluted 1:2
diluted 1:9
ug/ml plasma, Bk-eq
10
50
120
Incubation Time, Min

kinetics of turtle kinin release.

B. Effect of Glass Surface Area on Turtle Kinin Production

Since variation in glass surface area could be expected to affect kinin production, experiments were carried out to determine the relation of this parameter to the rate and yield of the kinin releasing reactions.

These experiments were also designed to test the glass-triggered reactions in turtle plasma against the model for kinin production in mammals (Figure 2). According to this proposed mechanism for activation of the endogenous kallikrein-kinin system, the amount of prekallikrein converted to kallikrein should be directly proportional to the amount of Hageman Factor activated, which in turn is probably directly related to the number of negative sites on the activating material (Margolis, 1963: Nossal, et al, 1968). If the mammalian and turtle systems were similar, variations in glass surface area/ml of plasma would be predicted to change the rate but not the yield of kinin producing reactions.

Method. Incubates were prepared as follows:

	Glass ballotini	Plasma	Saline	8-$HQSO_4$
a.	0.24 g (5 cm^2/ml)	2 ml	4 ml	2 mg
b.	0.96 g (20 cm^2/ml)	2 ml	4 ml	2 mg
c.	2.4 g (50 cm^2/ml)	2 ml	4 ml	2 mg
d.	4.8 g (100 cm^2/ml)	2 ml	4 ml	2 mg

These tubes were incubated at 20°C in the usual manner

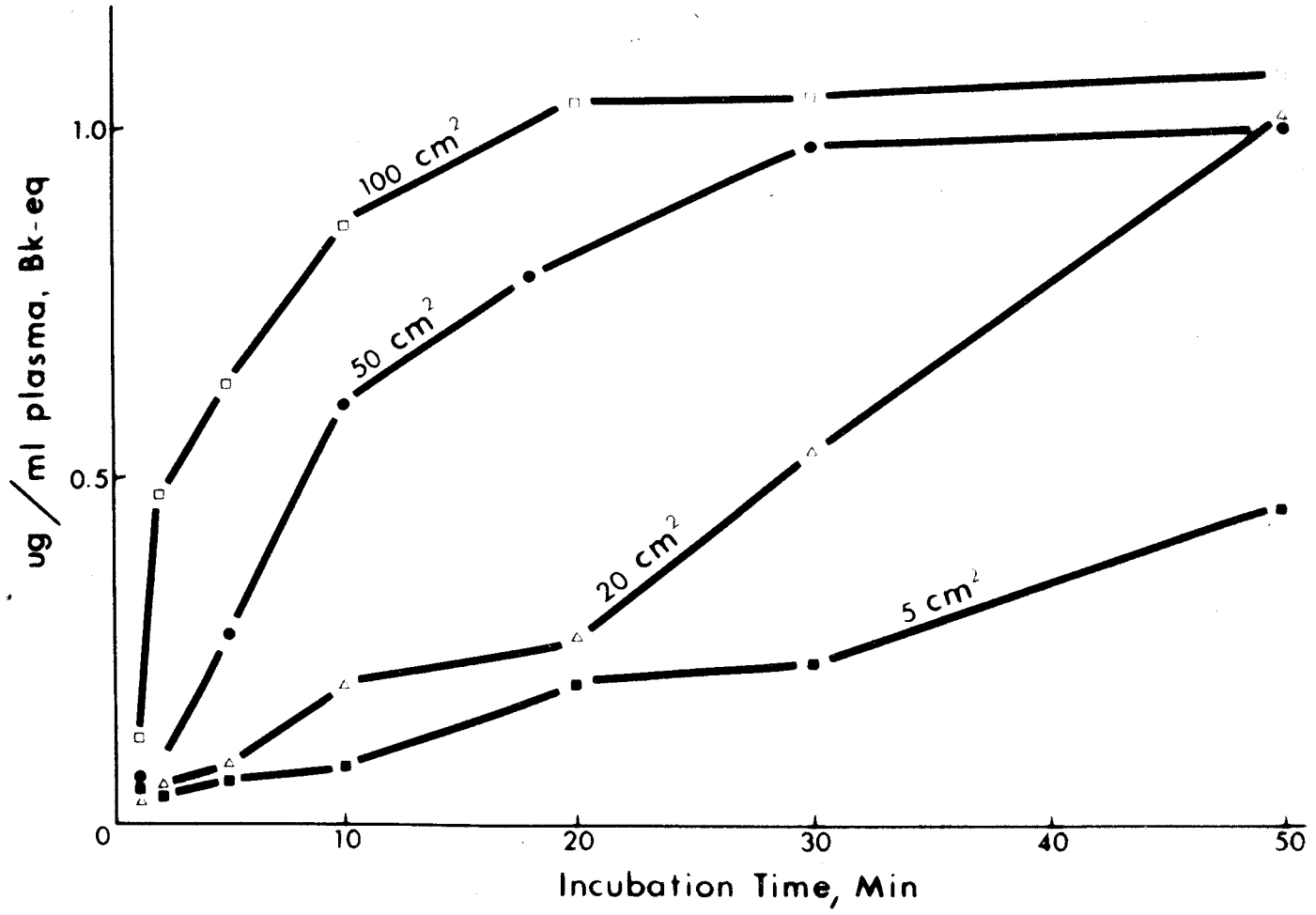
and sampled at T = 0, 1, 2, 5, 10, 20, 30, and 50 minutes, after the addition of the glass. Each experiment on the plasma of one turtle included all four incubates, as well as control incubates containing no glass. The whole experiment was repeated on plasma of different turtles.

Results. Data from 3 experiments on different plasmas are given in Figure 10. These results are expressed as the mean activity present at a particular sampling time. It is clear that the rate of kinin production was directly proportional to the amount of glass surface added. Maximum levels of kinin produced in 50 minutes did not differ between incubates containing 100, 50, and 20 cm^2 glass surface/ml. Kinin produced by 5 cm^2 glass/ml did not appear to have reached maximum levels even after 50 minutes of incubation, but would probably have done so in a longer incubation period. These results are exactly as would be predicted from the mammalian model (Figure 2).

Margolis and Bishop (1963) also investigated the relationship of glass surface area to bradykinin production in human plasma. Surprisingly, they found that 250 cm^2 glass/ml plasma gave a lower yield than 50 cm^2 glass/ml, and that kinin was released in both incubates at the same rate. As suggested by these workers, the lower yield at 250 cm^2 could have been due to adsorption of kinin to glass surface at this high

Figure 10. The effect of glass surface area on turtle kinin production at 20°C. Plasma diluted 1:2 with saline was activated with 5, 10, 50 and 100 cm^2 glass surface/ml undiluted plasma. Values are given as ug/ml plasma, BK-eq, and are means of 2 experiments.

Figure 10

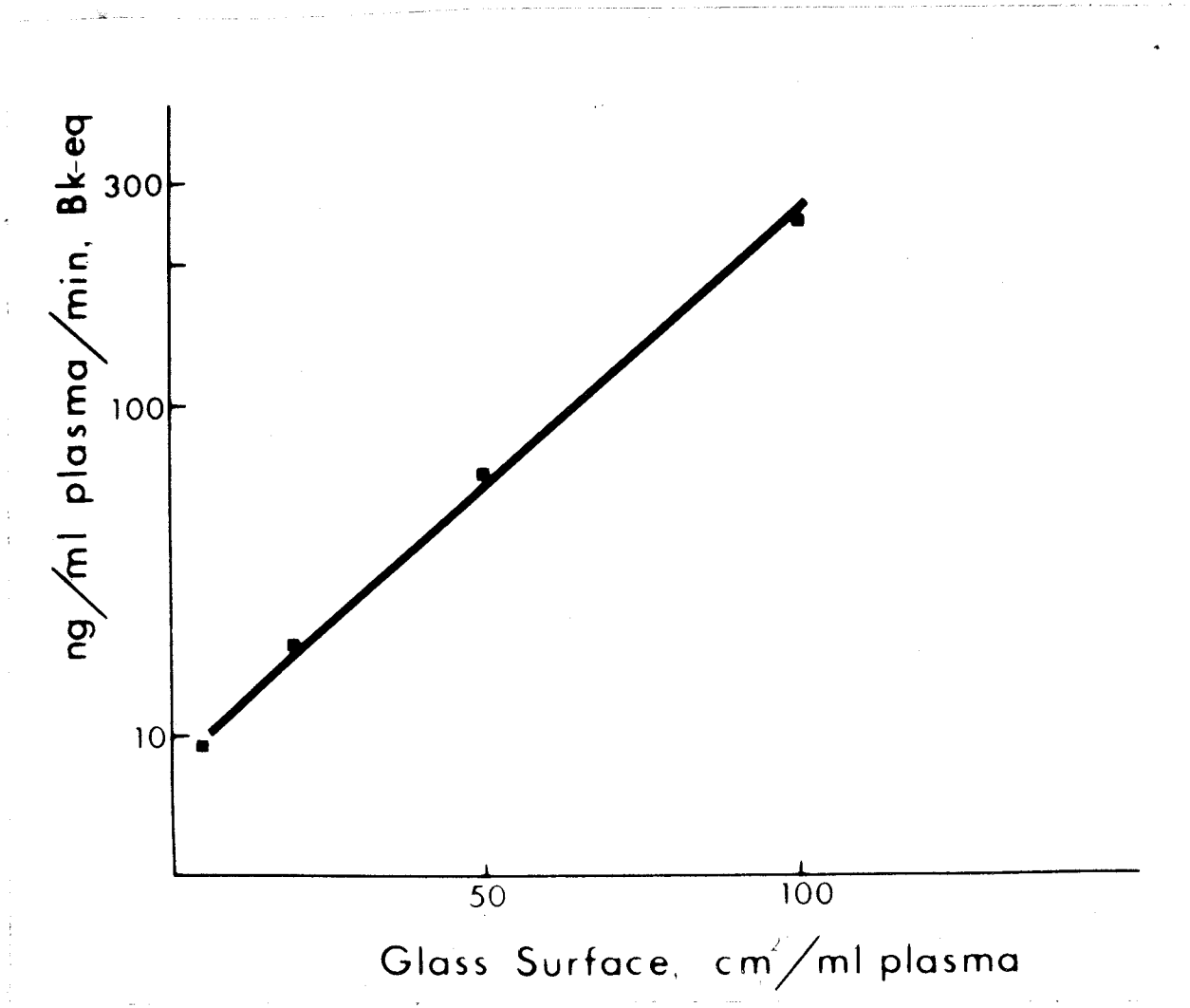

ug / ml plasma, Bk-eq
1.0
0.5
0
100 cm²
50 cm²
20 cm²
5 cm²
10
20
30
40
50
Incubation Time, Min

concentration. With regard to the similarities found in rates of kinin production, it seems possible that the higher incubation temperature (about 23°C) and the incomplete kininase inhibition by EDTA (Margolis and Bishop, 1963) obscured the relation between rate of production and glass surface area that should exist in this plasma.

Maximum velocity of kinin production was estimated from the straight portion at the beginning of each curve in Figure 10, and the relation between the log of this initial velocity and glass surface area is given in Figure 11. Over the experimental range the relation approximates a straight line, indicating that the kinetics involved are not first-order. That is, a doubling of the glass surface area more than doubles the rate of kinin formation, implying that the the enzymic reactions involved have a "multiplier effect", as can occur in "damped cascade" enzyme pathways (Hemker and Hemker, 1969). The slope of the line suggests that it would not pass through the origin, and that at 0 cm^2/ml plasma, the V_{max} would be about 7 ng BK-eq/ml/min. This cannot be the case, since controls with no glass did not develop kinin activity. Therefore, it is probable that this multiplier effect is greater when small amounts of glass surface (less than 5 cm^2/ml plasma) or some other activator are involved.

Figure 11. Effect of glass surface area on maximum velocity of turtle kinin production at 20°C. Values for V_{max} are given as ng/ml plasma/min, BK-eq, and were estimated from the initial straight portion of the curves shown in Figure 10.

Figure 11


ng/ml plasma/min, Bk-eq
300
100
10
50
100
Glass Surface, cm²/ml plasma

C. Activation and Decay of Plasma Kallikrein

It seems reasonable to assume that any plasma which releases a kinin on contact with glass contains some type of kallikrein in a pre-enzyme state. However, the relative concentrations of the pre-enzyme in the plasma, its rate of activation, and the rate of decay of the activated enzyme are all important variables in the system, and could markedly affect its ability to function in a physiological role. These aspects of kinin release were therefore studied in turtle plasma, and compared directly to the equivalent events in rat plasma.

The method used in this study had features in common with those described by Margolis (1960) and Briseid et al (1968) for human plasma, and it is based on the preparation of a plasma depleted of substrate for plasma kallikrein, but which still contains pre-kallikrein.

It has been shown that trypsin releases more kinin from turtle plasma than is released by glass (Table 3). For convenience, that portion of the total kininogen which releases kinin to glass activated enzymes will be referred to as Substrate 1 (see Chapter 1).

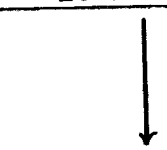
1. Preparation of Substrate 1-depleted plasma. The object of this experiment was to incubate plasma with glass ballotini for a period just long enough to allow the release

of all kinin in Substrate 1. This plasma could then be used as a source of prekallikrein.

Method. Turtle plasma was incubated in several experiments with 50 and 100 cm^2 glass surface/ml plasma, in the absence of a kininase inhibitor. At selected time intervals samples of this plasma were separated from the glass and incubated alone for a further two hours at 20°C; this allowed time for a complete inactivation of any kinins produced, and for the normal loss of the kallikreins which had been responsible for developing them. A portion of each incubate was then removed, its enzymes were inactivated in boiling water, and the product assayed as a control. The remainder of the mixture was used to measure the amount of Substrate 1 still present in these glass-depleted samples. This was determined by subsequent incubation of each sample with the kininase inhibitor 8-$HQSO_4$ and fresh acid-washed glass for one hour at 20°C. Samples from these tubes were boiled and assayed in the usual manner.

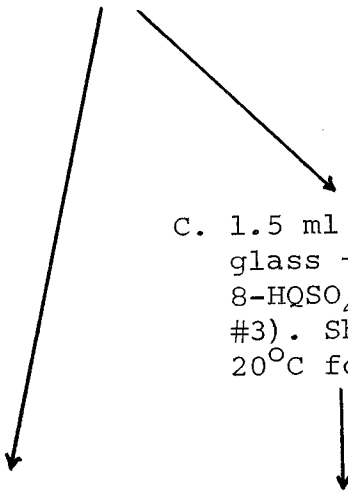
Also included in each experiment was a control incubate which was treated exactly as described above, except for the addition of 8-$HQSO_4$ in the initial incubation. These samples always contained some kinin activity. Details of each experiment can be shown schematically as follows:

	Plasma	Saline	Glass	8-$HQSO_4$	Purpose
1.	5 ml	10 ml	50 cm^2/ml	-	To deplete Substrate 1 by glass activation.
2.	5 ml	10 ml	100 cm^2/ml	-	
3.	5 ml	10 ml	100 cm^2/ml	5 mg	



A. 2 ml removed from each incubate at T = 0, 2, 5, 10, 20, and 30 minutes, and incubated separately for 2 hours at 20°C. *

To allow kinin and kallikrein to be destroyed by natural mechanisms.



C. 1.5 ml + 0.6 g glass + 0.5 mg 8-$HQSO_4$ (except #3). Shaken at 20°C for 60 min.

To convert residual Substrate 1 to active kinin by glass activation.

B. 0.5 ml sampled and assayed.

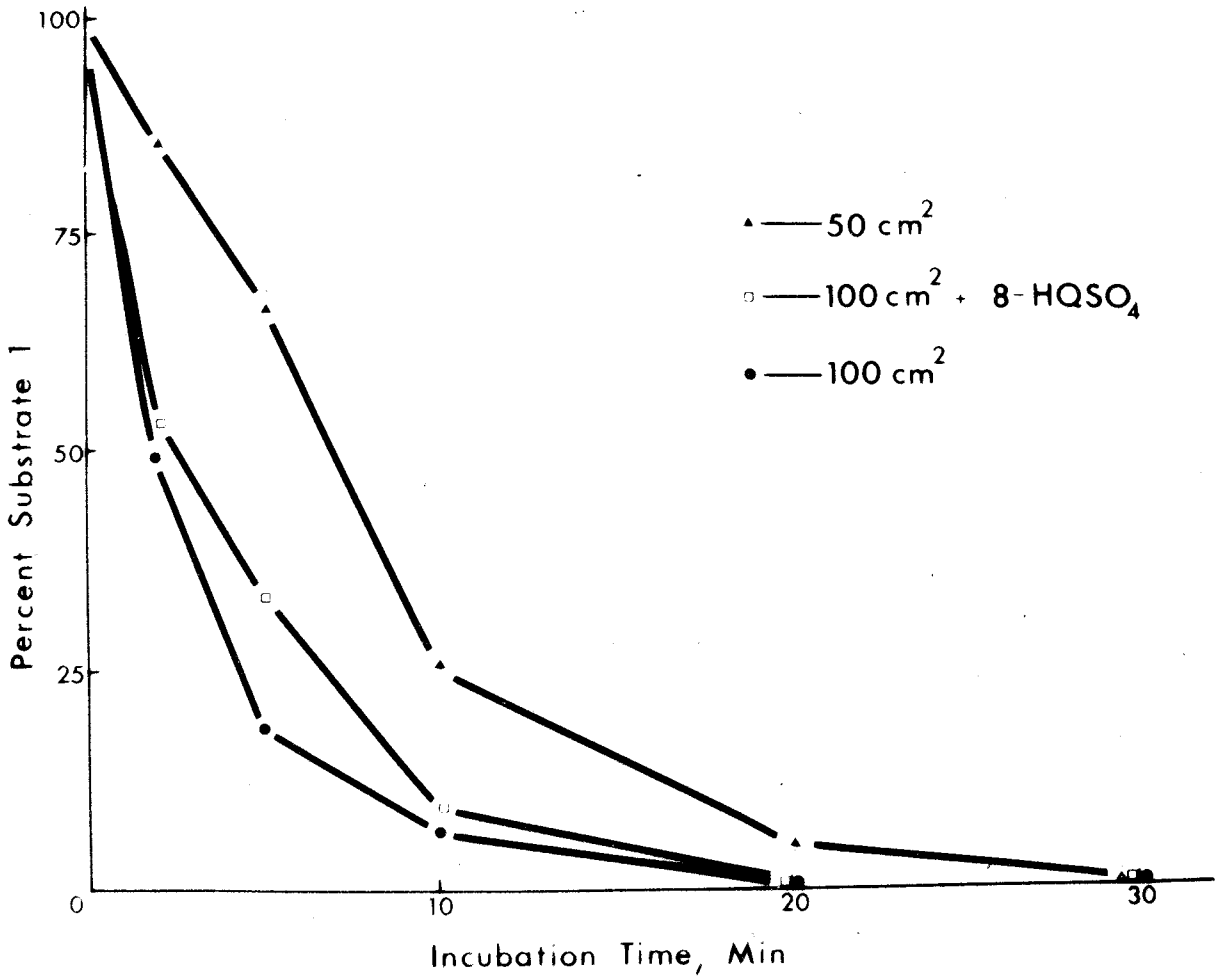
D. 1.0 ml sampled and assayed.

*... Although kallikrein continues to release kinin after removal of glass, the effect is short-lived, as will be shown in section 3, this chapter.

Results. A summary of experimental results given in Figure 12 shows the progressive decline of Substrate 1 on incubation with glass. For all incubates the activity contained in sample D (see above) at T = 0 represented 100% Substrate 1. For

Figure 12. Rate of depletion of substrate for glass activated enzymes (Substrate 1) in turtle plasma, by 50 and 100 cm^2 glass surface/ml. Details of methods are given in the text, p 66.

Figure 12


100
75
50
25
0
10
20
30
Percent Substrate 1
Incubation Time, Min
50 cm2
100 cm2 + 8-HQSO4
100 cm2

incubates #1 and #2 activity in subsequent samples (D) was expressed as a percent of this maximum activity. For incubate #3, which contained kininase inhibitor, the kininogen remaining was calculated as the maximum activity possible for that plasma (value of D at T = 0) minus the activity in sample B at a particular time, expressed as a percent.

Incubates #1 and #2, without kininase inhibitor, never had kinin activity after 2 hours' incubation in the absence of glass (sample B).

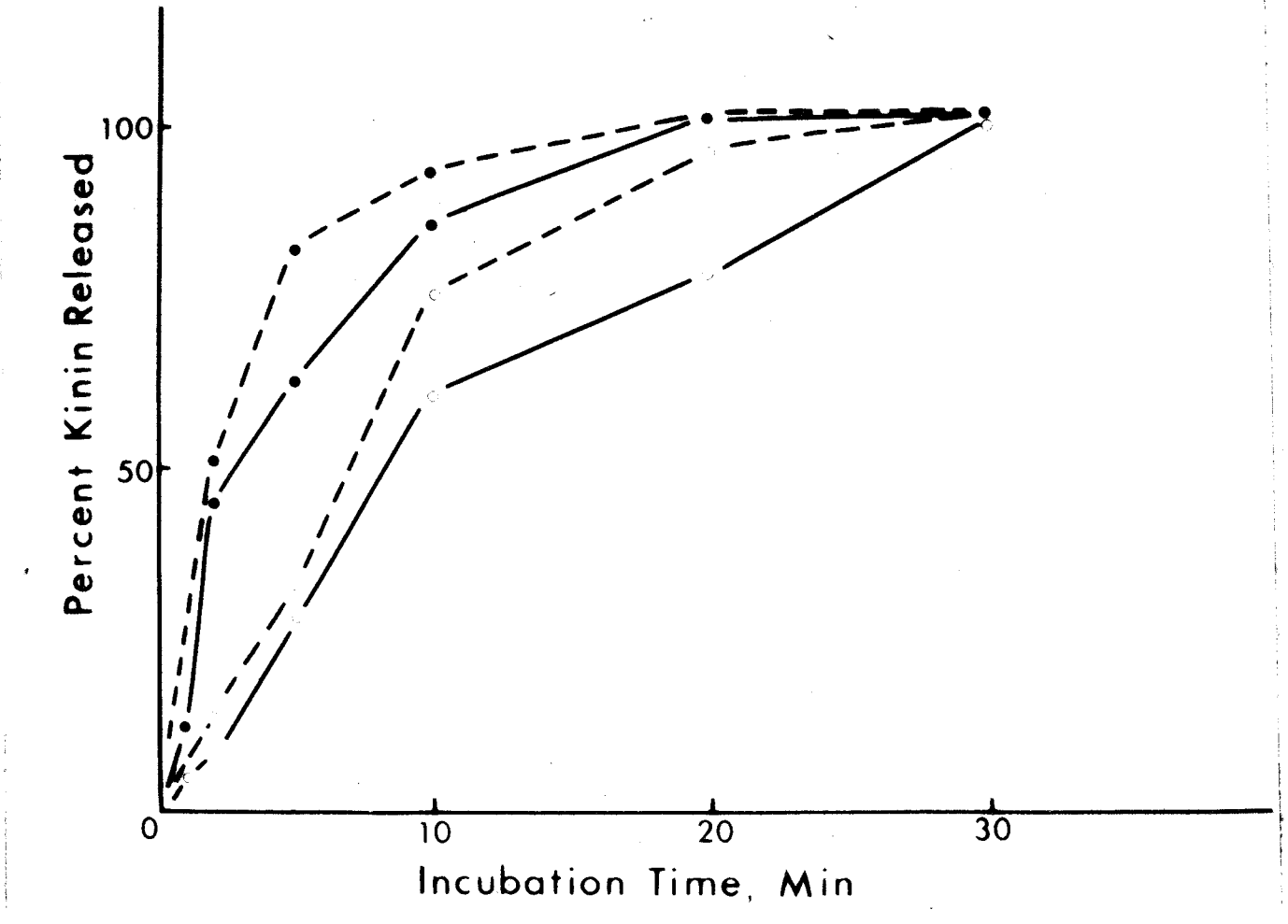
10 minutes' incubation with 100 cm^2 glass/ml plasma, or 20 minutes' incubation with 20 cm^2/ml was sufficient to degrade virtually all Substrate 1. 90% of this substrate was depleted by 5 minutes' incubation with 100 cm^2, or 10 minutes with 50 cm^2 glass/ml. Turtle plasma treated in this fashion, if similar to human plasma, should still contain large amounts of pre-kallikrein, capable of activation by addition of fresh glass surface.

It is apparent from Figure 12 that there is little difference in rates of kininogen depletion in the presence or absence of 8-$HQSO_4$. This suggests that the amounts of kininase inhibitor used throughout this work did not significantly modify the kinetics of turtle kinin release.

All three curves for kininogen depletion are the inverse of the corresponding curves for kinin production. Figure 13

Figure 13. Effect of a 2 hour incubation of samples after removal of glass on the proportion of kinin released from turtle plasma by 100 cm^2 (solid dots) and 50 cm^2 (hollow dots)/ml plasma. Solid lines indicate samples which were inactivated immediately, and dashed lines indicate those samples which were incubated for 2 hours at 20°C after the removal of glass. Details are given in the text, p 68, 70.

Figure 13


Percent Kinin Released
100
50
0
10
20
30
Incubation Time, Min

compares the proportion of kinin produced by the two methods, at different time intervals. Solid lines are based on information presented in Figure 10. Data for percent Substrate 1 depleted in Figure 12 were translated into kinin produced, and are connected by dashed lines in Figure 13. The control incubate in Figure 12 (with 8-$HQSO_4$) was not included. It is clear that those samples incubated for 2 hours after removal of glass (dashed lines) contained only slightly more activity than those which were boiled immediately upon removal of the sample (solid lines). This implies a rapid inactivation of plasma kallikrein; otherwise a much larger portion of the kininogen would be degraded after the removal of glass, by kallikrein released in the initial incubation.

2. Activation of plasma kallikrein in turtle and rat plasma. The purpose of this experiment was to compare relative amounts, and rates of activation, of prekallikrein in turtle and mammalian (rat) plasma. Glass activation of prekallikrein preparations was followed by periodically removing samples and reacting them with a standard amount of substrate. It was assumed that the amount of kinin thus produced would be proportional to the amount of active plasma kallikrein added to the substrate.

Method. The prekallikrein preparation used here was Substrate 1

depleted plasma; it was prepared as described in the previous experiment. Turtle plasma was depleted for 5 and 10 minutes with 100 cm^2 glass/ml, and rat plasma was exposed to 100 cm^2 glass/ml for 10 minutes only. The amount of Substrate 1 remaining in these preparations after depletion was determined by the procedure outlined on page 66 (samples C and D).

A standard substrate was prepared by diluting intact turtle or rat plasma 1:2 with the appropriate saline, which also contained 1 mg 8-$HQSO_4$/ml plasma. At T = 0, 10 ml of each prekallikrein preparation was activated in a 50 ml polypropylene centrifuge tube with 16 g glass beads (200 cm^2/ml). At the desired time intervals 0.5 ml samples were withdrawn and rapidly injected into 1.5 ml of the substrate preparation, in 5 ml plastic test tubes. These mixtures were incubated at 20°C for 60 minutes to ensure completion of the reaction, then sampled and assayed in the usual manner (Chapter 2).

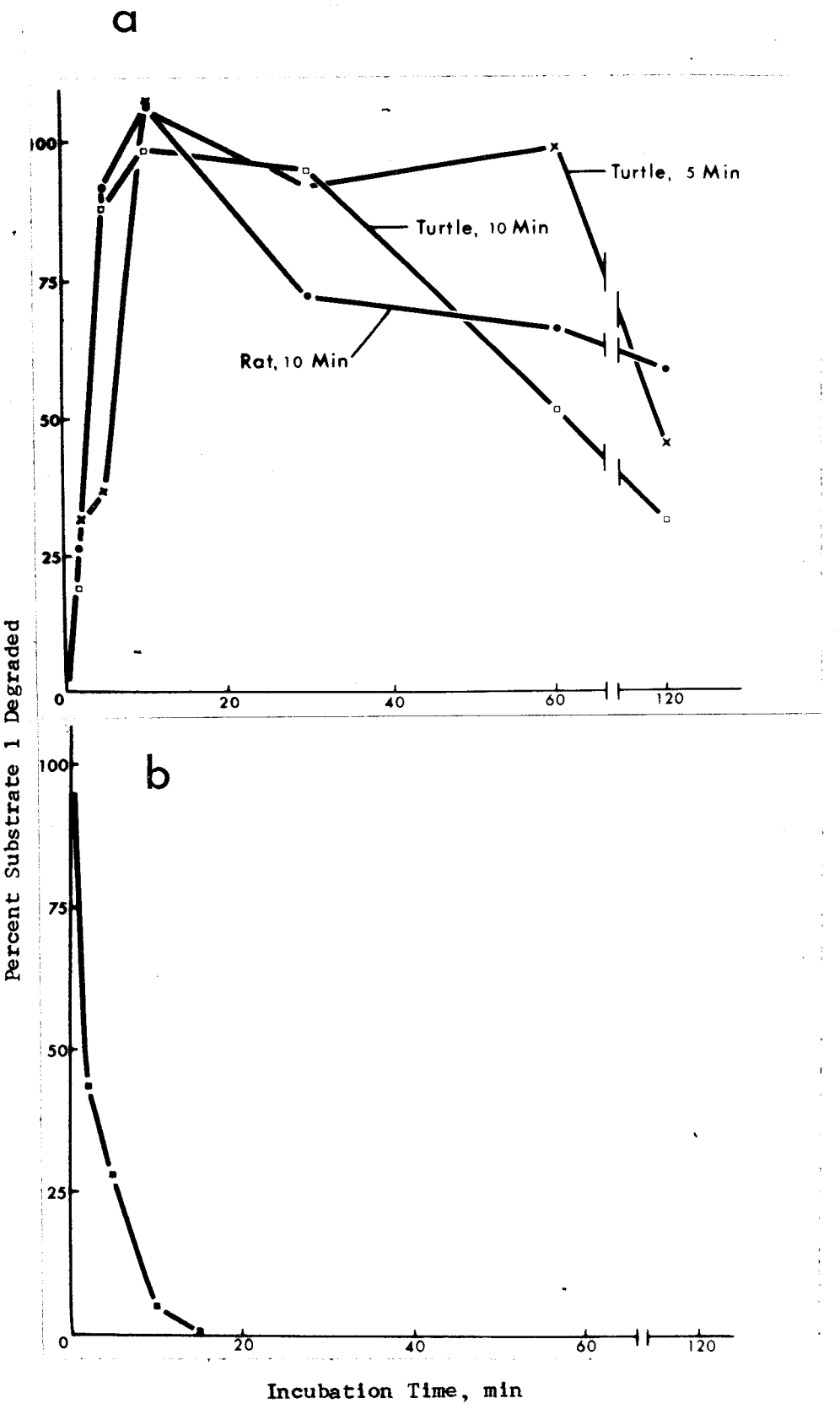
The maximum yield of the substrate preparations was determined in each case by a separate incubation with glass and kininase inhibitor, for 60 minutes, at 20°C. In cases where the prekallikrein preparation itself contained residual Substrate 1 (up to 10% in 5 minute-depleted turtle plasma) the maximum yield of the substrate was increased to include this amount.

Results. Data from 8 experiments are given in Figure 14 a

Figure 14 a. The release of active kallikrein from rat and turtle prekallikrein preparations activated with 200 cm^2 glass surface/ml plasma. Results are expressed as percent Substrate 1 in the standard substrate preparations degraded by aliquots of the activated kallikrein at different incubation times. Details of the method are given on page 71. Values for rat and turtle (5 min) are means of 2 experiments, and values for turtle, 10 min, are means of 4 experiments.

b. Decay of active turtle kallikrein. Kallikrein was released from a prekallikrein preparation by activation with 200 cm^2 glass/ml plasma for 10 minutes. Disappearance of activity after removal of the glass was followed by reacting samples with standard amounts of substrate. Values are means for two experiments.

Figure 14


a
b
Turtle, 5 Min
Turtle, 10 Min
Rat, 10 Min
100
75
50
25
0
20
40
60
120
Percent Substrate 1 Degraded
Incubation Time, min

expressed as the percent of the theoretical maximum activity released from the substrate by each of the activated samples. Controls with non-activated prekallikrein and substrate plasma did not contain kinin activity.

Margolis (1958) and Briseid et al (1968) found that human plasma contained prekallikrein far in excess of the amount required to degrade all kininogen present at any time. It is evident from Figure 14 a that this situation also exists in both rat and turtle plasma. Activation by 200 cm^2 glass/ml plasma rapidly released kallikrein, and at 10 minutes the concentration was sufficient to degrade all Substrate 1 in the substrate samples. This level or greater was maintained for about 40 minutes, after which there was a relatively slow decrease in kallikrein activity. After 120 minutes there was still enough enzyme present to release about half the available kinin. The fact that prekallikrein was present in excess is underlined by the following facts:

a) Part of the supply had already been used in depleting Substrate 1 in the prekallikrein preparation (see p 66);

b) In any test incubation the substrate preparation was derived from three times as much plasma as the kallikrein used to activate it;

c) High concentrations of active kallikrein existed despite simultaneous inactivation.

Mammalian plasma has more than one type of kallikrein held in an inactive form (Schachter, 1969). If turtle plasma is similar, it is possible that the kallikrein activity assayed in the above experiments is due to more than one enzyme, and that the relative proportion of these enzymes changes with continued activation. For example, it cannot be ruled out that the kallikrein responsible for kinin release at 120 minutes might be entirely different from that which causes the rapid kinin production when glass beads are added to fresh plasma (e.g., Figure 10).

3. Decay of plasma kallikrein. According to Schachter (1969), the plasma or serum of all vertebrates tested to date, as well as the hemolymph of some invertebrates, has the ability to inactivate or inhibit all varieties of kallikrein. There are at least two of these inactivators in mammalian plasma, one of which may be an enzyme (Werle and Schmal, 1968). The efficiency of these inhibitors is obviously an important variable in a kallikrein-kinin system, since the concentration of kallikrein in the plasma at any time will depend on the rates of activation and des truction of the enzyme. It was the object of this experiment to characterize the decay of turtle plasma kallikrein, in the absence of simultaneous production of the enzyme.

Method. Substrate 1 depleted plasma was prepared as a source

or prekallikrein, free of extra kininogen. 5 ml were re-activated with 8 g glass beads (200 cm^2/ml plasma) for 10 minutes. This ensured that sufficient kallikrein would be released to produce maximum amounts of kinin from the substrate (according to Figure 14 a).

At zero time, 4 ml of the supernatant plasma were removed in a 5 ml plastic syringeand placed in a 50 ml polypropylene centrifuge tube. At the desired time intervals 0.5 ml samples were withdrawn from this mixture in sterile 1 ml syringes and added to 1.5 ml samples of substrate (as in experiment 2). After one hour these mixtures were sampled and assayed. Maximum values for kininogen were determined in a separate incubation. Results. Means for two experiments on the plasma of different turtles are shown in Figure 14 b. Enough kallikrein was present initially to degrade all its substrate. Decay was rapid in the absence of glass; after 15 minutes at 20°C , the activity was so reduced that no kinin was released from the substrate. This rapid inhibition is of the same order as that described for human plasma (Margolis, 1958).

The potency of these inactivators in turtle plasma, combined with the rapid liberation of plasma kallikrein (compared in Figures 14 a & b) suggests that the conversion of prekallikrein to kallikrein is very rapid indeed, and that amounts of prekallikrein may be even greater than the previous experiment seemed to suggest.

D. Effect of Temperature on Kinin Production

Many poikilotherm enzymes have been found to possess, at low temperatures, different kinetics than their mammalian counterparts. It seemed possible that at low temperatures kinin production in the turtle, a poikilotherm, might be more efficient than in the rat, a homeotherm. Kinin production in plasma from these animals was therefore tested at three different temperatures; 37°C, a normal mammalian temperature; 20°C, a normal temperature for this turtle (Carr, 1952); and 5°C, a temperature also encountered by the turtle.

Method. 5 ml of plasma from each animal was diluted 1:2 with saline (Appendix B) containing 1 mg 8-$HQSO_4$/ml undiluted plasma. This mixture was placed in 50 ml polypropylene test tubes in a water bath previously equilibrated at 5, 20 or 37°C. At time zero 6 g glass beads (50 cm^2/ml) were added to each tube and subjected to continuous shaking. At the desired intervals, samples were withdrawn, inactivated by boiling, and stored for assay.

Control incubates containing a) no glass, and b) no inhibitor, were included for both plasmas in each experiment.

Results. Absolute values for kinin production in rat and turtle plasma are given in Table 4. Rat plasma usually produced higher concentrations of plasma kinin. Therefore, to allow direct comparison of the kinetics of kinin release, data are

Table 4. Kinin production in rat and turtle plasma at different temperatures, by 50 cm^2 glass/ml plasma.

Temperature	Species	Activity (ug/ml plasma, BK-eq) at different sampling times (min)						
		0	1	2	5	10	30	60
37°C	Rat(2)*	0.01	0.7	1.5	1.7	1.7	1.6	1.6
	Turtle(2)	0.01	0.5	1.2	1.3	1.3	1.2	1.2
20°C	Rat(2)	0.01		0.3	0.8	1.2	1.5	1.5
	Turtle(3)	0.01	0.05	0.1	0.3	0.7	1.0	1.0
5°C	Rat(2)	0.01	0.01	0.01	0.07	0.4	1.2	1.3
	Turtle(3)	0.01	0.01	0.01	0.06	0.4	1.7	1.6

*... values in parentheses indicate the number of animals used in different experiments to obtain the mean values presented in this table.

expressed in Figure 15 as percent of the maximum amount of kinin that could be released. Comparison of these curves does not indicate that there was a difference in the relative rates at which kinin was produced in rat and turtle plasma, at any of the temperatures.

Maximum velocities of kinin production at each temperature were calculated from Figure 15, using data from Table 4. The Q_{10} values for rat and turtle kinin production did not differ, being 1.9 over the range 20 to 37°C, and 1.3 from 5 to 20°C. The former value is in good agreement with Q_{10} values calculated by Margolis (1966) for human plasma over the same range. I could find no published values for Q_{10} of kinin production rate in the 5 to 20°C range.

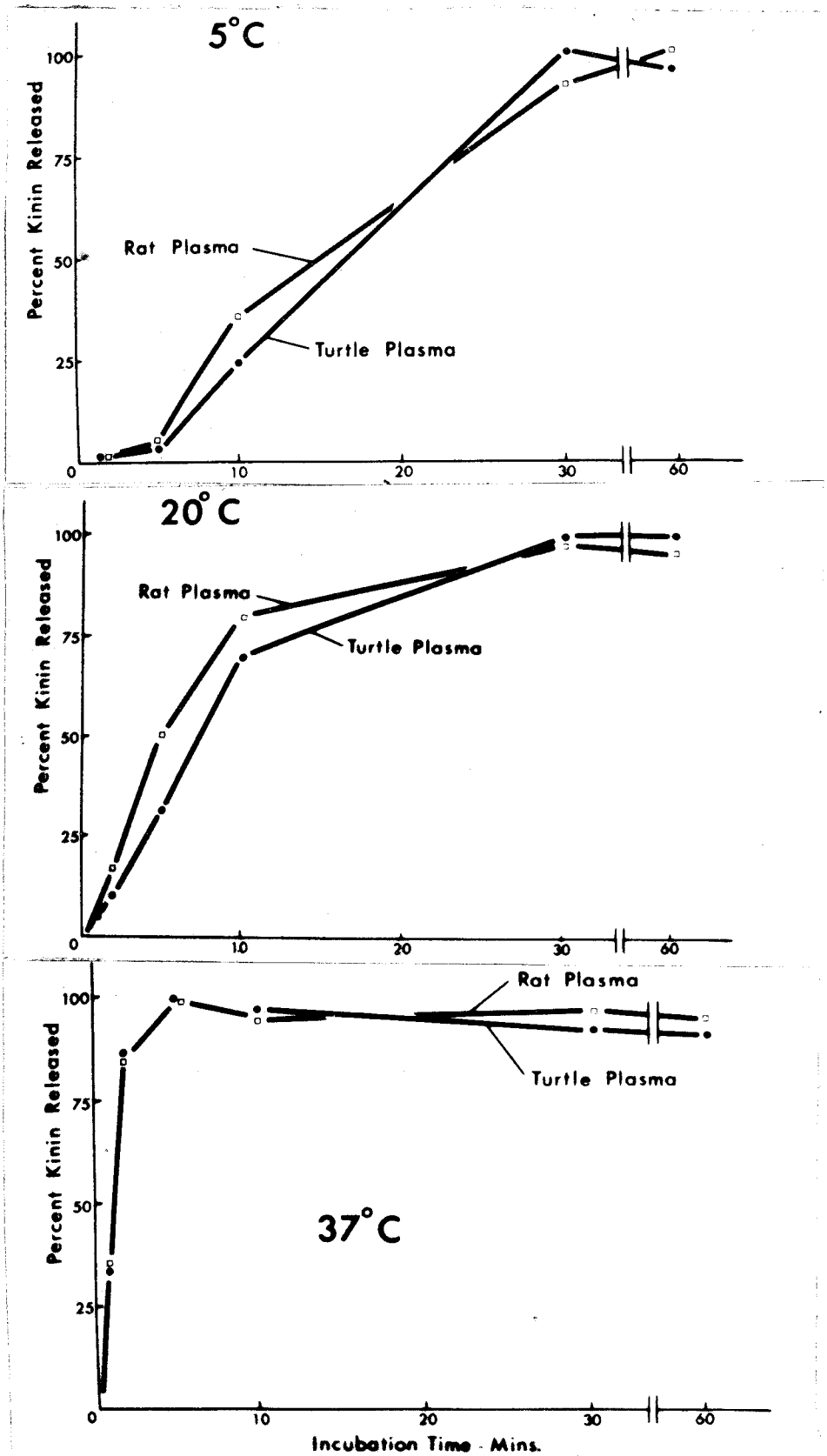
Kinin production in both plasmas had a definite lag at 5°C; no kinin appeared until at least 5 minutes of incubation, after which there was a relatively rapid release of kinin. This was reflected in the low Q_{10} for maximum rate of kinin release at low temperatures. This lag, or transient phase, is a predictable characteristic of a damped cascade enzymic process (Hemker and Hemker, 1969), and further supports the hypothesis that the kallikrein-kinin system of both turtle and mammalian plasma may fit this model.

E. Kininogens in Turtle Plasma

It has been mentioned (Chapter 1) that there are at least

Figure 15. Effect of temperature on glass triggered kinin production in rat and turtle plasma. Both plasmas were tested at 5, 20, and 37°C. Kinin released at a particular sampling time is expressed as percent of the maximum possible, based on data given in Table 4. Values are means of 2 or 3 experiments, as indicated in Table 4.

Figure 15


5°C
Percent Kinin Released
100
75
50
25
0
Rat Plasma
Turtle Plasma
10
20
30
60
20° C
Percent Kinin Released
100
75
50
25
0
Rat Plasma
Turtle Plasma
10
20
30
60
Rat Plasma
Turtle Plasma
Percent Kinin Released
100
75
50
25
0
37°C
10
20
30
60
Incubation Time - Mins.

two kininogens in mammalian plasma, and that the relative efficiency of a wide number of kallikreins and kininogenases in releasing kinins from these precursors is a complex topic, currently a subject for much research (Hamberg, 1969). Since it was the aim of this study to characterize kinin release by the entire kallikrein-kinin system, an exhaustive study of the kininogen complex per se in turtle plasma was not carried out. The experiments with trypsin and glass surface activation described in this section were conducted to determine the proportion of the total kininogen which is available to surface activated endogenous enzymes, and to establish more clearly whether the different amounts of kinin released by trypsin and glass (Table 3) were due to different kininogens.

1. Effect of trypsin concentration on turtle kinin release in intact plasma. Low concentrations of trypsin (0.1 mg/ml plasma) release kinin from human plasma in an initial rapid phase, followed by a period of slower production. Margolis and Bishop (1963) have taken this phenomenon to indicate the presence of two kininogen moieties, one a better substrate for trypsin than the other. Therefore, experiments similar to those of Margolis and Bishop were conducted with turtle plasma, and the results compared to this data for human plasma.

Method. Plasma was incubated at 37°C with saline, 8-$HQSO_4$ and

trypsin, in the following proportions:

	Trypsin	Plasma	Saline (pH = 7.4)	8-$HQSO_4$
1.	2 mg	2 ml	4 ml	2 mg
2.	0.2 mg	2 ml	4 ml	2 mg

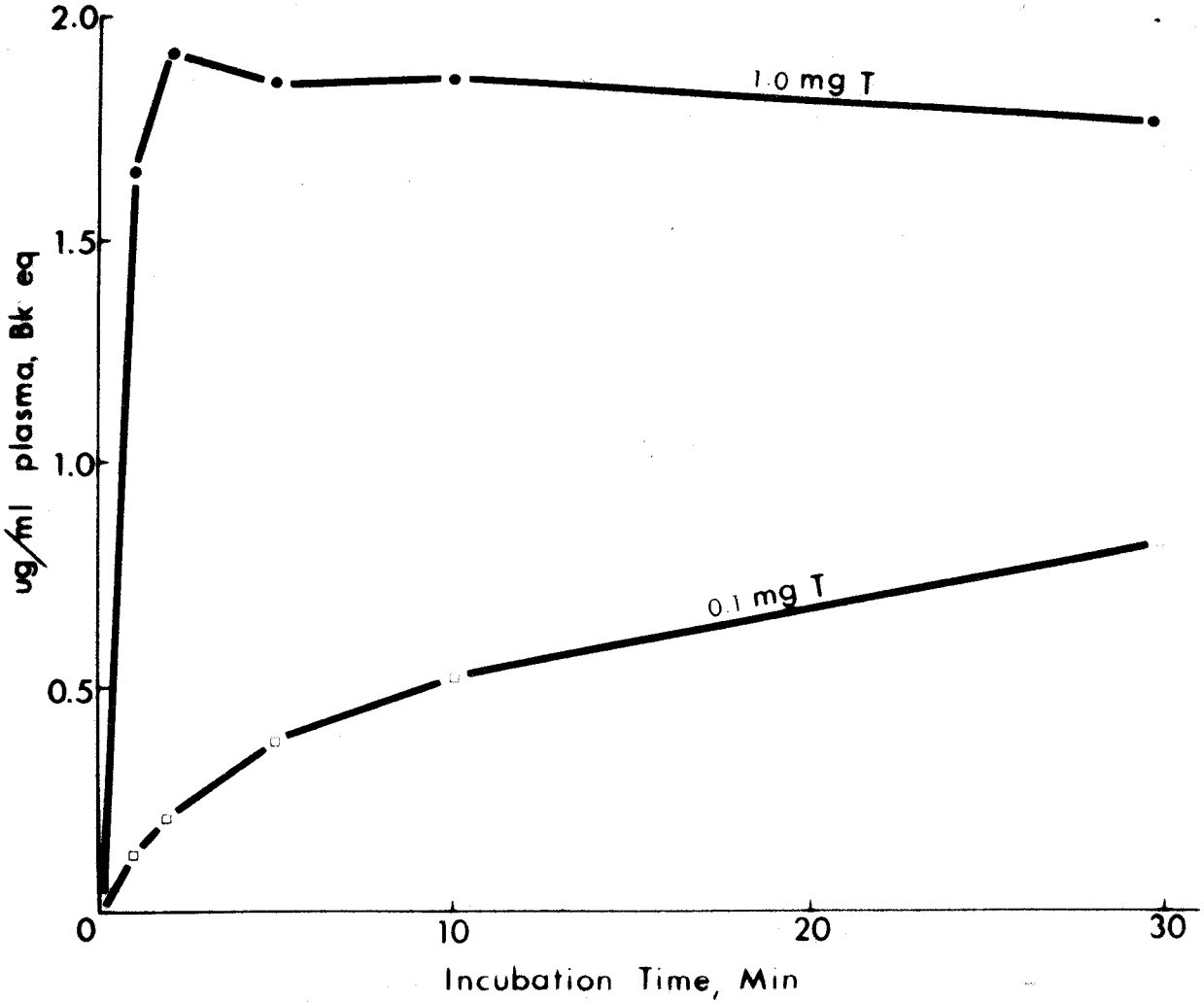
At appropriate times samples were removed from each tube, boiled and stored for assay (Chapter 2). The experiment was repeated on plasma from three turtles, and controls containing a) no kininase inhibitor, or b) no plasma, or c) no trypsin, but otherwise similar to the test incubates, were included in each experiment.

Results. None of the controls contained biological activity as measured on the isolated rat uterus. Figure 16 shows kinin production in the experimental incubates, with the two concentrations of trypsin. Each point is a mean, based on three values. The results are similar to those obtained for acid-treated human plasma by Margolis and Bishop (1963). 0.1 mg trypsin released kinin much more slowly than did 1.0 mg trypsin/ml.

The curve for turtle kinin production by 0.1 mg trypsin/ml plasma appears to be relatively smooth, and does not show any sharp inflection point, as found by Margolis and Bishop. Nonetheless, the initial rapid rate of production followed by later slower kinin release (after 10 minutes), might be interpreted as support for the two kininogen hypothesis.

Figure 16. Comparison of rate and yield of kinin production in unheated plasma by two concentrations of trypsin; 1.0 and 0.1 mg/ml undiluted plasma. Values are means for three experiments.

Figure 16

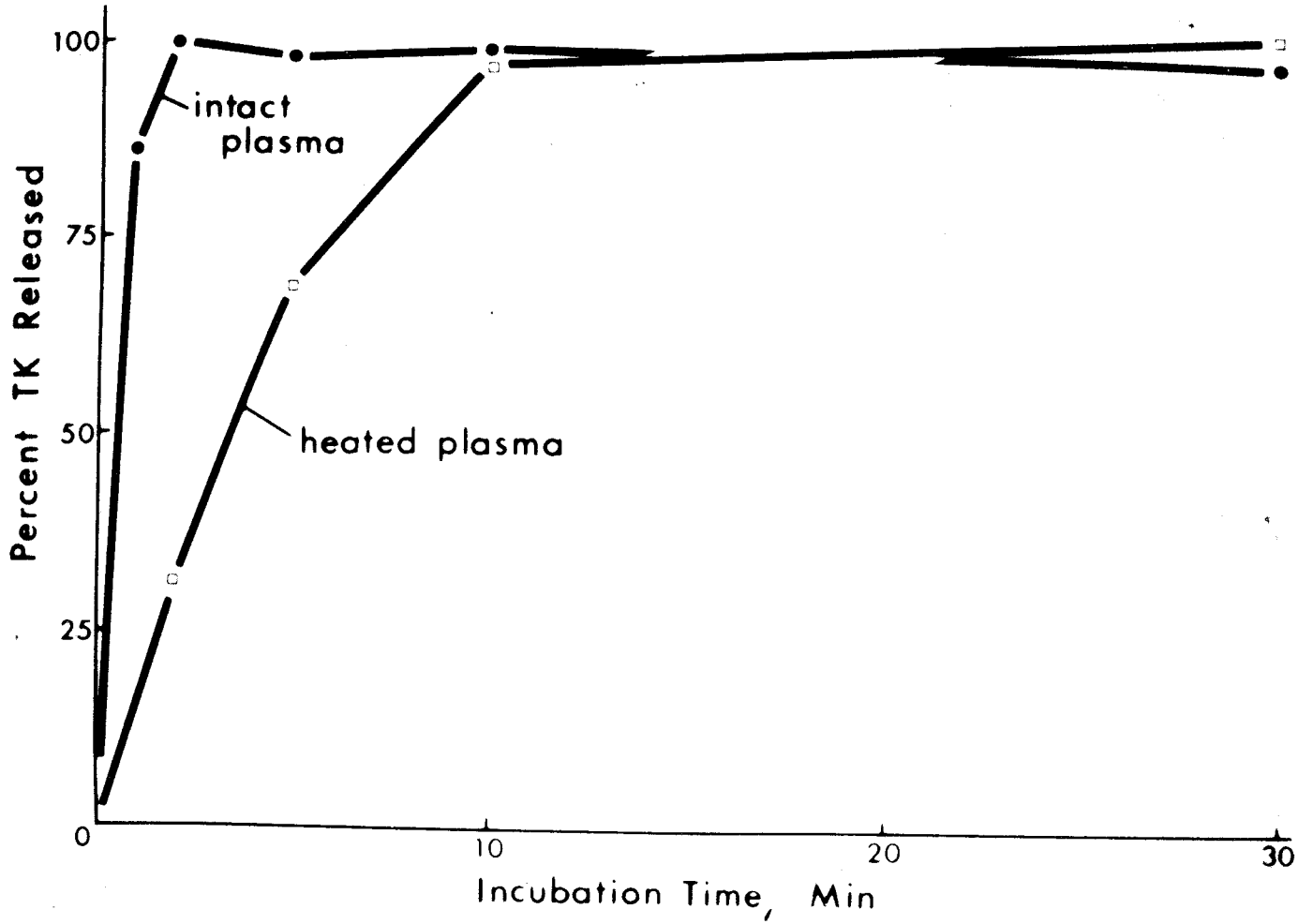

2.0
1.5
1.0
0.5
0
ug/ml plasma, Bk eq
1.0 mg T
0.1 mg T
10
20
30
Incubation Time, Min

According to Figure 2, trypsin activates plasma kallikrein in intact plasma (Webster, 1968), in addition to releasing kinin directly from kininogen. In heated plasma, on the other hand, all enzymic components have been inactivated, and trypsin acts only on kininogen. Therefore, if turtle plasma is similar, equivalent concentrations of trypsin should release kinin more rapidly from intact than from heated plasma. Figure 17 compares rates of kinin release from heated and unheated turtle plasma by 1.0 mg trypsin/ml plasma. Values for heated plasma were taken from Figure 3 a, and those for unheated plasma from Figure 16. In order to aid in comparison all data are expressed as percent of the maximum kinin released. Six different animals were involved. It is clear that turtle kinin was released more rapidly from intact plasma. The results in this Figure therefore suggest that turtle plasma may be similar to mammalian plasma in that trypsin converts prekallikrein to kallikrein in unheated plasma.

2. Effect of trypsin on Substrate 1-depleted plasma. Although previous experiments had established that trypsin always released more turtle kinin than did glass surface from a given sample of turtle plasma, the two kininogen hypothesis could be tested more directly by determining whether plasma depleted of substrate for glass activated kallikreins could still release turtle kinin to trypsin. The experiment described

Figure 17. Comparison of rate and yield of kinin production in unheated and heated (to 65°C) turtle plasma, by 1.0 mg trypsin/ml undiluted plasma. Values are means for three experiments.

Figure 17


100
75
50
25
0
Percent TK Released
intact
plasma
heated plasma
10
20
30
Incubation Time, Min

here was designed to carry out this test, and to compared the response of turtle plasma directly to that of rat plasma.

Method. In each experiment every sample of plasma was treated in 6 different ways:

1. Maximum kinin released to trypsin. This was determined by incubating 2 ml plasma + 4 ml saline + 2 mg 8-$HQSO_4$ + 2 mg trypsin in siliconed glass test tubes at 37°C. At 30 minutes, these were sampled and assayed in the usual manner.

2. Maximum kinin released by glass. 2 ml plasma were incubated with 4 ml saline + 2 mg 8-$HQSO_4$ and shaken at 20°C with 2.4 g glass beads (50 cm^2/ml plasma). After 50 minutes these tubes were sampled and assayed.

3. Kinin released by trypsin from glass-depleted plasma.

a) 2 ml plasma was diluted with 4 ml saline and shaken at 20°C with 2.4 g glass beads for 50 minutes. No 8-$HQSO_4$ was added.

b) 4 ml of the mixture obtained from #3 a was placed in a siliconed glass tube and incubated with 1.3 mg 8-$HQSO_4$ and 1.3 mg trypsin, at 37°C. After 30 minutes this incubate was sampled, and assayed.

4. Kinin released by trypsin from trypsin-depleted plasma.

a) 2 ml plasma was diluted with 4 ml saline and reacted with 2 mg trypsin in a siliconed glass tube at 37°C. No 8-$HQSO_4$ was added.

b) After 30 minutes, 4 ml of the mixture from #4 a was placed in a fresh glass tube, and re-incubated at 37°C with 1.3 mg 8-$HQSO_4$ and 1.3 mg trypsin. After 30 minutes this tube was sampled and assayed.

5. Kinin released by glass from trypsin-depleted plasma. 4 ml of trypsin-depleted plasma preparaed as in #4 a was placed in a 50 ml polypropylene tube with 1.3 mg 8-$HQSO_4$ and 1.6 g glass beads and shaken at 20°C for 50 minutes, sampled and assayed.

6. Kinin released by glass from glass-depleted plasma. 4 ml of glass-depleted plasma, prepared as in #3 a was incubated with 1.3 mg 8-$HQSO_4$ and 1.6 g glass, as described in #5 b.

Results. Means for 7 experiments on turtle plasma and 5 experiments on rat plasma are given in Figure 18. The results are expressed as percent of maximum kinin released, based on the value of turtle kinin developed by trypsin (incubate #1, above) from any plasma. In both plasmas kinin released by glass (G-1) formed a relatively constant proportion of the total released by trypsin; about 75% in the rat, 65% in the turtle. Plasma depleted of substrate for glass-activated enzymes always released additional kinin when re-incubated with trypsin. This amount of kinin, about 29% in the rat, and 40% in the turtle, was approximately equal to the difference between relative amounts

Figure 18. Relative effects of trypsin and glass in releasing kinin from intact turtle and rat plasmas. Conditions of incubation are described in the text, p 85-86. Values for turtle and rat plasma are based on 7 and 5 experiments respectively.

T-1.....trypsin

G-1.....glass surface

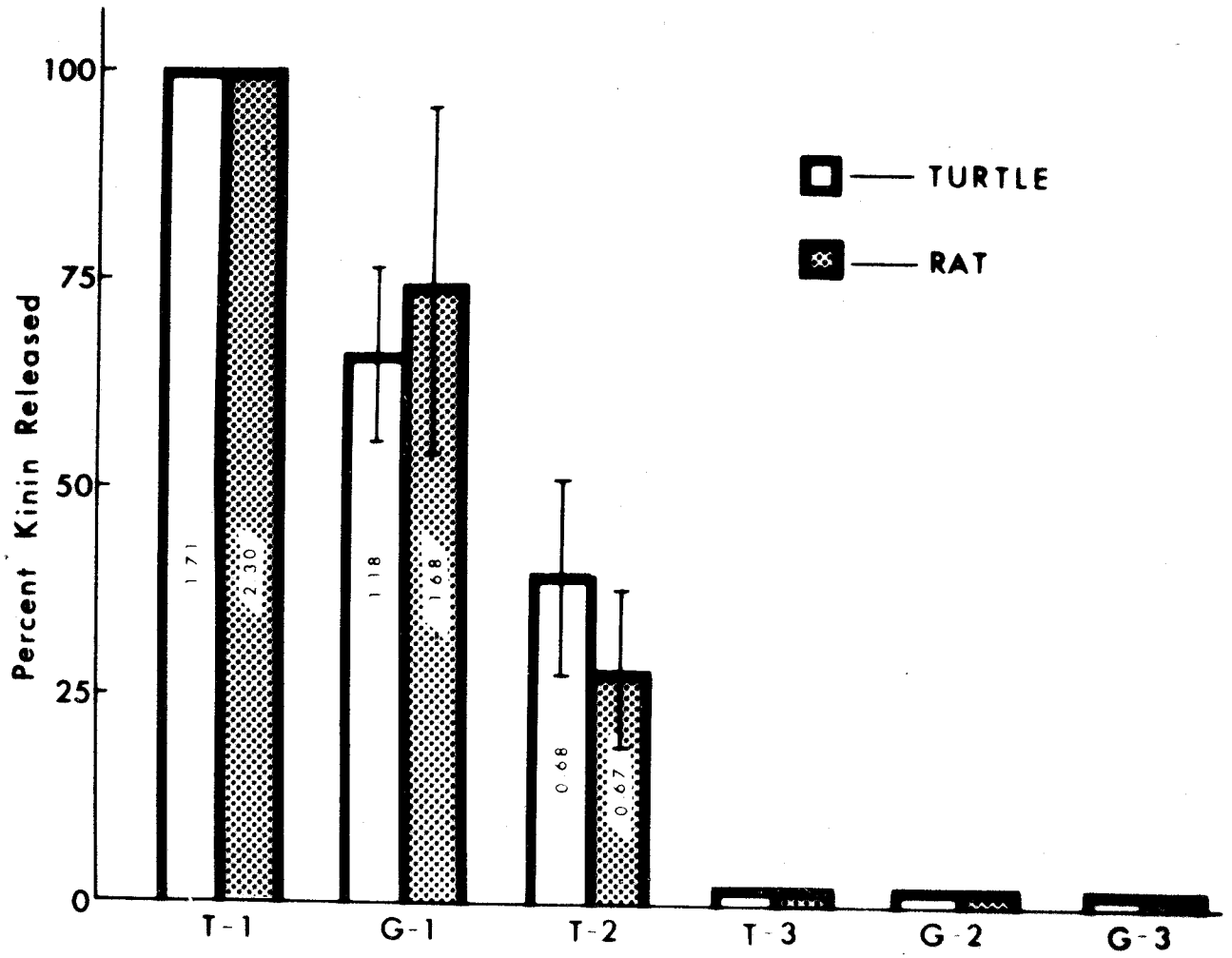
T-2.....glass surface followed by trypsin

T-3.....trypsin followed by trypsin

G-2.....trypsin followed by glass surface

G-3.....glass surface followed by glass surface

Figure 18


100
75
50
25
0
Percent Kinin Released
TURTLE
RAT
1 7 1
2 3 0
1 1 8
1 6 8
0 6 8
0 6 7
T-1
G-1
T-2
T-3
G-2
G-3

of trypsin and glass released kinin (column T-1 minus column G-1). Although there were no significant differences in these proportions between rat and turtle plasma, it is possible that a larger sample size might indicate that turtle plasma releases relatively less kinin to glass than rat plasma. In both plasmas the residual kininogen (column T-2) was significantly less than the amount degraded by glass activated enzymes (column G-1).

On the other hand, glass depleted plasma developed an insignificant amount of extra kinin (column G-2) when re-incubated with fresh glass. Also, trypsin depleted plasma did not yield significant kinin activity when re-incubated with glass (column G-3) or with fresh trypsin (column T-3).

These facts suggest that in both plasmas some kinin may exist in a precursor linkage which is not available to glass activated enzymes; this appears to support the hypothesis that turtle plasma, like mammalian plasma, contains at least two molecular species of kininogen.

However, it is not possible on the basis of these experiments alone to eliminate an alternative hypothesis to explain the partial exhaustion of kininogen by glass; i.e., that some factor essential to the reaction may exist in a limited supply, and disappear rapidly on activation with glass. However, this is not the case for mammalian plasma

(Margolis and Bishop, 1963).

F. Discussion and Conclusions

The significance of each of the experiments described in this chapter has been discussed individually, and the general conclusions will be summarized in Chapter 7. Briefly, turtle plasma was tested qualitatively against a model of the mammalian kallikrein-kinin system as shown in Figure 2. None of these direct or indirect tests suggested that the model was not equally applicable to turtle plasma. Certain other aspects of the system, such as the amount of prekallikrein, the kinetics of the inactivation of kallikrein, the response of reaction rate to temperature, and the nature of the kininogen complex were compared directly to mammalian plasma, and no qualitative differences were noted.

It may be concluded that turtle plasma, in contrast to snake and bird plasma, has a fully developed kallikrein-kinin system capable of producing up to 3.0 ug/ml plasma, bradykinin-equivalent, a level comparable to some mammals, and which has the potential for endogenous activation.

In further work, more detailed studies of the various reactions investigated, with the use of purified enzymes and substrates, would be the most fruitful approach. Nonetheless, the basic work presented here must be extended to a wide variety of other species before any generalizations can be made.

CHAPTER 5. CHEMICAL AND PHARMACOLOGICAL TESTS ON PURIFIED TURTLE KININ

A. Purification of Glass Released Turtle Kinin

The experiments described in Chapter 4 indicated that the endogenous enzymic mechanisms for release of turtle kinin were closely similar to those of mammals. However, this did not imply that the kinins produced were identical, and therefore purification of the glass released factor was carried out, with a view to conducting pharmacological and chemical tests on the peptide. This method of release was chosen since the kinin is produced by endogenous enzymes, rather than by artificially added enzymes.

A wide variety of methods have been employed to release, extract and purify mammalian plasma kinins. These were summarized by Erdos (1966) and by Schachter (1969). Many analyses of purified kinins have been made, but to date there has been no amino acid sequence or analysis of glass released kinin (Schachter, 1969). This seems to be a strange ommission, in view of the generally accepted opinion that this method of release may parallel physiological release of these hypotensive peptides.

1. Method. In 1967 Brocklehurst and Zeitlin published a method for the determination of low levels of free kinin in

small blood samples. Recovery of artifically added bradykinin was close to 80% in trial experiments. The procedure also had the advantage of excluding a wide variety of interfering substances such as adrenaline, histamine, oxytocin, and 5-hydroxytryptamine. This method is presented in its original form in Appendix C. A number of preliminary purifications of turtle kinin were carried out, according to this method, using small amounts of plasma (less than 200 ml). These resulted in the adoption of a number of modifications of the butanol extraction technique, mainly to accomodate the larger amounts of plasma required. The exact procedure followed during the final purification is presented below; it combines features of the methods described by Brocklehurst and Zeitlin (1967) and Zacest and Mashford (1967), and is similar to that of Habermann (1966).

Through all extraction and purification procedures, kinin activity was followed by means of the isolated rat uterus bioassay.

a. Release of kinin. 900 ml of plasma was obtained from 30 turtles by the standard method (Chapter 2) and stored not more than 18 hours at $0^{o}C$ in siliconed 2 L beakers covered with parafilm. Prior to activation, the plasma was diluted with 900 ml turtle saline (Appendix B) which contained 900 mg 8-$HQSO_4$ (1 mg/ml undiluted plasma). 450 ml of this fluid were placed in each of 4 siliconed 1 L Erlenmeyer flasks and warmed

to 25°C on a magnetic stirrer (Fisher Flexa-Mix). 135 g of acid washed glass ballotini (25 cm^2/ml undiluted plasma) were added to each flask, and the entire mixture was stirred at a moderate speed, with a magnetic stirrer.

b. Extraction procedure. After 50 minutes of incubation the supernatant from each flask described above was poured into 900 ml of boiling 95% ethyl alcohol, in siliconed 2 L beakers. The glass beads remaining in the flask were twice washed with 100 ml of 80% ethyl alcohol, and the washings were added to the original supernatant.

The ethanol extract was boiled for 10 minutes, cooled, and filtered through Whatman #1 filter paper on a Buchner funnel, under water aspiration. The precipitate and filter paper were periodically washed with 100 ml 80% ethanol and discarded, and the washings were added to the filtrate.

The pooled, opalescent green filtrate, now about 2.5 liters, was reduced to about 50 ml in a rotary flash evaporator, at 40°C, under a reduced pressure of 0.2 - 0.5 mm Hg. The concentrated solution was acidified with 1.5 ml glacial acetic acid and twice extracted with 2 to 3 volumes of di-ethyl ether. This ether extract was reduced to dryness in a siliconed flask and stored at - 20°C for later estimation of kinin content. Previous trial experiments had indicated that under the above conditions little kinin is extracted by ether, though

many impurities are removed. These experiments, and the rationale for including this step in the method, are described in detail in Appendix E.

After ether extraction, the aqueous phase which contained the turtle kinin was reduced to dryness under vacuum, at 40°C. The dried sample was then taken up in 25 ml of warm (60°C) saturated NaCl solution, acidified to pH 1.5 with HCl. This solution was extracted at 60°C with 50, 25, and 25 ml of redistilled 1-N butanol in a 125 ml separating funnel. The pooled butanol extract was rapidly reduced to dryness in a flash evaporator, initially at 0.2 mm Hg and 40°C, and in the final stages, at 0.1 mm Hg and 80°C, in order to remove the last traces of butanol. The residue, which contained considerable NaCl, was stored dry at - 20°C until assay, and further purification. The presence of NaCl was a necessary condition, since the efficiency of the extraction in the absence of NaCl was very low (see Appendix F).

c. Column chromatography.

i. Gel filtration. The dry powder from the butanol extract was taken up in 16 ml of 0.02 M ammonium acetate buffer, pH = 5.0 (Appendix B). 0.3 ml was removed for bioassay, and for estimation of the total peptide content by the method of Lowry et al (1951). The remainder was subjected to gel filtration on a 90 x 2.5 cm Sephadex G-25 column built as described in Chapter 2. The buffer in the column above the gel was

allowed to sink to the level of the filter paper and the 16 ml of extract were applied to the column in a 20 ml plastic syringe through attached polyethylene tubing (Intramedic, 190). This fluid was also allowed to sink to the level of the filter paper. The column was then shut off and fresh buffer was added slowly to the top of the column, which was subsequently connected by plastic tubing to a 1 L reservoir flask containing 0.02 M buffer. Flow through the column was adjusted to 12-18 ml/hour, and the eluate was fed to an LKB fraction collector set at 5 ml/tube.

Fractions eluted after the void volume were tested for biological activity on the rat uterus, for "Lowry" peptide, and for ionic strength by conductivity measurements. The active fractions in the single peak obtained from this column were pooled, and a sample was removed from this pool for later assay.

ii. Ion exchange. The combined activity, now in 70 ml, was loaded at 6 ml/hour on to a 20 x 0.9 cm CM-Sephadex column (Chapter 2) in 0.02 M ammonium acetate buffer. An additional 50 ml of buffer were washed through the column, and collected, to be tested for possible biological activity. No kinin was detected, thereby ensuring that the turtle kinin had attached to the resin, while other impurities would have washed through. An ionic gradient to 1.0 M of the same buffer

was then initiated, and the eluate from the column was collected in 5 ml fractions. Biological activity was estimated on the rat uterus bioassay after neutrilization of the samples with small amounts of $NaHCO_3$. The ionic strength of the fractions was measured, and "Lowry" peptide was determined on that fraction which contained peak activity.

2. Results. A summary of the purification is given in Table 5. The increase in purification at each step was calculated from the specific activity, which was based on the total ug bradykinin-equivalent/total mg "Lowry" peptide. On this criterion, overall purification was 1475 fold over the original plasma concentration.

The elution pattern of turtle kinin on Sephadex G-25 is presented in Figure 19. The active material was clearly separated from a major protein peak, and was eluted just before the salt. All butanol extracts had considerable NaCl, since conditions of extraction required a saturated NaCl solution. This was not a disadvantage, since the salt peak served as a convenient marker to help locate kinin activity, and the G-25 column was an effective desalting mechanism.

Turtle kinin was also eluted from CM-Sephadex in a single peak, although one fraction contained a relatively high level of kinin (Figure 20), causing a "shoulder" before the main

Table 5. The purification of glass released turtle kinin.

Stage of purification	Total volume, ml.	Total peptide, mg "Lowry" peptide.[a]	Total rat uterus oxytocic activity, ug BK-eq.[b]	Recovery,%.	Specific activity, ug BK-eq/ mg "Lowry" peptide.	Factor of purification[c].
Original plasma	900	468.0	2490 $\pm$ 250	-	0.53	1
Butanol extract	16	60.0	531 $\pm$ 18	21.3	8.9	16.8
Sephadex G-25 pooled eluates	75	1.95	370 $\pm$ 40	69.7	189	357
CM-Sephadex pooled eluates	25	0.45	352 $\pm$ 26	94.5	782	1475

a...Peptide determined by the method of Lowry, et al (1951).

b...Oxytocic activity determined by the 4-point method of Holton (1948), with errors expressed as confidence limits, p = 0.05.

c...The factor of purification = specific activity of the preparation/specific activity of the original plasma.

Figure 19. Purification of glass-released turtle kinin on a 90 x 2.5 cm Sephadex G-25 gel filtration column, built in 0.02 M ammonium acetate buffer, pH = 5.0.

Figure 19

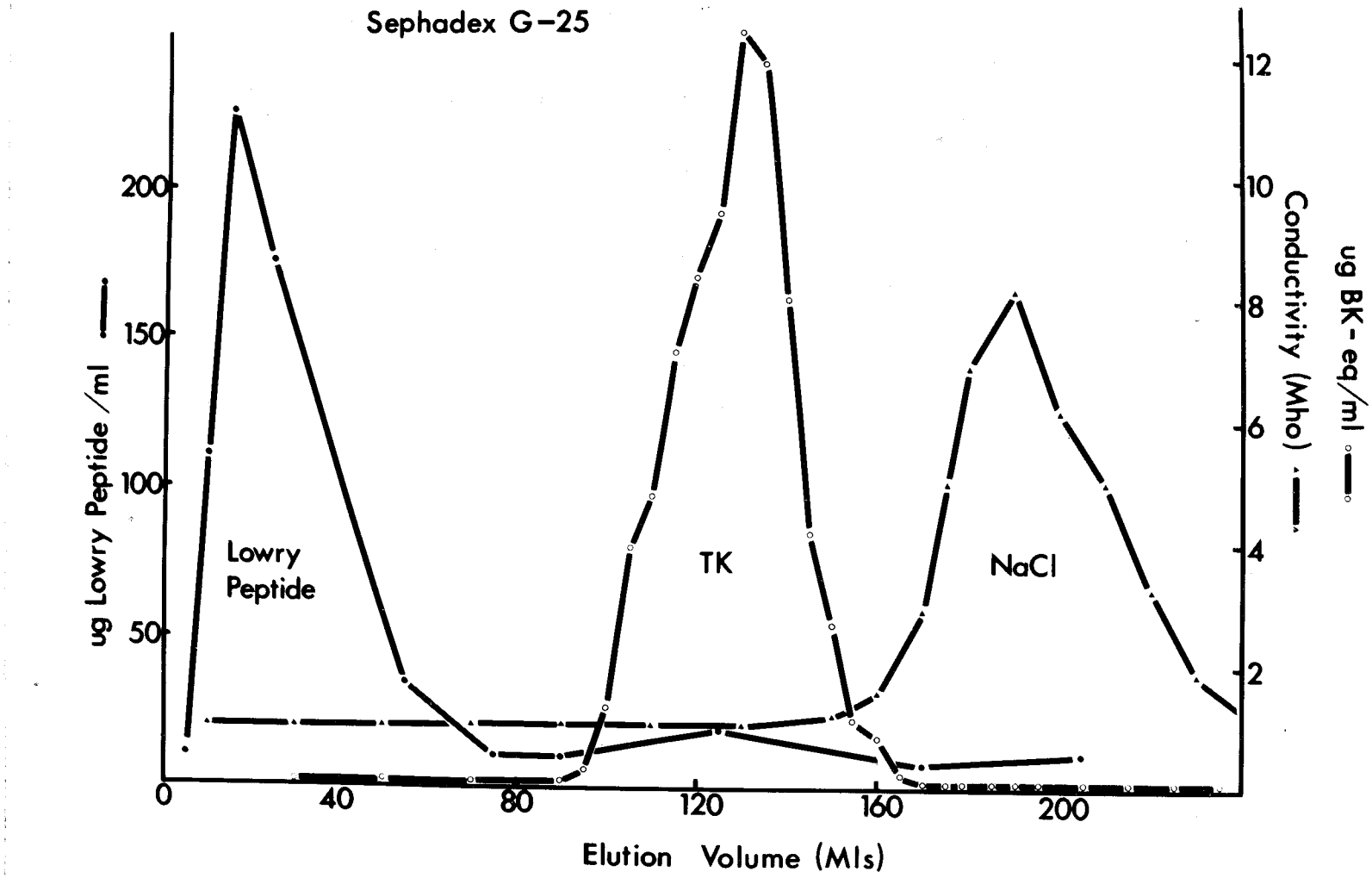
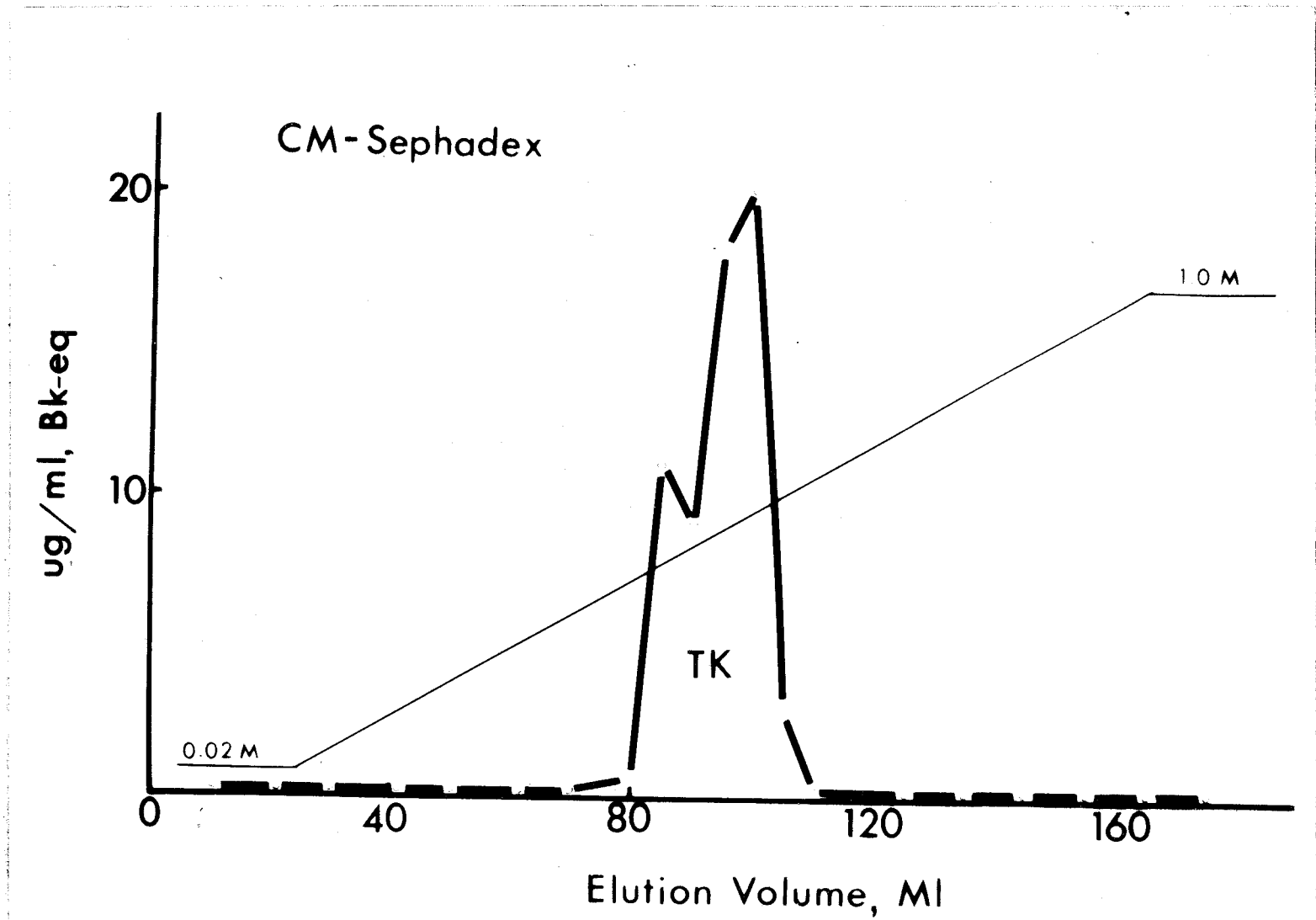

Sephadex G-25
ug Lowry Peptide /ml
200
150
100
50
0
40
80
120
160
200
Elution Volume (Mls)
Lowry Peptide
TK
NaCl
Conductivity (Mho)
ug BK - eq/ml
12
10
8
6
4
2

Figure 20. Purification of glass released turtle kinin on a 20 x 0.9 cm CM-Sephadex ion exchange column. The column was built in 0.02 M ammonium acetate buffer, pH = 5.0, and developed with a concentration gradient to 1.0 M ammonium acetate, pH = 5.0.

Figure 20


CM-Sephadex
20
10
ug/ml, Bk-eq
1.0 M
0.02 M
TK
0
40
80
120
160
Elution Volume, Ml

peak. The turtle kinin in this fraction was later tested for amino acid content (this chapter, p 125) and it did not differ from the kinin in the remainder of the peak.

3. Discussion and Conclusions. If turtle kinin is as active on the rat uterus as bradykinin, and if "Lowry" peptide is an exact measurement of peptide content, then a specific activity (rat uterus activity/mg "Lowry" peptide) of 1000 would indicate a pure preparation. While there is no basis for assuming that either of these conditions is strictly true, the experimental value for specific activity of the CM-Sephadex eluate, which was 782, suggests that the preparation was approaching purity. This observation was later corroborated by amino acid analysis (p 127).

The behaviour of the glass-released turtle kinin in column chromatography was very similar to that observed by Habermann (1966) for the glass released "contact" kinin of beef serum. His data gave no indication that more than one active peptide was present in the extract. As Habermann points out, this is not conclusive proof that only one peptide is released from kininogen by plasma kallikreins, since there is considerable evidence that both lys-bradykinin and met-lys-bradykinin are rapidly converted to bradykinin in intact plasma (Figure 1), and that serum kallikrein itself splits met-lys-bradykinin into the dipeptide met-lys, and bradykinin.

In any case, the method described in this section succeeded in providing about 350 ug bradykinin-equivalent of purified turtle kinin. This was ample for amino acid analysis and other chemical and pharmacological tests, and was derived from an amount of turtle plasma (900 ml) which was small compared with the volumes used by other workers in studies of mammalian plasma kinins (e.g., Elliott et al, 1961; Pierce and Webster, 1961).

B. Pharmacological Studies

The mammalian plasma kinins bradykinin, lys-bradykinin, and met-lys-bradykinin have qualitatively the same action in a wide variety of bioassays (Erdos, 1966). However, some of these assays will distinguish between these related peptides on a quantitative basis. Turtle kinin from crude extracts and purified eluates was therefore assayed against synthetic bradykinin in several bioassays which show qualitative differences between bradykinin and lys-bradykinin. These were the following; the contraction of the guinea pig ileum, the relaxation of the rat duodenum, and the depression of rat and rabbit blood pressure (Sturmer and Berde, 1963).

Edery and Grunfeld (1969) have shown that treatment of the rat uterus with chymotrypsin specifically sensitized the preparation to bradykinin, and to a few closely related peptides; therefore this bioassay was also used as a pharmacological test.

1. Methods.

a. General.

In all assays the bradykinin-equivalent of an active eluate was estimated by the statistical method of Holton (1949). The responses of the test preparation to high and low doses of the standard and unknown were matched in four-point groups. In each assay, 3 to 5 of these groups were obtained, and application of Holton's statistical method allowed 95% confidence limits to be assigned to the calculated value for potency. Where the amounts of unknown were not limiting (i.e., for the more sensitive bioassays), 5 groups were always used, to increase accuracy.

Concentrated turtle kinin at various degrees of purification was assayed. The butanol extracts (17 fold purification) and Sephadex G-25 eluates (357 fold purification) were from pilot purification attempts, involving relatively small amounts of kinin. CM-Sephadex eluates (1475 fold purification) were from the final purification described in this chapter.

All solutions to be tested were adjusted to pH 7.0 - 7.5 with small amounts of saturated NaOH. The concentration of the buffers containing the turtle kinin did not interfere with the rat uterus, guinea pig ileum, or rat duodenum bioassays, since these were all sufficiently sensitive to allow significant

dilution of the buffer. However, the ammonium acetate buffer containing the turtle kinin from CM-Sephadex columns was about 0.8 M, and frequently gave small pressor responses in blood pressure bioassays. This was overcome either by lyophilyzing the samples, or by placing the standard in an equimolar solution of the buffer.

b. Bioassays.

The rat uterus bioassay was carried out as described in Chapter 2. Other assays are briefly described below.

i. Rat uterus sensitized with chymotrypsin. In this assay a value was obtained for the potency of the unknown exactly as described for the rat uterus bioassay. The same uterus was then treated with 500 ug chymotrypsin for 60 seconds (Edery and Grunfeld, 1969). The assay was then repeated on this sensitized preparation.

ii. Relaxation of the rat duodenum. This assay was based on a description by Antonio (1968). Rats (Wistar strain) of either sex, weighing 200-250 g were sacrificed as described for the rat uterus bioassay. The abdomen was opened and the duodenum was removed, flushed with Tyrode's solution and placed in a 5 ml organ bath connected to a 2 liter reservoir in a thermally regulated water bath. The temperature for most assays was 37^{o}C. The supporting fluid throughout was Tyrode's solution as used by Antonio, which was composed as follows:

Chemical	Amount (g/L)	Chemical	Amount (g/L)
NaCl	8.00	$NaHCO_3$	1.00
KCl	0.20	$NaH_2PO_4 \cdot H_2O$	0.055
$CaCl_2$*	0.22	Glucose (Dextrose)	1.00
$MgCl_2 \cdot 6H_2O$	0.20		

*...Antonio showed that the concentration of Ca^{++} affected the sensitivity of the preparation, and that 1 mM Ca^{++}, or 0.22 g $CaCl_2$/L allowed the greatest relaxation response to bradykinin.

Both the organ bath and reservoir were bubbled with air. Responses were recorded exactly as described for the rat uterus assay, except that measurements were taken to the nearest 0.1 mm.

iii. Contraction of the guinea pig ileum. Adult male guinea pigs (500 g) were killed by a blow on the head, bled, and the ileum was removed through an abdominal incision. The tissue was flushed with Tyrode's solution, and a piece (5 cm in length) was suspended in the 5 ml organ bath. The remainder of the assay was carried out as described for the relaxation of the rat duodenum, except that the temperature of the bath was regulated between 30 and 35°C, depending on the spontaneity of the preparation. Lowering the assay temperature usually decreased spontaneous responses.

iv. Depression of rat blood pressure. The technique used in this assay was based on a method described by van Dyke et al (1955). Male Wistar rats (220-260 g) were anesthetized

in 45 to 60 minutes by subcutaneous injection of urethane (175 mg/100 g body weight). A small sagittal incision was made in the ventral surface of the neck. Using blunt dissection techniques, the jugular vein was located and freed of connective tissue. Two ligatures were placed around the vein, and the one distal to the heart tied. The vessel was punctured with a 22 gauge needle and cannulated with PE 10 tubing (Intramedic), which was tied in with the two ligatures. In the same manner, the carotid artery was dissected free of surrounding tissue and cannulated with PE 50 tubing.

1 ml of 50 mg% Heparin solution (in 0.9% NaCl) per 100 g body weight was immediately given intravenously, to prevent the formation of blood clots during the assay.

Injections of the active agents were given into the jugular vein by means of siliconed glass syringes, and washed through with 0.9% NaCl. The total volume of the injection never exceeded 0.2 ml. The depressor responses were measured in mm Hg from the carotid artery, which was connected to a Statham P 23AA pressure transducer coupled to a Beckman dynograph recorder.

Potency of the unknown sample was estimated by the usual four-point assay. In every assay control injections of 0.2 ml of the buffer (usually ammonium acetate adjusted to pH 7.0 with NaOH) supporting the unknown were given. Sensitivity to

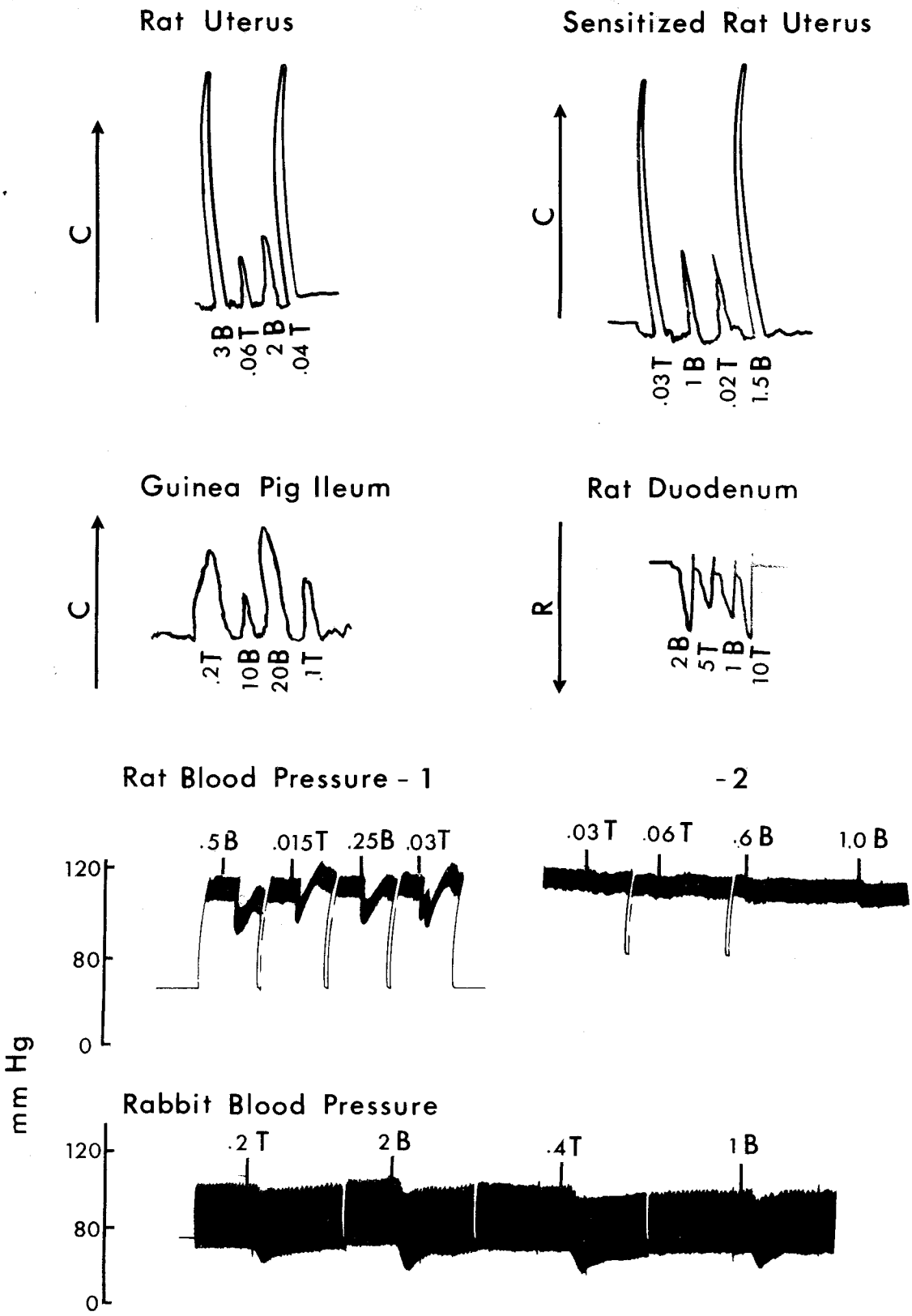
this vehicle varied between animals. Responses to kinins were regarded as valid when these control injections elicited no response in the same preparations, or when the kinins were placed in equimolar solutions of buffer.

It is standard procedure in this type of bioassay to treat the animal with an adrenergic blocking agent such as dibenzylene (van Dyke, et al, 1955) to stablilize blood pressure, and to prevent interference by changes in catecholamine levels. It has also been reported that this treatment potentiates some blood pressure responses to bradykinin (Al-Katib, et al, 1969). However, I found that these preparations became very insensitive to bradykinin after administration of 0.5 mg dibenzylene/100 g body weight (Figure 21). Therefore no blocking agent was used in these bioassays.

v. Depression of rabbit blood pressure. Male rabbits (1.5-2.0 kg) were injected intraperitoneally with 155 mg urethane/100 g body weight, in 0.9% saline, and in 2 hours anesthesia was deep enough to allow surgery. The jugular vein and carotid artery were exposed and cannulated as described for the rat blood pressure bioassay. Injections of bradykinin and turtle kinin were given by the intravenous route, in a total volume not exceeding 0.4 ml. Responses to agonists and controls were recorded as described in the previous assay, and potency of the unknown was calculated in the usual manner,

Figure 21. Responses typical of synthetic bradykinin (B) and purified turtle kinin (T) in six bioassays. A single four-point group is given for each assay, except for the rat blood pressure bioassay, where the two groups show the response of one rat to bradykinin and turtle kinin before (#1) and after (#2) administration of 0.5 mg dibenzylene/100 g body weight. Arrows with a "C" indicate contraction, those with an "R" indicate relaxation. Drum speed for all smooth muscle bioassays was 2 mm/min, and for blood pressure assays was 24 mm/min.

Figure 21


Rat Uterus
C
3B
.06T
2B
.04T
Sensitized Rat Uterus
C
.03T
1B
.02T
1.5B
Guinea Pig Ileum
C
.2T
10B
20B
.1T
Rat Duodenum
R
2B
5T
1B
10T
Rat Blood Pressure - 1
-2
.5B
.015T
.25B
.03T
.03T
.06T
.6B
1.0B
120
80
0
mm Hg
Rabbit Blood Pressure
.2T
2B
.4T
1B
120
80
0

using three four-point groups.

2. Results and Discussion

In all bioassays the purified turtle kinin gave responses typical of mammalian plasma kinins, as shown in Figure 21. It contracted the rat uterus and guinea pig ileum after a typically long latent period, it relaxed the rat duodenum, and it lowered the blood pressure of rat and rabbit. Chymotrypsin sensitized the rat uterus 2 to 5 times to both bradykinin and turtle kinin, and this suggested a marked similarity between the two active agents.

A summary of the quantitative results obtained in each assay is given in Table 6. Values in the final column are ratios of the rat uterus activity/test bioassay activity, for each of the bioassays listed in the first column.

Synthetic bradykinin was used as standard in all bioassays. Therefore, if turtle kinin were chemically identical to bradykinin, none of these ratios would be significantly different from one, providing the same turtle kinin preparation was used for both assays involved in any ratio. This was true for the rat uterus/sensitized rat uterus ratio, and for the rat uterus/rat duodenum ratios. Also, the ratio of rat uterus/rat blood pressure, at 1.3 ± 0.2. was only slightly greater than one, and could not be taken to indicate any difference. However, the ratios of rat uterus/guinea pig ileum activity, and rat uterus/

Table 6. Pharmacological studies; the ratios of different biological activities of turtle kinin, assayed against synthetic bradykinin.

Method of Assay	Turtle kinin preparation assayed[a].	Assay value; BK-eq, ug/ml.	Rat uterus activity; BK-eq, ug/ml.	Ratio; rat uterus/ biological activity assayed
Rat uterus, sensitized with chymotrypsin	Butanol extract	2.5 ± 0.1[b]	2.3 ± 0.2	0.9 ± 0.1
Rat duodenum	Sephadex G-25 eluate	0.16 ± 0.03[c]	0.17 ± 0.01	1.0 ± 0.1
		0.19 ± 0.03[c]	0.19 ± 0.01	1.0 ± 0.1
Rat blood pressure	CM-Sephadex eluate	10.1 ± 1.5	13.2 ± 1.0	1.3 ± 0.2
Guinea pig ileum	Sephadex G-25 eluate	0.10 ± 0.04	0.19 ± 0.01	2.0 ± 0.8
	CM-Sephadex eluate	5.2 ± 0.7	13.2 ± 1.0	2.5 ± 0.4
Rabbit blood pressure	CM-Sephadex eluate	0.95 ± 0.15	6.7 ± 0.8	7.0 ± 1.8

a...The stages of purification, and degrees of purity are inidcated in Table 5.

b...The 4-point method of Holton (1948) was used in all bioassays; errors are expressed as confidence limits, p = 0.05.

c...Values obtained during two separate runs through Sephadex G-25.

rabbit blood pressure activity were markedly different from one. Compared to values obtained in rat uterus assays, bradykinin was 2.5 times more active than turtle kinin on the guinea pig ileum, and 7.0 times more active than turtle kinin on the rabbit blood pressure. These data strongly indicated that turtle kinin and bradykinin were chemically different.

These results may be compared to those of Erdos, Miwa and Graham (1967) who reported that alligator and turtle plasma activated with glass beads released a substance oxytocic to the rat uterus, and concluded that the active agent was a peptide pharmacologically indistinguishable from mammalian plasma kinins, possibly a mixture of bradykinin and lys-bradykinin. Closer examination of these findings reveals that they are not incompatible with results given in this thesis. Firstly, these workers did pharmacological studies only on alligator plasma, showing that the active principle contracted the isolated guinea pig ileum, caused vasodilation in the dog hind limb, and caused a depression of rabbit blood pressure. Secondly, ratios of activities for the alligator kinin were calculated only for rat uterus/guinea pig ileum and rat uterus/dog hind limb blood flow. Furthermore, no data were given for any of these assays. If alligator kinin is identical to turtle kinin, the rat uterus/guinea pig ileum activity should have been different from one,

but Erdos et al only remark that the ratios were similar to bradykinin.

C. Chemical Studies

Turtle kinin was compared to bradykinin in a wide variety of specific and indirect tests.

1. Miscellaneous Tests.

During the processes of release, extraction and purification, turtle kinin was subjected to a number of treatments which would inactivate or exclude other substances known to occur in plasma, and which are oxytocic to the rat uterus.

a. Boiling. Bradykinin is not inactivated by boiling at neutral or acid pH (Rocha e Silva, 1949). The glass-released turtle kinin studied here was boiled for at least 10 minutes at neutral pH in all experiments (Chapters 4 and 5) and maintained full activity (e.g., Figure 10). Oxytocin and its analogues are inactivated by this process.

b. Trypsin. Synthetic bradykinin is only very slowly inactivated by incubation with trypsin at 37°C (Oates and Melmon, 1966). Many experiments with turtle plasma indicated that the activity released by a relatively high concentration of trypsin showed no detectable loss of activity after 30 minutes of incubation at 37°C (e.g., Figure 17). In contrast, Substance P is completely inactivated under these

conditions (Pernow, 1955).

c. Butanol extraction. The butanol extraction at low pH described in Chapter 5 has been shown by Brocklehurst and Zeitlin (1967) to extract bradykinin from the aqueous layer, while largely excluding histamine, adrenaline and 5-hydroxytryptamine. These workers found that only 0.4% of the histamine, and 3.2% of the 5-hydroxytryptamine were taken up in the butanol. The present work with turtle kinin indicated that this active peptide was extracted by the same procedure, and subsequent column chromatography (Figures 19 and 20) suggested that only one active factor had been extracted.

In all of the indirect tests described above, turtle kinin was indistinguishable from mammalian plasma kinins, but differed from many other active principles contained in vertebrate plasma.

2. Paper Chromatography.

Turtle kinin obtained from Sephadex G-25 columns was compared to synthetic bradykinin (Sandoz) and lys-bradykinin (Schwarz) in three paper chromatograms, using a butanol-acetic acid-water solvent system.

Method. The technique used has been described by Perks (1966) and by Vizsolyi (1967), and is given in detail in Appendix D. Three experiments were carried out, one a 12

hour run, the others 24 hours. In each experiment the turtle kinin sample was run beside both bradykinin and lys-bradykinin. Biological activity in each R_f division or in arbitrary divisions (Appendix D) was estimated by assay against synthetic bradykinin.

Results. Figure 22 is a representation of results from the 12 hour experiment. Most bradykinin and turtle kinin ran at R_f 0.3 - 0.4. This is in agreement with published values for R_f of bradykinin in this solvent system (Oates and Melmon, 1966). Most lys-bradykinin ran at R_f 0.2 - 0.3, and was therefore partially separated from bradykinin and turtle kinin.

It was the purpose of the two 24 hour chromatograms (Figure 23) to increase the possible resolution of the kinins by increasing the length of the run. Although the solvent front ran completely off the paper during this time, all biological activity was sufficiently retarded to remain on the paper. The arbitrary units were obtained by dividing the distance from the origin to the bottom of the paper into 10 sections, and eluting each section separately. These units are therefore not comparable to R_f values, but are a good measure of the distance moved from the origin. The results in Figure 23 show that lys-bradykinin was sharply separated (by one complete unit) from both bradykinin

Figure 22. Paper chromatography of lys-bradykinin, turtle kinin, and bradykinin in butanol-acetic acid-water (4:1:5) on Whatman 3MM chromatography paper; a 12 hour run.

Figure 22

12 Hr Chromatogram

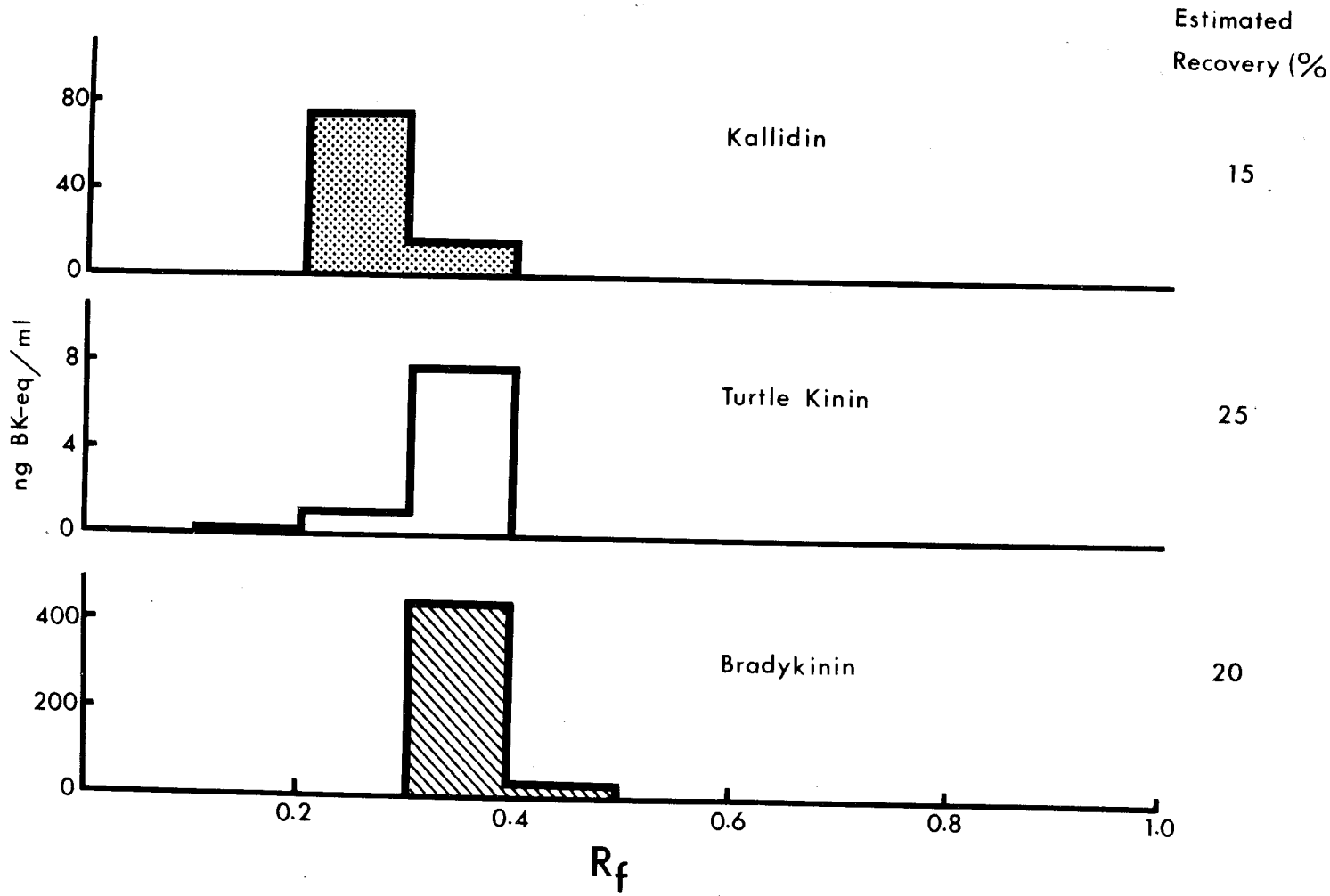
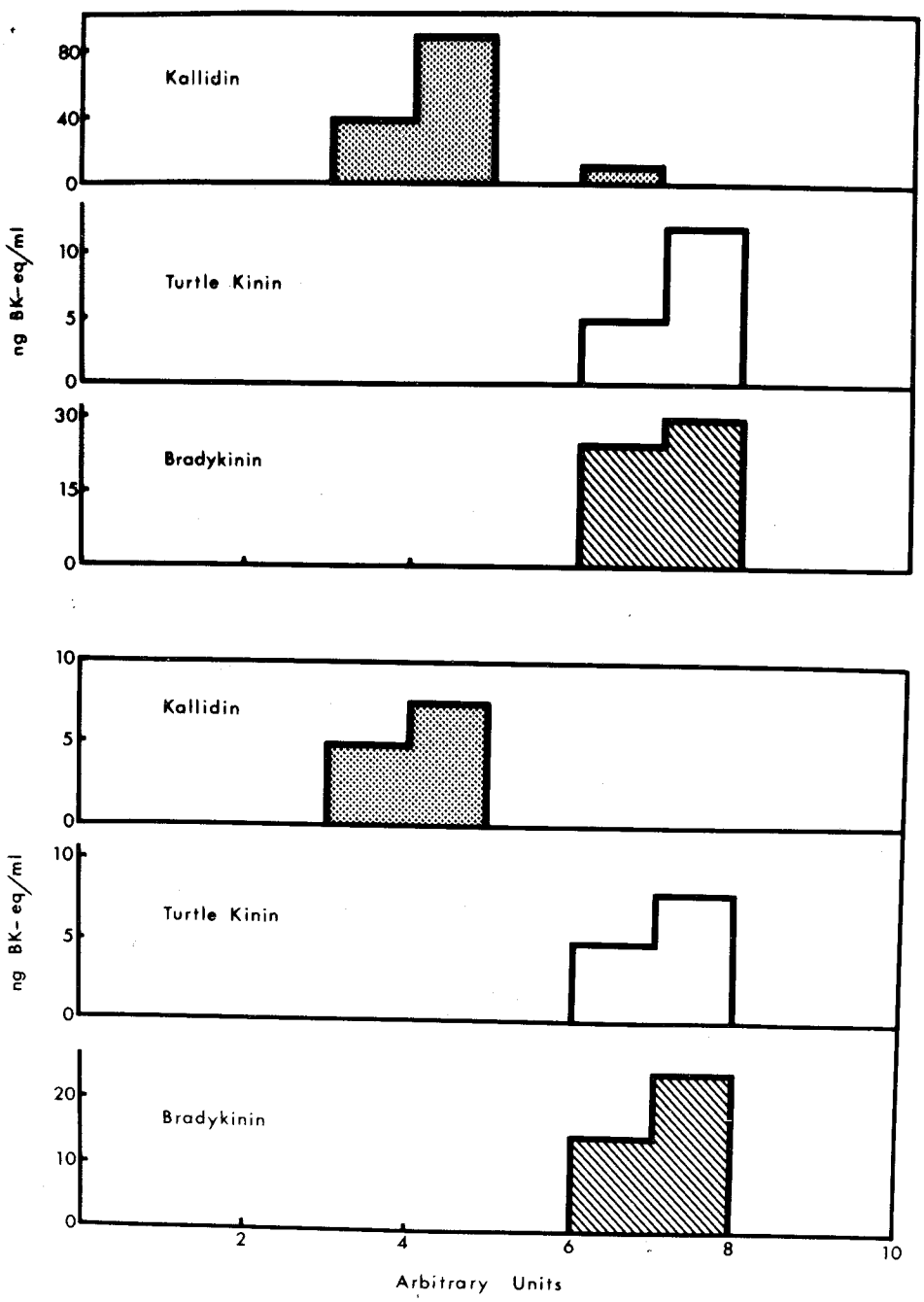


Figure 23. Paper chromatography of lys-bradykinin, turtle kinin, and bradykinin in butanol-acetic acid-water (4:1:5) on Whatman 3MM chromatography paper; 24 hour runs. Since the solvent front ran off the paper during this time, results are given in arbitrary units (see p 112).

Figure 23

24 Hr Chromatograms



	Estimated Recovery (%)
Kallidin	29
Turtle Kinin	18
Bradykinin	14
Kallidin	13
Turtle Kinin	28
Bradykinin	35

and turtle kinin activity. There was no difference in the behaviour of bradykinin and turtle kinin in this sytem.

3. Gel Filtration on Sephadex G-25

The elution pattern of turtle kinin from Sephadex G-25 (Figure 19) was compared to that of bradykinin eluted under similar conditions.

Method. Synthetic bradykinin was mixed with blue dextran and NaCl, and applied to a 95 x 2.5 cm Sephadex G-25 column, which was built in 0.02 M ammonium acetate buffer, and eluted as described in Chapter 2. The experiment with turtle kinin was described in this chapter, p 93 - 94.

Results. The elution pattern of turtle kinin given in Figure 19 may be compared to that of synthetic bradykinin, shown in Figure 24. Although the columns differed slightly in size and elution volume, and in the amount of bradykinin-equivalent in the eluate, patterns of elution were similar, both active peaks coming just before the NaCl peak.

4. Ion Exchange Chromatography on CM-Sephadex

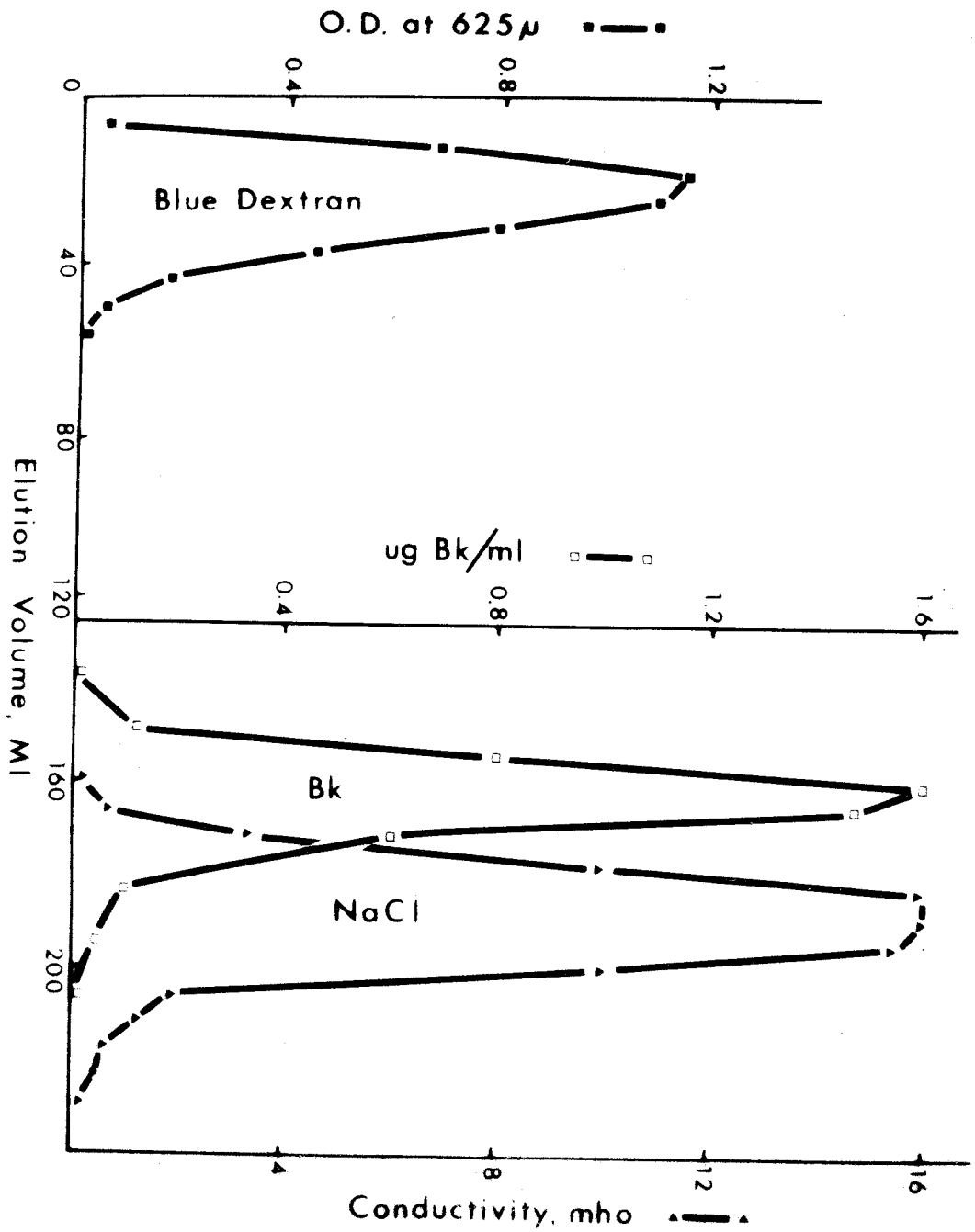
The ion exchange properties of turtle kinin, bradykinin, and lys-bradykinin were compared in a number of ways:

a) by elution of bradykinin and turtle kinin from columns developed with 0.5 M ammonium acetate buffer;

b) by separation of bradykinin and lys-bradykinin on a column developed with 1.0 M buffer, and comparison of the

Figure 24. Elution pattern of synthetic bradykinin on a 95 x 2.5 cm Sephadex G-25 gel filtration column, in 0.02 M ammonium acetate buffer, pH = 5.0.

Figure 24


O.D. at 625μ
0
0.4
0.8
1.2
Blue Dextran
40
80
ug Bk/ml
120
0.4
0.8
1.2
1.6
Elution Volume, Ml
Bk
160
NaCl
200
4
8
12
16
Conductivity, mho

elution of turtle kinin in the same system;

c) by a direct comparison, in which equal portions of bradykinin and turtle kinin were mixed and eluted from a CM-Sephadex column developed with 0.6 M buffer.

Methods.

a) Approximately 10 ug bradykinin-equivalent of glass released turtle kinin purified on Sephadex G-25 in a pilot experiment were loaded in 5 ml on to a small (17 x 0.9 cm) CM-Sephadex column built in 0.02 M ammonium acetate buffer as described in Chapter 2. An ionic strength gradient to 0.5 M buffer was applied, and the activity collected in different fractions was estimated on the rat uterus, after neutrilization with $NaHCO_3$. Two other columns of the same size, one loaded with 10 ug synthetic bradykinin, and the other with 10 ug bradykinin-equivalent of lys-bradykinin were developed in exactly the same manner.

b) A mixture of 10 ug bradykinin and 10 $\pm$ 1 ug lys-bradykinin were loaded in 2 ml on to a CM-Sephadex column built in 0.02 M ammonium acetate buffer. The column was developed using a gradient to 1.0 M buffer, exactly as described in Chapter 5 for the purification of turtle kinin. Activity of the 6.2 ml fractions was assayed, and the elution pattern was compared to that of turtle kinin in the same system.

c) 6.7 ug bradykinin was mixed with 6.6 $\pm$ 0.45 ug of

turtle kinin, bradykinin-equivalent (a sample from the CM-Sephadex eluate listed in Table 5), and adjusted to 3 ml 0.3 M ammonium acetate buffer, pH = 5.0. This mixture was loaded on a CM-Sephadex column also built in 0.3 M buffer. The column was then washed with 50 ml of the buffer, and the eluate was checked for biological activity. A concentration gradient to 0.6 M buffer, pH = 5.0, was then applied to the column in the usual manner, and 5 ml fractions were collected and assayed.

Results.

a) Figure 25 compares the elution of turtle kinin and bradykinin on separate columns, by 0.5 M buffer. The patterns are identical. No biological activity was detected in the eluate from the column loaded with lys-bradykinin, and it was concluded that a more concentrated buffer would be required to detach this peptide from the resin.

b) Figure 26 a illustrates the separation of bradykinin and lys-bradykinin by a gradient to 1.0 M buffer. The resolution of these peptides is very clear, the two peaks being separated by 57 ml of eluate. The elution pattern of turtle kinin in the same system is also included in this Figure. Since previous results established that bradykinin is eluted at the same ionic strength as turtle kinin (Figure 25), the first peak in Figure 26 a may be identified as bradykinin,

Figure 25. Elution of synthetic bradykinin and turtle kinin from separate CM-Sephadex columns built in 0.02 M ammonium acetate buffer, pH = 5.0, and developed with a concentration gradient to 0.5 M ammonium acetate buffer, pH = 5.0.

Figure 25

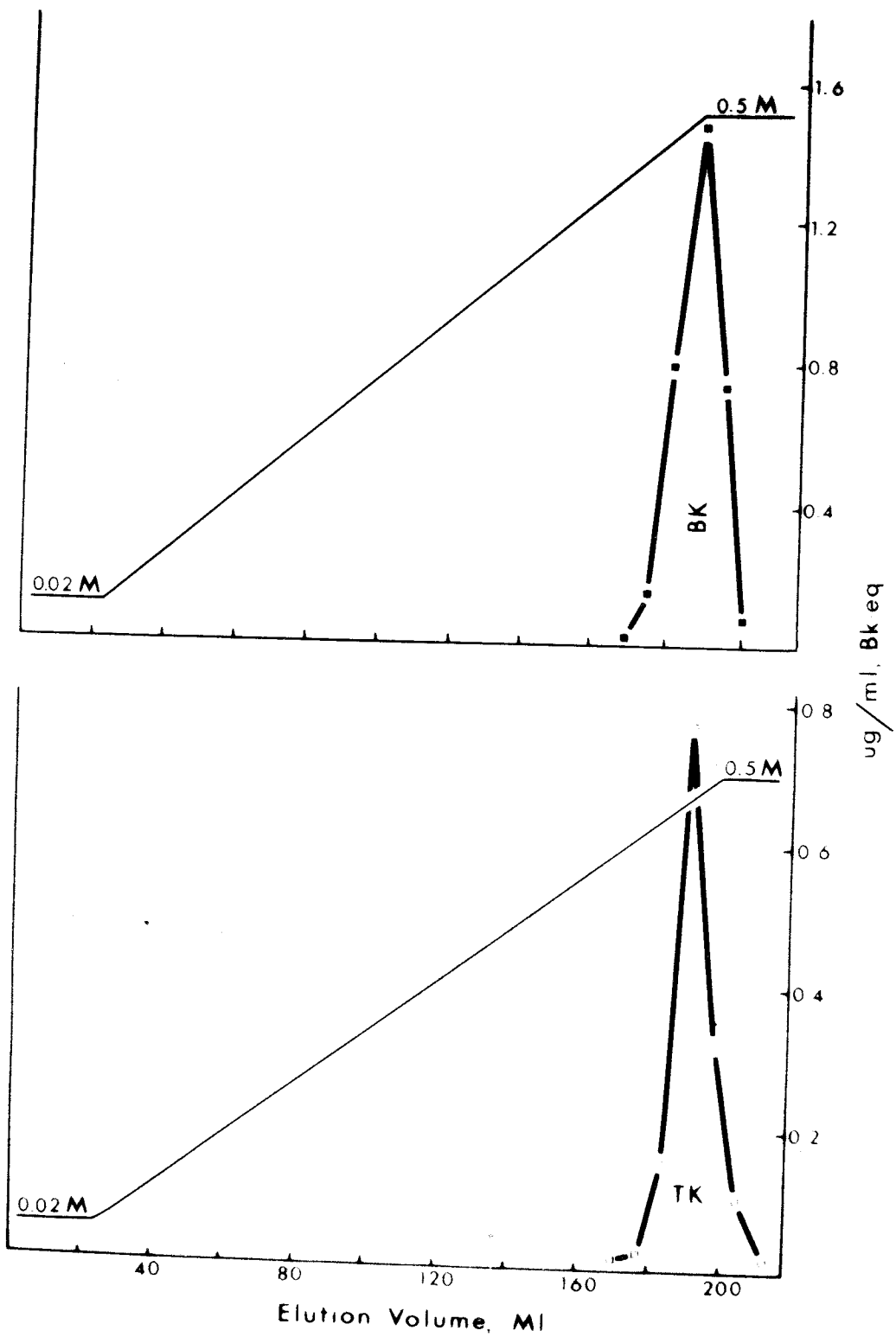
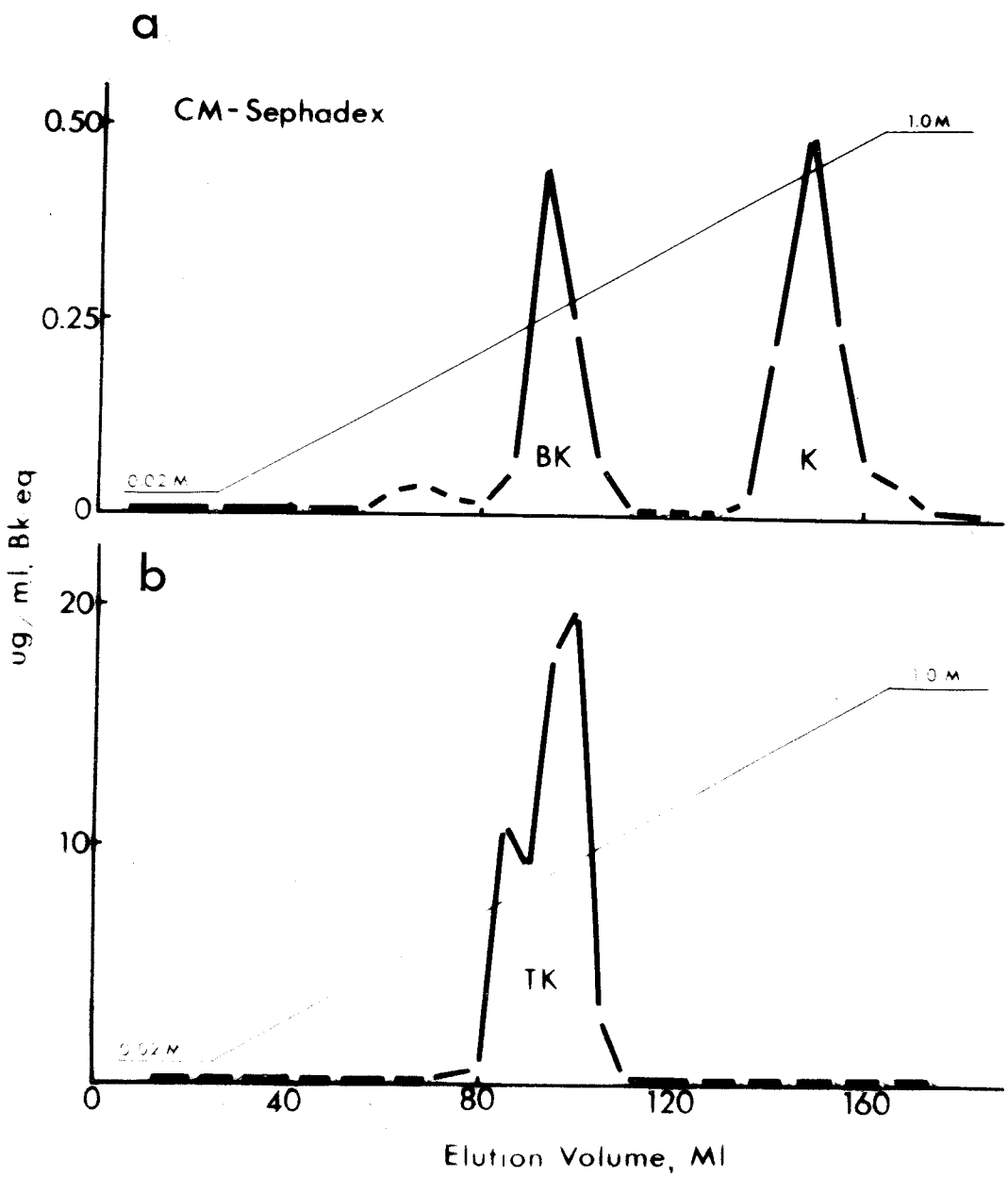

0.5 M
0.02 M
BK
1.6
1.2
0.8
0.4
0.5 M
0.02 M
TK
0.8
0.6
0.4
0.2
40
80
120
160
200
Elution Volume, Ml
ug/ml, Bk eq

Figure 26 a. Separation of synthetic bradykinin from synthetic lys-bradykinin on a CM-Sephadex column (20 x 0.9 cm) developed with a concentration gradient from 0.02 M to 1.0 ammonium acetate buffer, pH = 5.0.

b. The elution pattern of turtle kinin in the same system (from Figure 20), included for comparison.

Figure 26


a
CM-Sephadex
0.50
0.25
0
1.0M
0.02M
BK
K
b
20
10
0
1.0 M
0.02 M
TK
40
80
120
160
ug. ml. Bk eq
Elution Volume, Ml

and the second as lys-bradykinin.

Since met-lys-bradykinin runs close to lys-bradykinin in this sytem (Miwa, Erdos, and Seki, 1969) it can be concluded that turtle kinin is also different from met-lys-bradykinin.

c) The hypothesis that bradykinin is inseparable from turtle kinin in ion exchange chromatography on CM-Sephadex was tested more rigorously by the chromatography of a mixture of the two, using a more shallow gradient to increase resolution. Results of the test (Figure 27) indicate that a single peak of activity was eluted. Pooled activity from elution volume 90 - 140 ml was estimated at 10.9 $\pm$ 1.5 ug bradykinin-equivalent. This total is significantly greater ($p<.01$) than either the bradykinin (6.7 ug) or the turtle kinin (6.6 $\pm$ 0.45) initially loaded on the column. The peak must therefore contain both active principles. Assuming this to be true, the total recovery of biological activity from the column would be 10.9/13.3 = 74.4%, a normal recovery for CM-Sephadex.

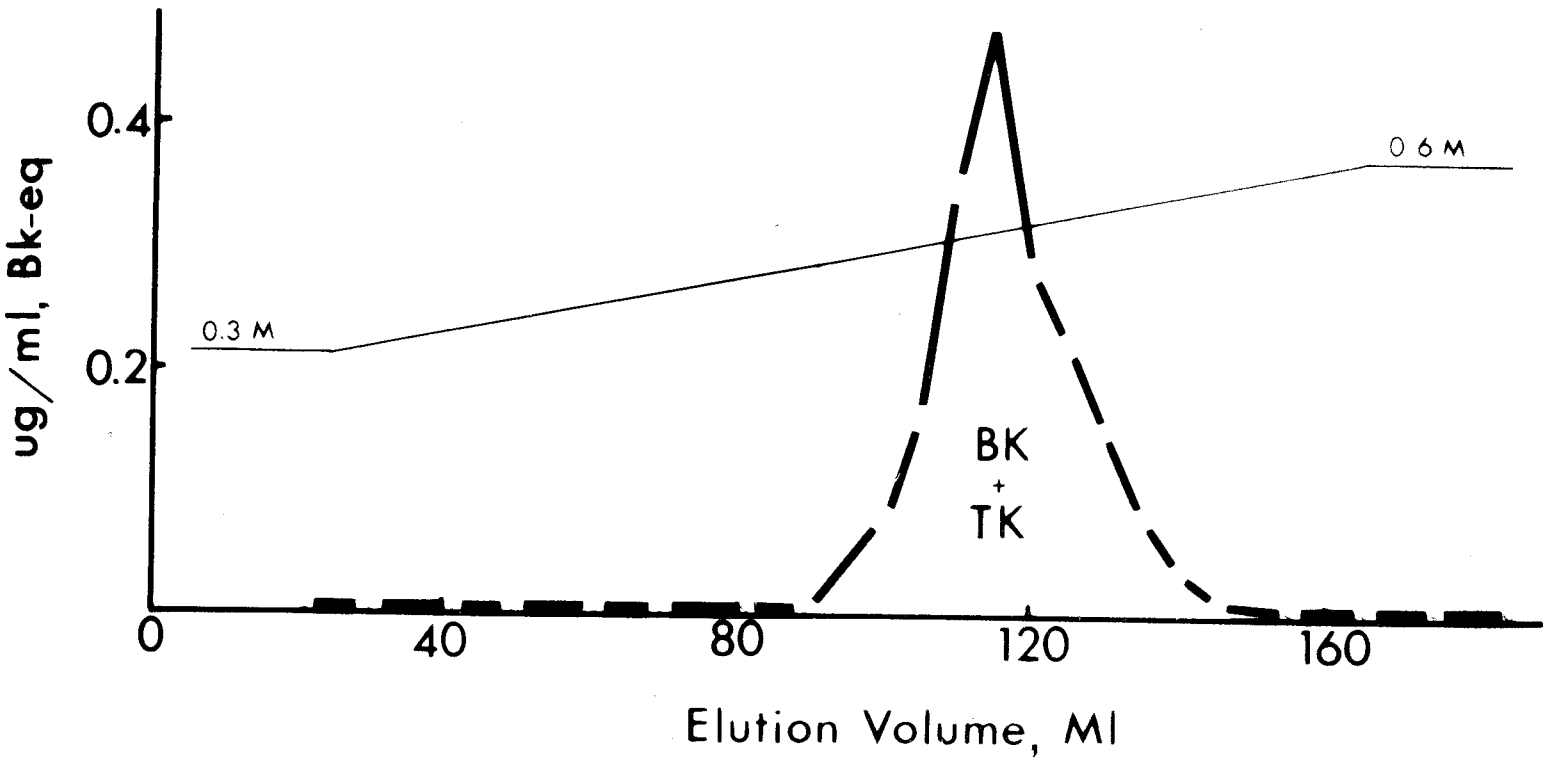
On the basis of the experiments described above it was concluded that the glass released turtle kinin is different from lys-bradykinin and from met-lys-bradykinin, but is very similar to bradykinin.

5. Amino Acid Analysis

Samples of purified turtle kinin and synthetic bradykinin

Figure 27. Elution pattern of a mixture of synthetic bradykinin and purified turtle kinin from a CM-Sephadex column (15 x 0.9 cm) developed with a shallow concentration gradient, from 0.3 M to 0.6 M ammonium acetate, pH = 5.0.

Figure 27


0.4
0.2
ug/ml, Bk-eq
0.3 M
0.6 M
BK
+
TK
0
40
80
120
160
Elution Volume, Ml

were hydrolyzed, and subjected to amino acid analysis.

Method. Three samples of purified turtle kinin were hydrolyzed separately. Two samples (100 ± 10 and 145 ± 10 ug BK-eq) were taken from the main peak in the CM-Sephadex eluate (Figure 20), and one from the "shoulder", at elution volume 85 ml (62 ± 4 ug BK-eq). One sample of synthetic bradykinin (Schwarz, 100 ug) was also hydrolyzed separately.

The samples of turtle kinin in 0.8 M ammonium acetate, and samples of synthetic bradykinin in 0.2 M acetic acid were lyophilized in 50 ml vacuum flasks. In each case the residue was dissolved in glass distilled water and re-lyophilized at least 3 times to reduce levels of ammonium acetate and acetic acid.

The residue was then taken up in 4 ml of 6 N triple distilled HCl, and transferred to an acid washed 6 ml hydrolysis bulb. Using PE 10 tubing (Intramedic), N_2 gas was bubbled through the liquid for 15 minutes to displace dissolved O_2. The fluid was then frozen by immersing the hydrolysis bulb in liquid N_2, and subsequently evacuated for 30 minutes at 0.01 mm Hg pressure. Using a natural gas flame, the neck of the bulb was sealed under the same vacuum, and the bulb was placed in an oven (Fisher Isotemp) at 107°C, for 24 or 36 hours, according to the sample.

After this time, the sealed hydrolysis bulbs were removed

from the oven, cooled, carefully opened, and the contents were transferred to acid washed 50 ml round bottom flasks. These flasks were cooled in ice for 15 minutes, then attached to a flash evaporator and rotated under vacuum in a water bath at 37°C. After 30 minutes, the dried residues were redissolved in 2 ml iced distilled water and the sample re-dried. This washing was repeated 3 times to remove the last traces of HCl. Samples were stored dry at - 20°C until analysis.

Amino acid analysis was performed on a Biocal 200 analyzer. The dried hydrolysates were dissolved in 1 ml of sodium citrate buffer, pH = 2.2. Aliquots containing the desired amount of material were removed with acid washed pipettes and placed on the column in a volume of 1 ml. After completion of the 5 hour analysis, the separated peaks obtained on the record could be quantified by comparison of the areas with those of standard amino acids. In calculation of molar ratios, arginine was used as reference (Arg = 2.00) since it is relatively stable under the conditions of acid hydrolysis.

Results. Table 7 summarizes the results of the analyses. Synthetic bradykinin hydrolyzed for 24 hours gave molar ratios of amino acids exactly as would be predicted from a consideration of its molecular structure. However, turtle kinin hydrolyzed for the same period gave different molar ratios.

Table 7. Molar ratios of amino acids in purified turtle kinin and in synthetic bradykinin hydrolyzed for 24 and 36 hours.

Amino acid	Experimental Ratios					Deduced Ratios	
	24 hour hydrolysis			36 hour hydrolysis			
	BK	BK	TK[a]	TK[b]	TK[a]	BK	TK
Arg	2.00	2.00	2.00	2.00	2.00	2	2
Pro	2.93	2.60	6.80	3.00	3.16	3	3
Gly	1.06	1.28	2.00	1.16	1.15	1	1
Phe	2.01	1.63	0.28	1.68	1.82	2	2
Ser	1.07	1.21	0.05	0.15	0.14	1	0
Thr	0.01	0.12	0.12	0.99	0.99	0	1

Amino acids present in trace amounts only were: Ala, Val, Lys, Asp, Glu, Met, Ile, Leu, Tyr, and His. All values were less than 0.25, most less than 0.10.

a...turtle kinin from main peak, elution volume 120 (Figure 20)

b...turtle kinin from "shoulder", elution volume 85 (Figure 20)

Compared to bradykinin, this turtle kinin hydrolyzed for 24 hours had large amounts of proline (M.R. = 6.8), and glycine (M.R. = 2.0), low levels of phenylalanine (M.R. = 0.28), and only traces of other amino acids. The high proline content, and lack of most other amino acids, suggested that the turtle kinin was either incompletely hydrolyzed, or markedly different from bradykinin.

Samples of purified turtle kinin were therefore hydrolyzed for 36 hours, and analyzed as before. Results in Table 7 show that the molar ratios of Arg (2), Pro (3), Gly (1), and Phe (2) were now exactly as would be predicted for bradykinin. However, the turtle kinin differed in the absence of one molar ratio of serine, and in the presence of one molar ratio of threonine. Apart from this discrepancy, the molar ratios for all amino acids were either very close to the whole numbers expected for bradykinin, or were present in trace amounts only; this suggested that the turtle kinin in the CM-Sephadex eluate was highly purified.

The turtle kinin samples from the "shoulder" in the CM-Sephadex eluate had the same analysis as the kinin from the main peak (Table 7).

Table 8 compares the biological activity of the bradykinin and turtle kinin preparations to the amount of peptide measured, for each analysis. The ratios for bradykinin measured/

Table 8. Relation of biological activity of bradykinin and turtle kinin to nM of kinin measured by amino acid analysis.

Peptide	Biological activity of kinin applied to analyzer (nM BK-eq)	Amount of kinin measured by analyzer (nM Arg/2)	nM measured/ nM applied
Bradykinin	37.7	30.4	0.806
	18.9	14.7	0.788
	14.2	12.4	0.873
Turtle kinin	58.5 ± 6.5*	45.0	0.769
	68.8 ± 2.0	66.6	0.968
	27.4 ± 2.0	26.1	0.952

*...these values were determined by rat uterus assay, using synthetic bradykinin, 0.1 mg/ml, as standard.

bradykinin applied indicated that the analyzer measured 80 - 85% of the peptide applied. Since turtle kinin was assayed for biological activity against synthetic bradykinin, the similar ratios obtained for turtle kinin provide good evidence that this kinin is fully as active as bradykinin on the rat uterus.

Serine and threonine run close to one another during amino acid analysis, and therefore a series of tests were carried out in order to eliminate any possible error or ambiguity in the important conclusion that turtle kinin contained threonine, but no serine. The identity and resolution of these two peaks was tested by analyzing the following mixtures;

a) an aliquot of bradykinin hydrolysate + 40 nM of L-threonine, and

b) an aliquot of turtle kinin hydrolysate + 40 nM of L-serine. Results shown in Figure 28 indicate clearly that each of these test mixtures differed from its simple hydrolysate control only in the appearance of approximately 40 nM of the extra amino acid. This result excluded any possibility of a confusion between the two closely related amino acids.

D. Discussion and Conclusions

Amino acid analysis of turtle kinin revealed molar ratios identical to bradykinin, except for the apparent substitution of threonine for serine. Since incompletely hydrolyzed turtle

Figure 28. Copies of traces from amino acid analysis of hydrolysates of synthetic bradykinin and purified turtle kinin showing a serine peak for synthetic bradykinin (BK), a threonine peak for purified turtle kinin (TK), and both threonine and serine peaks for bradykinin + synthetic threonine (BK + Thr) and for turtle kinin + synthetic serine (TK + Ser).

Figure 28

BK
Asp
Thr
Ser
Glu
TK
Asp
Thr
Ser
Glu
BK + Thr
Asp
Thr
Ser
Glu
TK + Ser
Asp
Thr
Ser
Glu

kinin contained only traces of threonine (Table 7), there is little doubt that the threonine is an integral part of the molecule.

The simplest conclusion consistent with all available evidence is that turtle kinin may be an analogue of bradykinin in which threonine replaces serine, and it is therefore 6-thr-bradykinin:

1 2 3 4 5 6 7 8 9

NH_2-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-COOH

Bradykinin

NH_2-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-COOH

6-thr-bradykinin, the proposed turtle kinin

Support for this hypothesis comes from the pharmacological properties of turtle kinin. 6-thr-bradykinin was synthesized and studied by Schröder and Hempel (1964), and although it was as active as bradykinin on the rat uterus, the ratio of rat uterus/guinea pig ileum activity was 3, and the ratio of rat uterus/rabbit blood pressure activity was 10. These data were obtained using simple comparisons of the threshold dose for rat uterus and guinea pig ileum activities, and single readings from log dose-response curves for rabbit blood pressure. No estimates of errors of these assays are possible. Therefore, the ratios presented by these workers must be regarded as approximate, and could easily have 95% confidence limits of $\pm$ 100%. Nonetheless, the values given contrast

with the wide range of ratios obtained for many other synthetic bradykinin analogues (Schröder and Lübke, 1966), and in these circumstances they may be regarded as being in good agreement with values obtained for turtle kinin (Table 6).

Turtle kinin is also similar to synthetic 6-thr-bradykinin in that both are fully as active as bradykinin on the rat uterus (Table 8; Schröder and Hempel, 1964).

CHAPTER 6. PRELIMINARY STUDIES OF THE EFFECT OF BRADYKININ ON BLOOD PRESSURE IN THE TURTLE

Once it has been established that turtle kinin differs in structure from bradykinin (Chapter 5), it becomes essential to examine thoroughly the effects of this new kinin on such aspects of turtle physiology as the circulation. Due to a lack of purified turtle kinin, it has not been possible to carry out more than preliminary studies with the use of synthetic bradykinin.

There is no published record of attempts to examine the effects of mammalian plasma kinins on blood pressure of any reptile, although it is known that high concentrations of bradykinin and lys-bradykinin cause little change in blood pressure of birds (Erdos, 1966) and fish (Vogel et al, 1969). These results are not surprising, since neither of these groups appear to have a complete plasma kallikrein-kinin system (Chapter 3). By contrast, turtle plasma does have a fully developed kinin producing enzyme system (Chapter 4) and investigation of the effects of bradykinin on the blood pressure of these animals might be a more promising line of enquiry, and would at least serve as a useful base for comparison in future experiments with 6-thr-bradykinin, the proposed turtle kinin.

Many workers have found that pre-treatment of a mammal with an alpha-adrenergic blocker (e.g., phenoxybenzamine) does not reduce the vasodilator and hypotensive effect of bradykinin and in most cases actually increases this response (Harrison et al, 1968; Lang and Pearson, 1968; Lloyd, 1962; Nakano, 1965; Rocha e Silva et al, 1960). The same is true of the venoconstrictor action of bradykinin in rabbits (Bobbin and Guth, 1968) and of bronchoconstriction in the guinea pig (Collier, et al, 1965). There are many reports of a biphasic response to intra-arterial injections of kinins (Lang and Pearson, 1968; Al-Katib and Baba, 1969); there is an initial hypotension, followed by a pressor response. The latter phase is in part due to the release of catecholamines from the adrenal medulla (Feldberg and Lewis, 1964) and can be abolished by blockade of the autonomic nervous system. All workers studying in vivo responses have found that intra-arterial, rather than intravenous injections, have the lowest threshold.

It was the object of these preliminary studies to characterize the response of turtle blood pressure to intra-arterial injection of bradykinin, and to determine whether adrenergic blockade would modify this response.

A. Method

Turtles (P. scripta elegans) 1.0 - 2.0 kg in weight were anesthetized by subcutaneous injection of sodium pentobarbital

(60 mg/kg). The animal was taped upside down to a polystyrene form, and a square about 5 x 5 cm was cut in the plastron with a bone saw (Desoutter Co.). Removal of this portion of shell, and careful dissection of underlying muscle revealed the heart, and major blood vessels leading from it. Using watchmaker's forceps and fine scissors, the arteries were freed of mesenteries and connective tissue. Cannulations were accomplished by placing a clamp on the vessel, making a small V-shaped incision in its ventral surface distal to the clamp, and inserting the beveled end of the polyethylene cannula (PE 60, 190 or 200, Intramedic) through the incision and towards the heart. The cannula was secured by a ligature previously placed around the artery. Where injections were to be given into the same vessel, a size #27 needle connected to PE 10 tubing was inserted into the cannula about ½" from the beveled end, prior to its placement in the vessel.

In each experiment two of the following arteries were cannulated; left aorta, right subclavian, left subclavian, and left pulmonary arteries (see White and Ross, 1966, for a diagram of the heart and major blood vessels of this turtle). Blood pressure was recorded in mm Hg by means of a Statham P 23BB transducer coupled to a 6 channel Beckman dynograph. Blood flow was also recorded from one of these vessels, using a Doppler ultrasonic flow meter coupled to the Beckman recorder.

The collar (¼" bore) was tied around the intact artery, and any extra space filled in with Aquasonic gel. The following arbitrary measure of mean blood flow was used; height of pulse peak (cm) x heart rate (beats/min). These values should be directly proportional to mean blood flow.

Injections of all agents were given intra-arterially. Active agents were dissolved in buffered turtle saline (Appendix B) and washed in with saline so that the total volume of the injection never exceeded 0.3 ml. Drugs used were bradykinin, L-epinephrine, acetylcholine HCl, phenoxybenzamine, propanolol, atropine, and sodium pentobarbital (Appendix A).

B. Results

A serious flaw in most experiments was the steady decrease in blood pressure, though blood loss was insignificant. Values indicating the magnitude of this change in 6 experiments are given in Table 9. Associated with the loss of blood pressure was a general decrease in sensitivity of the preparation to any drug. Nonetheless, all animals gave pressor responses to both adrenaline and bradykinin in relatively low concentrations. On a per weight basis adrenaline was always more potent than bradykinin. Threshold doses for bradykinin were as low as 0.5 ug/kg body weight, but were as high as 10 ug/kg in some preparations.

Typical responses to synthetic bradykinin and adrenaline

Table 9. Decrease in blood pressure of 6 turtles over the 5 - 10 hour experimental period.

Experiment	*Arterial Blood Pressure (mm Hg)	
	Initial	Final
1	36/29	14/11
2	28/10	5/1
3	26/21	10/5
4	18/9	9/3
5	16/13	11/5
6	29/25	14/7

*...measured in right subclavian artery.

are given in Figure 29 a. The reduced responses of the same animal to higher doses of the same agents after application of 1.2 mg phenoxybenzamine/kg and 5.4 ug propanolol/kg are shown in Figure 29 b.

Figure 30 is a summary of the effects of phenoxybenzamine on blood pressure responses to bradykinin and adrenaline, in three different animals. Injections of propanolol to 10 ug/kg had no effect on these responses, although the drug seemed to stabilize heart rate. All of these pressor responses were accompanied by a decrease in blood flow (e.g., Figure 29 a). However, minor changes in the position of the collar-shaped probe around the vessel directly affected the flow itself; hence "base line" activity was constantly changing, making these results unsatisfactory. In any case, a dose of phenoxybenzamine sufficient to reduce the effect of adrenaline always significantly lowered the activity of bradykinin. Treatment with 1.2 mg phenoxybenzamine/kg, or any larger amount, always resulted in at least a 10 fold reduction in responses to both compounds.

C. Discussion and Conclusions

White and Ross (1966) found that normal aortic blood pressure in P. scripta elegans was about 30 mm Hg at systole, a value similar to those obtained for initial pressures in the present study (Table 9). However, the continual loss of pressure in all preparations suggested that the anesthetic and/or surgical

Figure 29 a. Typical responses of turtle blood pressure and flow to intra-arterial injections of synthetic bradykinin (B) and adrenaline (Ad).

b. Reduced responses of the same animal to higher doses of bradykinin and adrenaline, after administration of 1.2 mg phenoxybenzamine/kg and 5.4 ug propanolol/kg.

Figure 29

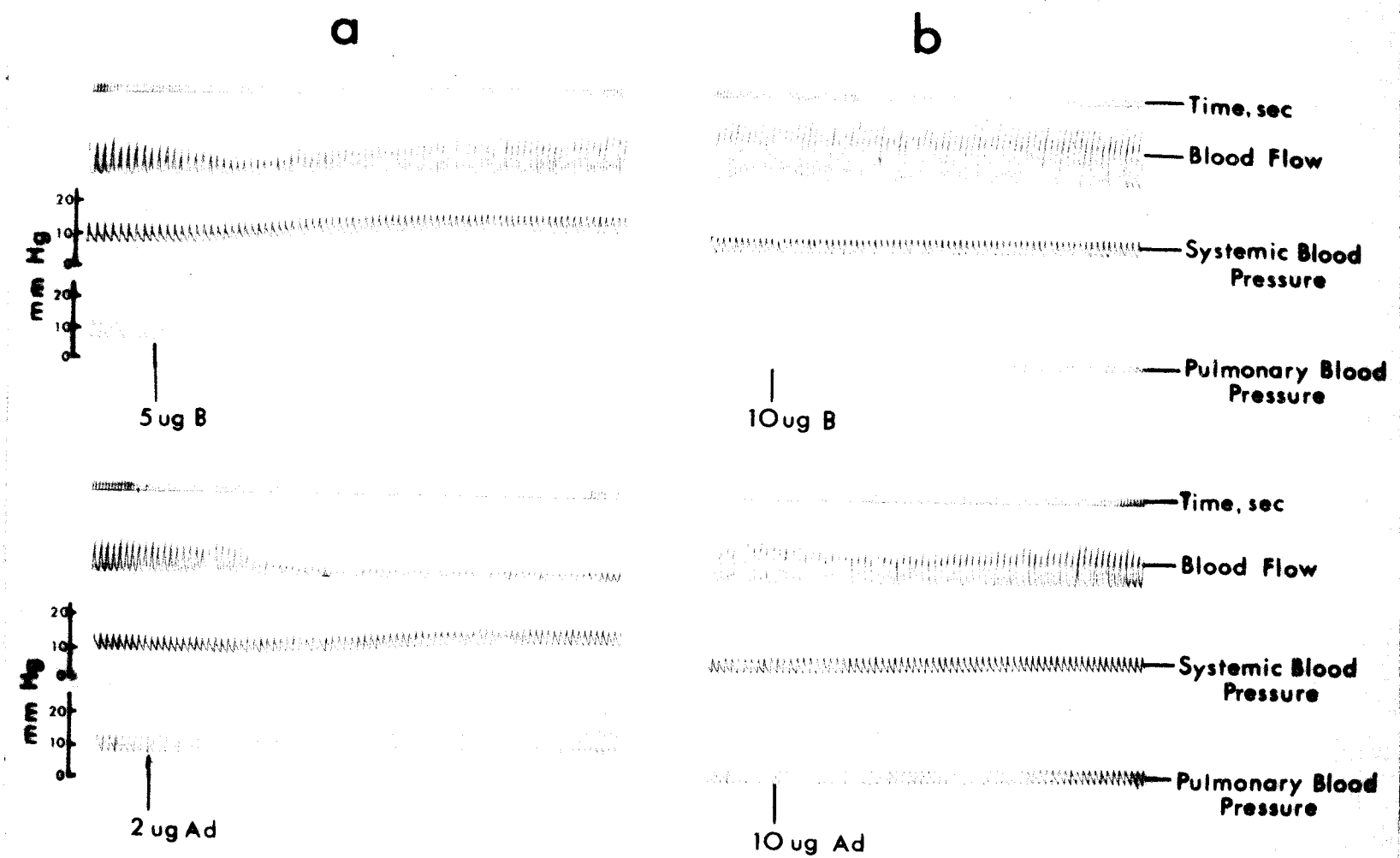
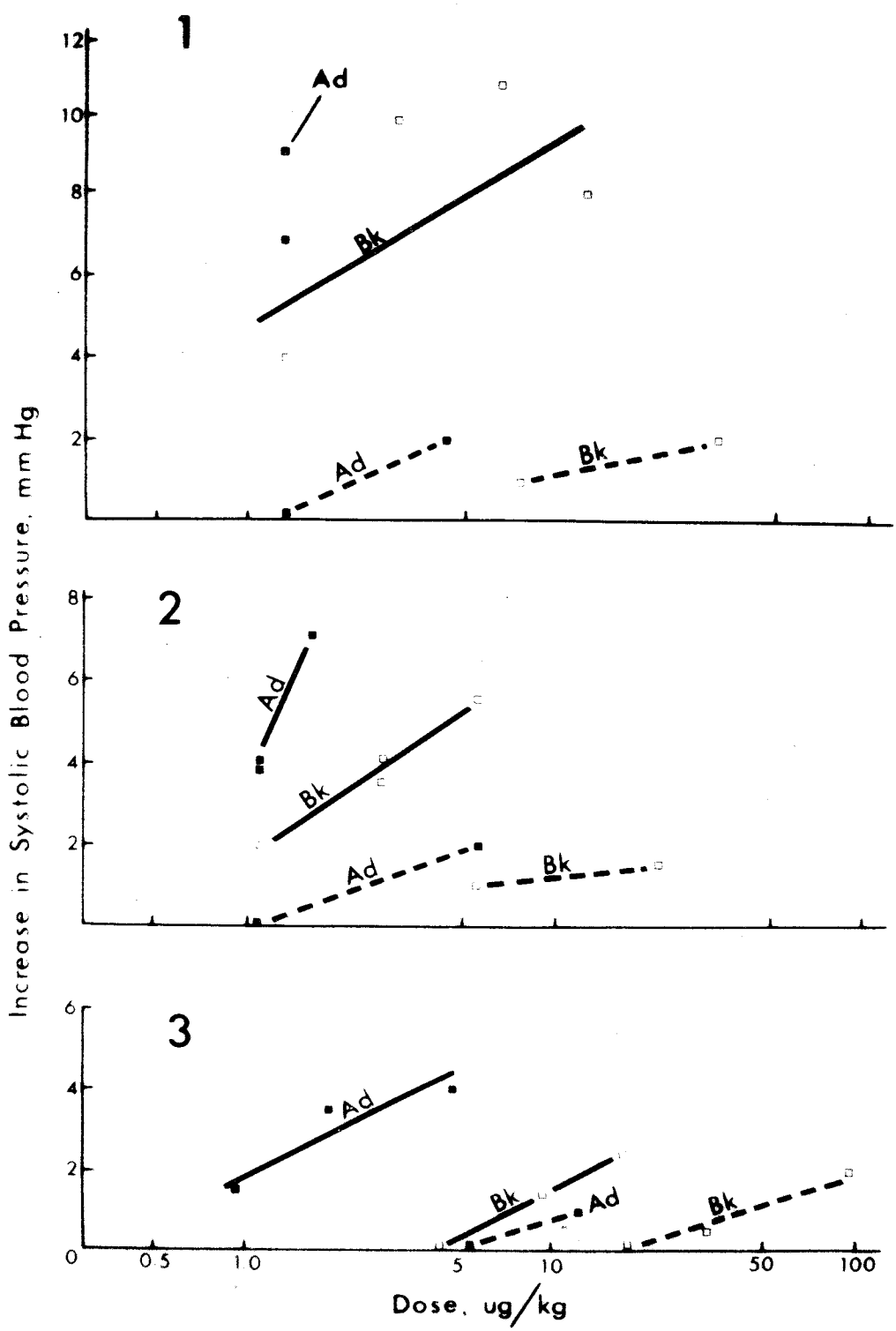

a
b
Time, sec
Blood Flow
Systemic Blood Pressure
Pulmonary Blood Pressure
mm Hg
20
10
0
5 ug B
10 ug B
2 ug Ad
10 ug Ad

Figure 30. Effect of adrenergic blockade on the pressor response to bradykinin in 3 different turtles. Solid lines are approximate log dose-response curves for synthetic bradykinin (BK) and adrenaline (Ad) prior to blockade. Dashed lines are approximate log dose-response curves after application of 1.2 mg phenoxybenzamine/kg.

Figure 30


1
2
3
Ad
Bk
Ad
Bk
Ad
Bk
Ad
Bk
Ad
Bk
Ad
Bk
12
10
8
6
4
2
8
6
4
2
6
4
2
0
0.5
1.0
5
10
50
100
Increase in Systolic Blood Pressure, mm Hg
Dose, ug/kg

treatment significantly altered the hemodynamics of these animals. The extremely low pressures encountered near the end of some experiments (e.g., #2, Table 9) probably indicate that the animals were near death; responses obtained from such preparations are of dubious value.

Lang and Pearson (1968), Pearson and Lang (1969), and Al-Katib and Baba (1969) found that the size of the pressor responses to intra-carotid injection of kinins was inversely related to arterial pressures in dogs and rabbits. At low pressures there was sometimes no hypotensive component to the response. Since the pressor response, which persisted in adrenalectomized animals (Lang and Pearson, 1968) could be reduced by adrenergic blockade or decerebrations, it was suggested that part of this response could be centrally mediated. However, other workers (Lecomte, et al, 1964) have found that pressor effects can be completely abolished by adrenalectomy in the rabbit.

The hypertensive effect of bradykinin in the turtle, which is also abolished by adrenergic blockade, therefore has certain similarities with the observations by the authors mentioned above. However, the turtle differed from all mammals studied in that no hypotensive response was ever evoked by any dose of bradykinin,up to 50 ug/kg body weight. It is therefore impossible to conclude that these preliminary studies provide

evidence that bradykinin has an effect on turtle blood pressure analagous to its effect in mammals.

CHAPTER 7. SUMMARY AND GENERAL DISCUSSION

Whole blood and plasma of various fish, amphibians, reptiles and mammals was compared with respect to the type of kallikrein-kinin system each contained (Chapter 3). The following components of the system (outlined in Figure 2) were tested as follows:

Component	Test
Kininogen, in whole blood	trypsin on denatured blood
Kininogen, in plasma	trypsin and hog pancreas kallikrein on denatured plasma
Kininase, in undenatured plasma	a. rate of inactivation of synthetic bradykinin in plasma
	b. effect of different concentrations of kininase inhibitor on kininase activity
Activatable plasma kallikreins in undenatured plasma	effectiveness of glass surface in activating kallikrein in the presence of kininase inhibitor
Kininogen, in undenatured plasma	effectiveness of glass surface, trypsin, and hog pancreas kallikrein in releasing kinin in the presence of kininase inhibitor

Tested plasmas were clearly separated into two groups:

Group 1. Mammalian and turtle plasmas. Heated plasma released relatively large amounts of kinin when incubated with

trypsin or hog pancreas kallikrein, always less with hog pancreas kallikrein. Kininase activity in intact plasmas was totally inhibited by low doses of 8-$HQSO_4$, and in the presence of this inhibitor, glass beads and trypsin caused the release of endogenous kinin.

Group 2. Trout, frog and Amphiuma plasmas. These plasmas were similar to those in group 1 in only two respects; a) the possession of kininase activity, and b) the release of biological activity from heated plasma, by hog pancreas kallikrein. Trypsin released only traces of a kinin-like factor from heated plasmas, while hog pancreas kallikrein liberated far larger amounts of activity. Kininase in intact plasmas was only partially inhibited by high doses of 8-$HQSO_4$, and no treatment (glass, trypsin, or hog pancreas kallikrein) caused the release of a kinin-like factor in undenatured plasma.

The suggestion that turtle plasma contained a kallikrein-kinin system similar to that of mammals was tested more rigorously by comparing the two systems in a variety of experiments (Chapter 4). Turtle plasma was found to be similar to mammalian plasma in the following characteristics:

1. Glass activation in the presence of 8-$HQSO_4$ produced kinin, the rate being directly proportional to the glass surface area.

2. Rates of activation and decay of plasma kallikrein were

similar.

3. Plasma kallikrein may be activated by trypsin, as it is in mammals.

4. Prekallikrein was present in amounts far larger than required to degrade all kininogen.

5. At least two types of kininogen were present in turtle plasma.

6. The effect of temperature on the rate of kinin release by plasma kallikreins was similar in turtle and rat plasma.

Mammalian plasma usually released more kinin/ml than did turtle plasma, but no qualitative differences in the mechanisms of release were noted.

Although no evidence had been obtained to suggest that the turtle plasma kallikrein-kinin system differed from that of mammals, the possibility remained that the active peptides released by the action of the enzyme systems might be different. Glass released turtle kinin was therefore subjected to a variety of pharmacological and chemical tests. Two pharmacological ratios, namely guinea pig ileum/rat uterus activity, and rabbit blood pressure/rat uterus activity, were significantly greater than one, being 2.5 and 7.0 respectively. Since the assays all used synthetic bradykinin as standard, these values strongly suggested that turtle kinin was chemically different from the mammalian kinin.

Purified turtle kinin was compared to synthetic bradykinin in gel filtration experiments (Sephadex G-25), in ion exchange chromatography (CM-Sephadex), and in paper chromatography in butanol-acetic acid-water. While these tests clearly distinguished between bradykinin and lys-bradykinin, turtle kinin and bradykinin were not separated. However, amino acid analysis of the purest preparation of turtle kinin established that it was chemically different. Repeated analyses indicated that molar ratios of proline (3), arginine (2), phenylalanine (2), and glycine (1) were identical to those of bradykinin, but that turtle kinin differed from the mammalian kinin in the presence of one molar ratio of threonine, and in the absence of one molar ratio of serine.

This evidence led to the hypothesis that turtle kinin was 6-thr-bradykinin, as follows:

NH_2-Arg-Pro-Pro-Phe-Gly-Thr-Pro-Phe-Arg-COOH

Proposed turtle kinin

Marked similarities in the pharmacological activity of synthetic 6-thr-bradykinin (Schröder and Hempel, 1964) and turtle kinin in certain bioassays offered support for this hypothesis.

Although final conclusions must await sequence analysis of the active peptide, the evidence presented in this thesis suggests that turtle plasma contains a kallikrein-kinin system

very similar to that of mammalian plasma, but based instead on threonine containing peptides, and culminating in the production of 6-thr-bradykinin as the natural plasma kinin of the turtle P. scripta elegans. It seems possible that treatment of turtle plasma with acid or glandular kallikrein could result in the production of met-lys-6-thr-bradykinin or lys-6-thr-bradykinin, in the same way that mammalian plasma gives rise to met-lys-bradykinin and lys-bradykinin (see Pierce, 1968).

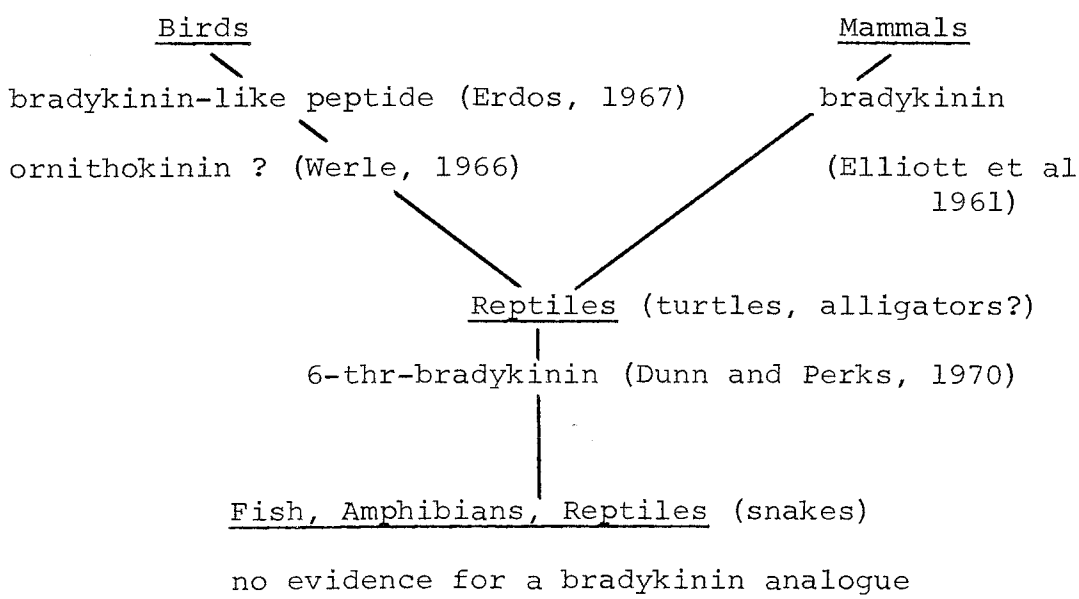
Non-mammalian kinins closely related to bradykinin have already been identified (Pisano, 1968), but to date all modifications are by addition of C or N terminal groups to the bradykinin moiety (Collier, 1968; Schachter, 1969; Pisano, 1968). This report is the first evidence to suggest that 6-thr-bradykinin occurs in nature, and it is the first example of a naturally occurring analogue of bradykinin in which there has been a replacement within the fundamental nonapeptide chain.

As a result of an exhaustive study of structure-activity relationships in synthetic analogues of bradykinin, Schröder and Hempel (1964) tentatively classified individual amino acid positions of the bradykinin molecule according to their relative influence on biological activity; the series, in order of descending importance, is as follows:

$$7,8 > 1 > 9 > 5 > 4 > 7 > 2 > 3 > 6$$

Changes in the residue at position 6 have the least effect on biological activity. Therefore, a mutation which could cause the interchange of serine and threonine at this position is a reasonable possibility, since it might well leave the molecule, and therefore the animal, physiologically functional. In addition, the interchange of these two residues requires but a single base change in the genetic code (Vleigenthart and Versteeg, 1967). This raises the possibility that other mutations could have produced yet different analogues in other vertebrate plasmas, and it is possible that further work could reveal a pattern of molecular evolution throughout the vertebrate tree.

The distribution of plasma kinins in major vertebrate groups is summarized in the following diagram:



Observations in the plasmas of birds, reptiles and fish are pitifully scarce. Therefore, connecting lines in this diagram do not imply evolutionary pathways for plasma kinins.

A discussion of the evolution of plasma kinins and the enzyme systems which release them is difficult at this time, since so few species have been examined. However, the fact that some reptiles (turtles and alligators) can produce an active plasma kinin, while other reptiles (snakes) cannot do so, encourages speculation that the plasma kallikrein-kinin system may have arisen within this group. However, investigations throughout this diverse class of vertebrates, and a more complete survey of amphibian and fish plasmas must precede any meaningful discussion of the origin of this enzyme system, and the active polypeptides produced by it.

Further studies of bird plasma are required to elucidate the nature of its kinins. Is the bradykinin-like factor described by Erdos et al (1967) bradykinin, 6-thr-bradykinin, or another closely related peptide? The answer to this question must be known in order to position the avian plasma kallikrein-kinin system relative to that of reptiles and mammals.

In birds, the nature of ornithokinin is a special problem, since its structure and pharmacology are entirely different from bradykinin. It is therefore not a true kinin (see Definitions and Abbreviations, p xvi). This type of system could

exist in many vertebrate plasmas, and be undetected at this time, since ornithokinin is inactive on mammalian tissues (Werle, et al, 1966) and would not be noted in standard bioassay procedures.

It should be pointed out that the bioassays used to characterize kinins from all sources (except ornithokinin) have involved mammalian tissues. Assays using tissues from the animal which produced the bradykinin analogue might result in a ranking of amino acid positions quite different from that presented on page 147 for bradykinin. Therefore, treatment of poikilotherm plasmas with trypsin and glass (and other kinin-producing agents) might appear to release no kinin simply because the factors liberated were not active on the isolated rat uterus.

Similar problems were involved in methods used in preliminary studies of the physiology of kinins in turtles (Chapter 6). The brief experiments on the effect of bradykinin on turtle blood pressure do not begin to suggest a physiological role for bradykinin, if only because bradykinin is not the natural plasma kinin of this turtle. Although results were encouraging in that low doses of bradykinin usually caused blood pressure changes, future experiments should investigate the effects of 6-thr-bradykinin on the turtle itself. Responses might be enhanced by the use of <u>in vivo</u> kininase inhibitors

(Erdos and Yang, 1966). The use of perfused organs, such as the blood perfused lung (after Hauge, et al, 1966) might also be a fruitful approach.

In the end, it is entirely possible that kinins have little effect on the vasculature of the turtle, and of other poikilotherms, and that their real physiological function lies in a hitherto unsuspected role. This problem is also of great concern in the numerous studies of mammalian plasma kinins, since many workers feel that the most important effects of the peptides may still be unknown. In any case, the data presented in this thesis shows that the physiological function in the turtle would depend on the actions of a new plasma kinin, 6-thr-bradykinin.

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APPENDIX A

Chemicals Used

Bradykinin (Sandoz Pharmaceuticals, BRS 640, 0.1 mg/ml)

Bradykinin (Schwarz Bioresearch, lot #6901)

Lys-bradykinin (Schwarz Bioresearch, lot #6902)

Trypsin (Nutritional Biochemicals Corp., 2X)

Kallikrein (Nutritional Biochemicals Corp., 10 K.U./mg)

Alpha-chymotrypsin (Nutritional Biochemicals Corp.)

8-hydroxyquinoline sulfate (Matheson, Coleman and Bell)

3-mercaptoproprionic acid (Sigma Chemical Co.)

2-mercaptoethanol (Sigma Chemical Co.)

Ethylenediam tetraacetic acid, sodium salt (Matheson, Coleman and Bell)

L-epinephrine (Sigma Chemical Co.)

Phenoxybenzamine hydrochloride (Smith, Kline, and French)

Atropine sulfate (Matheson, Coleman and Bell)

Acetylcholine chloride (Sigma Chemical Co.)

Propanolol (Inderal, I.C.I. 45,520, Ayerst Laboratories, Mtl)

Bromolysergic acid diethylamide (BOL #148, Sandoz)

Heparin (Nutritional Biochemical Corp.)

Sodium pentobarbital (Nembutal, Abbott, Mtl)

Urethane (Ethyl Carbamate, Matheson, Coleman and Bell)

MS-222 (Tricane methanesolfonate, Kent, Chemicals Ltd)

Diethyl ether (Fisher)

Ethyl alcohol (95%, Reliance Chemical Co.)

1-N-butanol (Fisher)

Acetic acid (Fisher)

Hydrochloric acid (Fisher)

Ammonium hydroxide (Fisher)

Sodium Hydroxide (Fisher)

NaCl (Fisher)

KCl (Fisher)

Na_2HPO_4 (Fisher)

NaH_2PO_4 (Fisher)

$CaCl_2$ (Fisher)

$MgCl_2 \cdot 6H_2O$ (Fisher)

Dextrose (Fisher)

Sodium phenolsulfonphthalein (J.T. Baker)

L-serine (Sigma Co.)

L-threonine (Sigma Co.)

N.B....all chemicalsused were of Reagent grade.

APPENDIX B

Composition of Physiological Solutions, (g/liter)

	$NaCl$	KCl	$CaCl_2$	$MgCl_2$	$NaHCO_3$	Na_2HPO_4	NaH_2PO_4
Mammals	9.0	0.42	0.24	-	0.20	trace	trace
Turtle	8.0	0.20	0.20	0.05	1.00	trace	0.04
Amphibians	6.5	0.14	0.12	-	0.20	trace	trace
Fish	7.4	0.36	0.17	-	0.31	1.60	0.04

Ammonium Acetate Buffer, 0.02 M. pH = 5.0

1 M acetic acid = 60.05 g/liter, and

0.02 M acetic acid = 1.201 g.liter.

Since S_g acetic acid = 1.049, 0.02 M acetic acid = 1.145 ml/liter.

Therefore 1.145 ml acetic acid were added to 990 ml distilled water, and the pH adjusted to 5.0 with NH_4OH, and the total volume made up to 1000 ml.

Phosphate Buffer for Trypsin Incubations, Chapter 3

Two solutions were prepared as follows:

a) 4.543 g of Na_2HPO_4 were added to 1000 ml hot distilled water.

b) 1.270 g NaH_2PO_4 were added to 1000 ml of hot distilled water. Solutions a) and b) were combined so that the pH was 7.35; the final buffer was 0.02 M for anions.

APPENDIX C

Determination of Plasma Kinin and Kininogen Levels

The procedure described below was taken from a method described by Brocklehurst and Zeitlin (1967).

A. Collection of Sample

5.1 ml of whole blood was taken quickly into a chilled polystyrene syringe through a siliconed needle. 0.1 ml was used for microhematocrit determination, the remainder being immediately inactivated by forcibly ejecting it through the needle into 15 ml of chilled ethanol in a 50 ml Nalgene centrifuge tube. The sample could be covered with parafilm and stored on ice for at least two days.

B. Preparation and Assay

The coagulated protein was scraped from the sides of the centrifuge tube, and the sample was centrifuged at 2000 g for at least 30 minutes. The supernatant should contain the free kinin while the precipitate contains the precursor. The precipitate was washed with 10 ml 80% ethanol and re-centrifuged. The washings were added to the first supernatant and kept on ice.

1. Kininogen. The washed precipitate was suspended in 10 ml 80% ethanol and placed in boiling water for 10 minutes to give complete denaturation. The suspension was centrifuged at 4,000 g and the supernatant discarded. The precipitate was washed

twice with 10 ml distilled water, then suspended in 15 ml of 2.5 M NaCl solution. This suspension was homogenized in a ground glass blender, and 0.2 ml aliquots of the homogenate were incubated at 37°C for 30 minutes with 1 mg of pure trypsin, in 5 ml 0.02 M sodium phosphate buffer, pH = 7.35. The trypsin was then inactivated by heating in a boiling water bath for 10 minutes. These solutions were assayed in this state, or stored at - 20°C until assay, which involved comparison with synthetic bradykinin on the isolated rat uterus in the presence of atropine (1 ug/ml).

2. Free Kinin. The kinin-containing ethanol extract was placed in a boiling water bath for 10 minutes to destroy latent enzyme activity, then evaporated to dryness under reduced pressure. The sample was reconstituted in 11 ml warm distilled water (60°C) saturated with NaCl and acidified to pH 1.5 with HCl. This solution was extracted in a separating funnel with 10, then, 5 ml of 1-N-butanol, which was then pooled and evaporated to dryness, beginning at 15°C, and rising to 50°C, under reduced pressure. The pressure was finally reduced to 0.1 mm Hg and the temperature raised to 80°C for 5 minutes to remove the last traces of butanol. These dry samples were stored at - 20°C until estimation of biological activity on the rat uterus, in the presence of atropine (1 ug/ml) and a 5-HT antagonist (bromolysergic acid diethylamide, 0.5 ug/ml).

APPENDIX D

Paper Chromatography, in Butanol-Acetic Acid-Water

1-N butanol, acetic acid, and water in a 4:1:5 ratio, and in a total volume of 200 ml were periodically shaken in a 250 ml separatory funnel, over a 2 hour period. This solvent system was then allowed to separate into 2 layers. The aqueous layer was divided among four Petri dishes and placed at the bottom of a 12" x 12" x 24" glass chromatography tank. A glass lid was sealed on with Lubriseal, and the system allowed to equilibrate at room temperature for at least 1 hour.

Standards (synthetic bradykinin and lys-bradykinin) and unknowns (turtle kinin) were applied in 10 ul Hamilton syringes to a sheet of Whatman 3 MM chromatography paper cut to 20½" x 8 5/8". A stream of air supplied by a portable hair dryer was used to dry in the samples at the origin.

The chromatogram was then placed in the equilibrated chromatographic tank, and the system allowed to equilibrate for another hour. The butanol layer of the solvent was then added to the bath at the top of the tank through an opening in the glass lid which was immediately stoppered. This system was allowed to run for 12 or 24 hours. The chromatogram was then removed, the solvent front marked, and the remaining solvent driven off by a flow of air from a fume hood fan.

Longitudinal strips of the dry chromatograms were cut

at the width of the origin + 0.5 cm on either side, to allow for diffusion. These strips were in turn cut into 10 equal parts between the origin and the solvent front. In the case of the 24 hour chromatograms, where the solvent front ran off the paper, the distance between the origin and the bottom of the paper was divided into 10 equal parts. Therefore, these divisions were arbitrary units, while those obtained for the 12 hour chromatogram represented individual R_f's. The rectangles obtained in either case were folded and placed individually in siliconed 5 ml beakers containing 1 ml 0.02 M ammonium acetate, pH = 5.0. After 6 hours at 4°C, the eluate was recovered from the paper by wrapping each in parafilm and squeezing manually. Eluates were stored in the siliconed beakers at 4°C until assay on the isolated rat uterus bioassay.

APPENDIX E

A method for kinin extraction described by Zacest and Mashford (1967) included an ether extraction of the alcohol concentrate (see Chapter 5). It was the object of this experiment to assess the efficiency of this added step in removing kinin from the alcohol extract.

Method. The following solution was prepared: 50 ug synthetic bradykinin + 5 ml 95% ethyl alcohol + 1 ml glacial acetic acid + 44 ml inactive protein solution from turtle plasma.

This mixture was twice extracted with 2 volumes of diethyl ether. The extract was pooled, and evaporated to dryness under reduced pressure. The thick green precipitate was taken up in 10 ml Locke's solutions, the pH adjusted to 5.0 with $NaHCO_3$, and the bradykinin content estimated on the isolated rat uterus. The biological activity remaining in the aqueous layer was also measured at this time.

Results. As follows:

Solution	Total Bradykinin (ug)	Percent
Ether extract	0.5 ug	1.0
Aqueous layer	44 ± 7.4	88 ± 9.3

Discussion. It was concluded that the ether extraction removed an insignificant proportion of the kinin from the ethanol extract, while the treatment did extract considerable amounts of impurities (lipids, etc).

APPENDIX F

Efficiency of Butanol in Extracting Bradykinin

The object of this experiment was to determine whether the use of a saturated sodium chloride solution was an absolute requirement for the extraction of kinins by butanol, since I had originally considered it to be inconvenient to remove the extra NaCl from the extract.

Method. Two solutions containing synthetic bradykinin were prepared: a) 15 ug BK + 25 ml saturated NaCl (60°C, pH = 1.5)

b) 15 ug BK + 25 ml distilled water (60°C, pH = 1.5)

Both of these solutions were extracted with 50, then 25 ml of 1-N butanol. The extracts were pooled in each case, and reduced to dryness under low pressure. The dried samples were reconstituted in Locke's solution, and the bradykinin content determined by assay on the isolated rat uterus.

Results. As follows:

Test Solution	Bradykinin Added	Bradykinin Recovered
Saturated NaCl	15 ug	17 ± 4.2 ug
No NaCl	15 ug	0.33 ± 0.1 ug

Discussion. It was concluded that;

a) the extraction is extremely efficient in the absence of plasma, being close to 100%, and

b) saturation of the aqueous layer with NaCl is an absoluted requirement for efficient extraction of bradykinin by butanol.