RENOMEDULLARY VASODEPRESSOR LIPID

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To Jill Strong,

for giving so much,

Colin Strong,

today's joy and tomorrow's hope,

and

Ramona and Gordon Strong,

for their faith.

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REVIEW OF THE LITERATURE

I - INTRODUCTION.

There is considerable evidence to support the hypothesis that the normal mammalian kidney has an antihypertensive action, that the renal medulla is an important site of this action, and that extracts of renal medulla contain biologically active substances mediating this effect. This evidence will be reviewed.

Many groups of investigators are engaged in attempts to extract, purify, identify and pharmacologically characterize these factors. Some of this work is being carried out in the research departments of pharmaceutical companies. Intense competition is engendered by the knowledge that the firm that can identify, synthesize and produce in commercial quantities an agent which mimics the antihypertensive action of the kidney will reap considerable commercial rewards. While this competition acts as a stimulus to progress in some ways, it does not promote dissemination of the results of this work through publication. A review of the literature on this subject will, therefore, contain a disproportionate degree of attention to the work of grant-dependent investigators.

Among the vasodepressor compounds of known structure, only the prostaglandins will be reviewed in detail, because of their very close relationship to renomedullary vasodepressor lipid.

II - RENAL ANTIHYPERTENSIVE ACTION.

A. WHOLE KIDNEY:

In Goldblatt's original experiments with the production of renal ischemic hypertension in the dog (1), he found that when only one renal

artery was narrowed, the resulting hypertension lasted only 1 to 2 weeks. When the contralateral normal kidney was removed, however, a severe sustained hypertension evolved, indicating that the normal kidney had opposed the development of chronic hypertensive disease. This phenomenon was observed by several other groups of early investigators (2,3,4).

If hypertension is produced in the experimental animal by constricting a renal artery, removal of the ischemic kidney causes the blood pressure to return to normal within six hours. If, however, the normal kidney is removed prior to removal of the ischemic kidney, it takes five times as long for the blood pressure to return to normal (5). Furthermore, Katz et al (6) showed that in the dog with a uretero-caval fistula on the side of the normal kidney, removal of the ischemic kidney also produced a rapid return of blood pressure to normal. This was the first of many demonstrations that the antihypertensive effect of normal kidney does not depend upon its excretory function (7,8,9,10,11,12,13).

Pure reduction of renal mass to one-sixth of normal, without alteration of the renal arterial supply, gives rise to a situation compatible with life, but one in which the experimental animal becomes hypertensive and shows a rise in blood urea nitrogen (14). It is as if the smaller renal mass were unable to exert a full antihypertensive effect, allowing hypertension to occur.

The production of hypertension in dogs totally deprived of renal tissue was first reported in 1936 by Harrison et al (15). In these experiments one-sixth of the bilaterally nephrectomized animals developed hypertension. These results were made difficult to interpret, however, by the short survival time of the renoprival dogs. In 1949 Grollman et al (8) maintained bilaterally nephrectomized animals for periods of

weeks by intermittent peritoneal dialysis and these animals regularly developed hypertension. These results were consistent with the view expressed earlier by Grollman et al (16) that removal of kidney tissue leads to a deficiency of an antipressor substance, with consequent development of hypertension.

Renoprival hypertension does not develop in adrenalectomized animals (17). It is considerably augmented by a positive sodium and water balance. Similar degrees of excess body sodium and water do not produce hypertension in animals with intact kidneys (18,19,20). The development of renoprival hypertension clearly depends upon some associated increase in extracellular fluid (21,22,23,24).

In renoprival man, Merrill et al (25) found that the level of blood pressure depended almost entirely upon the state of hydration as evidenced by sodium balance and weight. If hypertension developed as a result of excessive hydration, dehydration brought the blood pressure down to previous normal levels (26).

Kolff and Page (19) found that inclusion of donor kidneys into the circulations of overhydrated renoprival dogs lowered the blood pressure. Similar inclusion of transplanted donor hind limbs did not lower the pressure significantly. The reduction of blood pressure with kidney transplantation was not related to urine volume or to loss of extracellular fluid or sodium. These authors concluded that the kidney exerts a specific, non-mechanical, non-excretory effect. This was confirmed by the observations of Muirhead et al (11).

Tobian et al (27) have demonstrated that the antihypertensive action of the normal kidney can be modified by changing the level of the perfusion pressure of that kidney. They connected normal kidneys into

the circulatory systems of rats with renal hypertension. When the transplanted kidney was perfused with the full force of the high arterial pressure, there was a distinct drop in the blood pressure of the hypertensive rat. When they interposed a variable resistance in the arterial supply of the transplanted kidney and lowered its perfusion pressure, there was no drop in the rat's arterial pressure. This appropriate behaviour, with the antihypertensive mechanism of the normal kidney being brought into play by a high perfusion pressure and dampened by a low perfusion pressure, suggested the existence of a biological servomechanism.

B. RENAL MEDULLA:

Muirhead et al have made great contributions to the understanding of the role of the kidney in the maintenance of normal blood pressure. They produced hypertensive cardiovascular disease in dogs by performing bilateral nephrectomy and feeding a diet high in protein and sodium. If, instead of bilateral nephrectomy, one kidney was left in situ with its ureter anastomosed to the inferior vena cava, the animal was protected against hypertension-inducing effects of large sodium loads (28,29). The kidney with the uretero-caval fistula rapidly became hypertrophic, the greatest mitotic activity occurring in the outer renal medulla, just below the vascular arcade (12).

The importance of the renal medulla, and especially the outer renal medulla, as the site of the renal antihypertensive action was further emphasized by the autoexplantation studies of Muirhead et al (30,31). In the autoexplantation technique, one kidney is removed and the part to be explanted is finely divided in a blender, washed twice, and injected intravenously or intraperitoneally. The other kidney is removed two to

seven weeks later. Subsequent examination of the explantation sites reveals that within the pulmonary vessels, or studding the peritoneum, there are clusters and groups of granular and clear cells, the latter predominating. They resemble tubular epithelial cells but they do not form an organized excretory arrangement. Explantation of whole kidney, whole renal medulla or outer renal medulla protects nephrectomized dogs from developing the accelerated hypertension with myocardial and arteriolar necrosis which would otherwise result when the animals are fed a diet high in protein and salt. Explantation of renal cortex, spleen and liver affords no such protection (28).

Muirhead et al (32) further showed that administration of a crude extract of renal medulla was similarly protective against the development of renoprival hypertension. Extracts of renal cortex, lung, erythrocytes, bladder, and muscle gave no protection under similar experimental conditions.

Some doubt regarding the medullary site of renal antihypertensive action has been expressed by Hirofumi and Grollman (33), who found that ablation of rat renal cortex caused a greater blood pressure rise than did ablation of renal medulla. They also presented evidence that renocortical extracts had antihypertensive properties, and that renomedullary extracts did not. These findings are contrary to the greater weight of experimental evidence suggesting that the renal medulla and extractable renomedullary substances are the most important factors in the antihypertensive action of the kidney.

III - ANTIHYPERTENSIVE AND VASODEPRESSOR TISSUE EXTRACTS.

The ability of a tissue extract to oppose the development of exper-

imental hypertension or to reduce elevated blood pressures in experimental animals or patients with hypertensive disease when administered chronically, either orally or parenterally, is its "antihypertensive effect". The term "vasodepressor effect" refers to the production of an acute blood pressure fall in a bioassay animal when the active material is injected intravenously. Although there is recent evidence (34) that the substances responsible for these two effects are separable by column and thin-layer chromatography, these functions and the factors responsible for them are closely related.

A. HISTORICAL ASPECTS:

Around the turn of the century workers in France, Britain and the United States began a systematic investigation of the effects on blood pressure of bioassay animals of injections of aqueous or alcoholic extracts of most tissues. In 1895 Schafer (35) demonstrated the depressor effects of thyroid extracts. In 1896 Schafer and Moore (36) and, later, Osborne and Vincent (37) reported that extracts of all parts of the nervous system produced a marked temporary fall in blood pressure, acting directly on the blood vessels and not through the vasomotor nerves. In 1899 Mott and Halliburton (38) suggested that the active substance in these extracts was choline. In 1899 Cleghorn (39) showed that extract of sympathetic ganglia produced a blood pressure fall on intravenous injection, acting "on the blood vessels themselves or possibly on their sympathetic vasomotor neurons".

In 1903 Vincent and Sheen (40) found both pressor and depressor substances in saline extracts of kidney tissue. They concluded that renal depressor extracts produced a blood pressure fall by dilatation of

vascular beds other than in the splanchnic area. They found depressor substances also, however, in saline extracts of liver, nervous tissue, spleen, testis, muscle, pancreas, ovary, lung, intestinal mucous membrane, whole intestine, thyroid, thymus and adrenal. They also found a vasodepressor substance in alcoholic extracts of posterior pituitary and this was later shown to be histamine (41).

In 1909 Pearce (42) produced extracts of dog and rabbit kidney which caused a fall in blood pressure on injection into a dog. When injected into a rabbit, however, both extracts were pressor!

In 1909 Abelous and Bardier (43) described a vasodepressor substance called "urohypotensine", extracted from urine. Later a substance called "depressan" or "detonin" was extracted from the urine of normotensive subjects and patients with renal hypertension (44). It was markedly diminished or absent in urine from patients with essential hypertension. When given by intramuscular injection, it decreased the blood pressure of patients with essential hypertension. "Depressan" and "urohypotensine" were similar in that both resisted boiling, were non-dialyzable, and were precipitated by alcohol or ammonium sulfate.

With rare exceptions (41), there had been very few attempts to isolate the active principle or principles and what little work had been done along these lines suggested that the tissue extracts owed their depressor activity to their content of histamine and choline. In 1926, however, Vincent and Curtis (45) showed that saline, alcohol and methylated-ether would extract vasodepressor substances from the tissues of cats, rabbits and guinea pigs and that the activity of these extracts was not due solely to their histamine and choline content.

In 1931 von Euler and Gaddum (46) described cold acid-alcohol

extracts of small intestine and brain ("substance P") which lowered the blood pressure of atropinized rabbits by peripheral vasodilatation. The extracts also stimulated the tone and rhythm of isolated rabbit intestine after atropine. The extracts were further investigated by Gaddum and Schild (47). "Substance P" was found to be readily dialyzable, soluble in absolute alcohol, and stable in weak acid solution. It was rapidly destroyed by strong acid or alkali. It was clearly distinguished from histamine and adenosine by its relative acid stability, relative alkali stability and solubility in acetone. "Substance P" has more recently been shown to be a regular polypeptide constituent of the central nervous system, autonomic ganglia and peripheral nerves of a variety of animals. It differs pharmacologically from bradykinin (48) and it can be separated from bradykinin, kallidin, and histamine by paper electrophoresis (49). In man, intravenous infusions of "substance P" produce facial flushing, increased blood flow in the muscles and skin, hypotension and tachycardia (50). Its effects on blood pressure are probably the result of peripheral vasodilatation (51).

In 1933 Goldblatt (52,53), working independently of von Euler, described a vasodepressor substance extracted from human seminal fluid. During the same period, von Euler (54) found adrenaline-like activity in extracts from the vesicular and prostate glands of man and several species of animals. He later found a potent vasodepressor substance in aqueous-alcoholic extracts of monkey vesicular gland, named it "vesiglandin", and showed that it was different from "substance P" (55). In 1936 von Euler gave the name "prostaglandin" to a substance which lowered blood pressure in rabbits, cats and dogs, dilated hind limb vessels of frogs and increased the activity of animal intestine <u>in vivo</u> and <u>in vitro</u> (56).

It was found to be extractable from the seminal fluid, prostate and seminal vesicles of man and the vesicular gland of sheep. It was soluble in water, alcohol, acetone, ether and chloroform. It was readily destroyed by free halogens and normal acid and alkali. It differed from vesiglandin in that the latter was even less stable in acid and alkali, and had weak to absent action on nonvascular smooth muscle. The blood pressure lowering effects of the two substances were found to be similar. Prostaglandin and vesiglandin were shown to be different in their physicochemical and biological properties from other depressor substances such as kallikrein, adenosine, acetylcholine, histamine and "substance P".

In 1938 Jablons (57) used an acid-alcohol extract of pork kidney, freed of proteins, peptides and lipids, in the treatment of patients with nephritis and hypertension. This extract contained a water-soluble factor which lowered the blood pressure in 50% of patients treated, simultaneously causing a marked diuresis and natriuresis.

Harrison et al lowered the blood pressure of rats (58) and dogs (59,60) with experimental renal hypertension by oral or parenteral administration of renal extract (61,62). Their "renal anti-pressor substance" did not lower the blood pressure of normotensive animals when given orally. It was shown to inhibit the pressor response to injected renin, ephedrine or epinephrine. The rats or dogs which had severe hypertension prior to treatment showed severe untoward reactions (apathy, weakness, vomiting, shock and increased blood NPN) and even death upon treatment. If the pre-treatment hypertension was mild, the decline in blood pressure was unassociated with these adverse reactions.

Page et al (63,64,65,66) and Murphy et al (67) extracted a renal substance capable of inhibiting the pressor effect of angiotensin <u>in vivo</u>. They showed that it had an angiotensin-destroying effect <u>in vitro</u>. The

extract decreased renal vascular resistance by dilating both afferent and efferent arterioles when injected into the renal artery of normal dogs. When the extract was given by daily intramuscular injection for several weeks to hypertensive animals and patients, it had a pronounced blood pressure lowering effect.

While these extracts were effective in the therapy of patients with advanced hypertensive cardiovascular disease (68), they were also very costly. Very large amounts of source tissue were required (doses equivalent to 0.5 to 2.0 kilograms of fresh kidney tissue were needed for each patient daily). Furthermore, the patients often experienced severely painful reactions at the sites of injection and dangerous anaphylactoid reactions sometimes occurred. When further purification of the extracts reduced the severity of the reactions, the material showed reduced therapeutic efficacy. Chasis et al (69) attributed the reduction of blood pressure by these extracts to the mild pyrogenic reaction they produced.

Treatment with these extracts did, nevertheless, greatly improve some patients. When the blood pressure was reduced, there was an accompanying increase in cardiac output and effective renal plasma flow. The ballistocardiographic and ECG patterns returned toward normal. Heart size was reduced. Vision was improved in many patients with the malignant syndrome and, in many instances, fundal hemorrhages, exudates and papilledema disappeared (68).

Despite the early therapeutic promise of these extracts, Goldblatt was later (1947) to write in review (5): "The fact that there has been little progress in the treatment of hypertension by renal extracts during the past six years is in itself an indication of the difficulties involved and possibly of the inadequacy of the method".

B. CURRENT RENAL EXTRACT OF:

1. Muirhead et al:

Muirhead et al are currently producing an extract from the renal medulla of man, pig and dog which contains an active principle with a molecular weight of less than 1000. It acts against canine renoprival hypertension in doses of 25-300 µg./kg./day (70). To obtain a lowering of blood pressure, the extract must be given for 1-5 weeks but the pressure reduction persists for 2-7 weeks following cessation of therapy (71, 72). This extract does not prevent the exaggerated acute response to injected renin, characteristic of the nephrectomized animal, but it does reduce the residual blood pressure elevation noted 24 hours after the renin injection (31). It appears to act directly on the arterial-arteriolar wall rather than by antagonizing the renin-angiotensin system (73). This extract gives partial protection against the tissue damage produced by the experimental hypertensive state: necrosis of arterioles, myocardium and gastrointestinal smooth muscle, fibrinoid deposits in the gastrointestinal mucosa, and visceral arteriolar thickening (74).

The active factor is obtained by cold aqueous ethanol extraction of ground quick-frozen renal medulla (porcine) or whole kidney (human) at pH 5.5 and 0°C. After overnight extraction and filtration, the filtrate is defatted with Skellysolve B. (The Skellysolve extract is discarded). The defatted aqueous solution is extracted with ethyl acetate. The ethyl acetate extract is evaporated and the residue is purified by Sephadex partitioning, silicic acid column chromatography and thin-layer chromatography. Muirhead et al (34) report that the factor responsible for the extract's antihypertensive effect can be separated from the fraction which gives a vasodepressor effect on rapid intravenous injection into

anaesthetized, heparinized, pentolinium-treated dogs.

2. Milliez et al:

Milliez et al (75,76,77) are extracting an antihypertensive and vasodepressive substance from pork kidneys. This principle is thermolabile, stable between pH 4 and pH 11, and dialyzable. It has a molecular weight less than 4500 (determined by Sephadex G 25 gel filtration). It is soluble in acetone, chloroform and water and insoluble in petroleum ether. The extraction method involves cold acetone extraction of the kidney tissue, filtration and evaporation of the filtrate. The crude extract thus obtained is then either dialyzed against distilled water (which then contains the active factor) or subjected to binary chloroform-methanol-water extraction, the active material remaining in the chloroform phase.

3. Grollman et al:

Grollman's antihypertensive agent of renal origin is active when administered orally. It reduces the blood pressure of rats with experimental chronic hypertension. This principle has not yet been isolated in pure form (78). Since it is active when given orally it is probably not a protein. It is readily dialyzable through collodion and cellophane membranes. The active agent is prepared (79) by overnight extraction of ground fresh porcine kidney with acid acetone. After filtration through gauze and re-extraction with aqueous acetone, the combined aqueous acetone filtrates are concentrated <u>in vacuo</u> at less than 30°C. The concentrate is defatted by triple extraction with one-tenth volumes of petroleum ether (which are discarded). Further concentration is carried out by countercurrent distribution between secondary butyl alcohol and water,

the aqueous fraction being discarded. The secondary butyl alcohol fraction is concentrated by evaporation, chilled in a refrigerator overnight and then filtered. The filtrate is diluted with an equal volume of water and then distilled until the organic solvent is removed. The aqueous extract is then mixed with the animals' food for bioassay or concentrated further by chromatography on activated charcoal and on cation-exchange resin (Dowex 50). The yield at this stage is approximately 100 mg. per kilogram of original pork kidney.

4. Hickler et al:

Hickler et al (80,81,82,83) stimulated our interest in this field by their reported extraction of an unsaturated, hydroxylated, myotropic fatty acid with potent vasodepressor properties from the renal medulla. Their extraction procedure, as recently modified (84), was that chosen for use in our work and it is outlined in detail in the Materials and Methods subsection of the Investigative Section of this thesis.

Initially, these investigators produced a protein-free ultrafiltrate of saline homogenate of rabbit renal medulla which effected a prolonged reduction in the mean arterial blood pressure of the bioassay rat and anaesthetized dog on intravenous injection. Similar extracts of rabbit renal cortex produced only short duration depressions of pressure, probably associated with their nucleotide content (85). The active principle retained its potency after treatment with carboxypeptidase, ribonuclease, phosphatase and panprotease. Activity was destroyed at pH 11 for 1 hour at room temperature. Sephadex chromatography indicated a molecular weight less than 4700.

The lipid nature of the vasoactive material was suggested by the observation that on acid chloroform extraction of the aqueous ultrafil-

trate, essentially all of the vasoactivity was detected in the chloroform phase (82). The polar nature of the lipid was demonstrated by its recovery at the front on paper chromatography in a solvent system of isopropanol:butanol.

Thin-layer chromatography on silica gel in a solvent system of petroleum ether 85%:diethyl ether 15%:acetic acid left all of the vasoactivity at the origin with the phospholipid standard, whereas triglycerides, fatty acids [including arachidonic acid which is vasodepressor in the bioassay rabbit (86)] and diglycerides moved up from the origin. The active principle was not a phospholipid either, however, because when phospholipids were removed by precipitation in cold acetone (87), all of the vasoactivity remained in the cold acetone-soluble non-phospholipid fraction. Microphosphate analysis of vasoactive material did not reveal any significant content.

The active material migrated toward the anode during block electrophoresis on "pevikon" (a copolymer of polyvinyl chloride and polyvinyl acetate) with a triethylamine buffer (pH 8.6) run at 80 volts, 10 milliamps and 0°C. Gas-liquid chromatographic analysis did not reveal any significant fatty acid content in the C_{12}-C_{20} series. Infrared absorption studies, carried out on crude extracts, revealed a long chain compound (about C_{26}) with high absorption in the range for keto and carboxylic acid radicals, and especially for free hydroxyl groups.

On thin-layer chromatography (silica gel) in a solvent system of chloroform:methanol (3:2), the vasodepressor lipid moved up from the origin but crystalline prostaglandin E-1 remained at the origin (83).

It was the view of Hickler et al (83) that renal vasodepressor lipid opposed the effect of injections of angiotensin or renin by a direct act-

ion on the vascular wall rather than by any neutralizing action on renin and angiotensin.

5. Lee et al:

During the course of our work, Lee et al (88) reported the isolation and characterization of three unsaturated acidic lipids, two of which were thought to be responsible for the sustained vasodepressor effect of extracts of renal medulla. They were thought to be closely related derivatives of prostanoic acid. The first, called "medullin", was a weak stimulator of nonvascular smooth muscle. The second, called "compound 2", was tentatively identified as prostaglandin E-1. It was both a potent vasodepressor and a strong stimulator of nonvascular smooth muscle. The third lipid, called "compound 1", possessed strong nonvascular smooth muscle stimulating properties but minimal to absent vasodepressor activity, characteristics similar to those of the prostaglandin F compounds.

"Medullin" could be separated from the other potent vasodepressor, "compound 2", by its behaviour on thin-layer chromatography. When chromatographed on activated silica gel, with and without incorporated 2% silver nitrate, "medullin" had approximately fifty percent greater mobility than "compound 2" in two systems, (benzene 80:dioxane 80: acetic acid 4 and trimethylpentane 120:isopropyl alcohol 40:acetic acid 1). This suggested that there were fewer polar (hydroxyl) groups on the molecule of "medullin". It was stained more heavily by iodine vapor than "compound 2" and was therefore considered to be more unsaturated.

Ultraviolet analysis of "medullin" revealed a spectrum closely resembling that of prostaglandin E-1, except that a methanolic solution of "medullin" showed an absorption maximum at 2780-2800 Å, a pattern not

displayed by prostaglandin E-1 in ethanol.

Intra-arterial and intravenous injections of "medullin" reduced peripheral resistance markedly and induced a compensatory increase in cardiac output. "Medullin" had no effect on cardiac rate or contractile force of the isolated rabbit heart. Pure "medullin" had no effect on isolated aortic strips whether relaxed or partially contracted with norepinephrine or angiotensin.

The general features of the extraction method for "medullin" are as follows: renal medullary tissue is homogenized in 0.005 M Na_2HPO_4. After centrifugation, the sediment is discarded. Ethanol is added to the supernant to a final concentration of 80%. The precipitate thus formed is removed by filtration and is discarded. The filtrate volume is reduced by distillation in vacuo and the pH of the remaining solution is adjusted to 7.5. This is then extracted three times with petroleum ether (which is discarded). The aqueous ethanol phase is evaporated in vacuo to 10-15% volume and chromatographed on DEAE-cellulose, gradient elution with Na_2HPO_4/NaH_2PO_4 effecting a ten-fold purification. The active fraction in 0.005 M Na_2HPO_4 is acidified to pH 1-2 and extracted three times with benzene, chloroform or diethyl ether. The organic phase is evaporated in vacuo and the residue is purified by thin-layer chromatography. The yield of "medullin" is approximately 1 mg. per kg. of original kidney medulla.

The close relationship between the prostaglandins and the vasodepressor lipids of Hickler and Lee forms a junction in the roads travelled by groups of investigators approaching this subject from points of view that seem to differ. On the one hand, there are those who have been searching for a naturally-occurring renal factor capable of maintaining

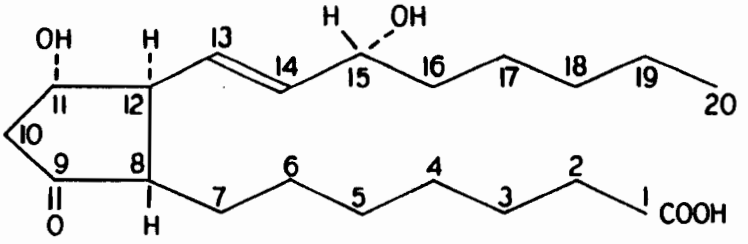
or producing normotension in hypertensive animals and patients, and, on the other hand, those who have been extracting, purifying, and chemically and pharmacologically characterizing the prostaglandins which have a wide range of biologically significant effects.

IV - THE PROSTAGLANDINS.

A. STRUCTURE AND NOMENCLATURE:

Prostaglandin is the name given by von Euler (56) to the acidic, lipid-soluble, smooth muscle stimulating and blood pressure lowering factor which he and Goldblatt (52,53) had independently extracted from human seminal plasma and sheep vesicular glands. A number of crystalline active compounds have recently been isolated from prostaglandin extracts (89,90,91) and the structures of many have been determined, beginning with that of prostaglandin E-1 by Bergstrom et al (92,93) in 1962. This structure is illustrated in Figure 1.

PROSTAGLANDIN E-1



(11α, 15-DIHYDROXY-9-KETO-PROST-13-ENOIC ACID)

PGE-2 AS ABOVE PLUS DOUBLE BOND Δ_5

PGE-3 AS ABOVE PLUS DOUBLE BONDS Δ_5 AND Δ_{17}

PGF-1,2,3 HYDROXYL GROUP REPLACES KETO GROUP ON CARBON 9

Figure 1.

Structure of Prostaglandin E-1.

The prostaglandins are derivatives of a parent C_{20} acid which has been named "prostanoic acid", and there is a systematic nomenclature based on this name:

Table I

Systematic Nomenclature of the Prostaglandins.

Prostaglandin E-1 = 11α,15-dihydroxy-9-keto-prost-13-enoic acid.
Prostaglandin E-2 = 11α,15-dihydroxy-9-keto-prosta-5,13-dienoic acid.
Prostaglandin E-3 = 11α,15-dihydroxy-9-keto-prosta-5,13,17-trienoic acid.
Prostaglandin F-1α = 9α,11α,15-trihydroxy-prost-13-enoic acid.
Prostaglandin F-1β = 9β,11α,15-trihydroxy-prost-13-enoic acid.
Prostaglandin F-2α = 9α,11α,15-trihydroxy-prosta-5,13-dienoic acid.
Prostaglandin F-2β = 9β,11α,15-trihydroxy-prosta-5,13-dienoic acid.
Prostaglandin F-3α = 9α,11α,15-trihydroxy-prosta-5,13,17-trienoic acid.
Prostaglandin F-3β = 9β,11α,15-trihydroxy-prosta-5,13,17-trienoic acid.

The E-compounds differ from one another in the number of double bonds present in the molecule, as do their like-numbered counterparts in the prostaglandin F series. The E-compounds have a keto group on carbon-9, whereas the F-compounds have a hydroxyl group on carbon-9. All the naturally occurring F-compounds have the hydroxyl group at C-9 in the α position. (It is chemical reduction of the keto group of prostaglandin E at C-9 with borohydride which gives the two trihydroxyacids epimeric at C-9).

B. OCCURRENCE:

The prostaglandins are present in a variety of tissues, secretions and species. They occur in highest concentrations in the seminal vesicular gland (90,91), which contains PGE-1, PGE-2, PGE-3 and small amounts of PGF-1, and seminal plasma (94,95), which contains PGF-2, in addition to the four previously mentioned compounds. Prostaglandin F-2 has been isolated from lung tissue of sheep, pigs, guinea pigs, monkeys and humans.

By an isotope dilution technique, the concentration of PGF-2 in sheep lung tissue was determined to be 0.5 µg./g. (96). Prostaglandin E-2 has recently been identified in sheep lung tissue (96), and prostaglandin F-3 has similarly been identified in bovine lung tissue (97). Calf thymus contains only prostaglandin E-1 in a concentration of 0.8 µg./g. (98).

Extracts of iris (99) and brain (100,101) contain smooth muscle stimulating compounds with properties similar to those of the prostaglandins. An alkali-stable principle similar to the prostaglandins has been found in perfusates of cat cerebellum, and ox brain tissue extracts were found to contain prostaglandin F in a concentration of 0.8 µg./100 g. tissue (102,103). Prostaglandin F-2 has been found in bovine brain in a concentration of about 0.3 µg./g. tissue (104).

Prostaglandin E-2, prostaglandin F-2 and prostaglandin precursors have been demonstrated in menstrual fluid (105) and endometrial curettings (106).

C. BIOSYNTHESIS:

It has been shown that essential fatty acids can act as precursors of the prostaglandins. Bergstrom et al (107) and Van Dorp et al (108) reported that tritium-labeled arachidonic acid is converted into labeled PGE-2 by sheep seminal vesicular gland homogenates. Homogenates of guinea pig lung also convert tritium-labeled arachidonic acid into prostaglandins (mainly PGF-2, but also PGE-2 and metabolites of PGE-2) (109). Homo-γ-linolenic acid may be converted into PGE-1 (110,111) and PGF-1α (112). Similarly, PGE-3 may be synthesized from 5,8,11,14,17-eicosapentaenoic acid by homogenates of sheep seminal vesicular glands (113).

D. METABOLISM:

Administration of tritium-labeled PGE-1 (prepared by the selective reduction of the Δ5 double bond of PGE-2 with carrier - free tritium gas and palladium as catalyst) to rats led to recovery of about 50% of the administered tritium in the urine and about 10% in the feces (114). Bile and urine contained equal amounts. High concentrations of tritium were found in the kidneys and the liver following administration of labeled PGE-1. The lungs, pituitary gland, adrenals, ovaries and uterus had tritium concentrations slightly above blood level. In sheep, following tritium-labeled PGE-1 administration, tritium concentrations were high in the organs mentioned, with significant concentrations occurring also in the vesicular gland and ampulla ductus deferens in the male, or Fallopian tubes, uterus and ovaries in the female.

Prostaglandin E-1 is metabolized into more polar derivatives. Lung tissue converts PGE-1 into 11α,15-dihydroxy-9-keto-prostanoic acid (by reduction of the Δ13 double bond) and 11α-hydroxy-9,15-diketo-prostanoic acid (by reduction of the Δ13 double bond and oxidation of the secondary alcohol group at C_{15}). The responsible enzymes occur in the particle-free fraction of lung homogenate and they have been demonstrated in several species including man, sheep, rabbit and rat. In the guinea pig the same transformations take place in the kidney and small intestine (115). That these transformations occur *in vivo* in the rat has been shown by the demonstration of the metabolites in blood (114). These metabolites are not present in urine, indicating that they are further metabolized before being excreted. PGE-2 and PGE-3 are metabolized in lung tissue in the same manner as PGE-1 (113,115).

E. BIOLOGICAL EFFECTS:

Knowledge of the widespread occurrence of the prostaglandins has prompted investigation into their effects on the various organ systems. The investigators have been seeking to link occurrence with physiological function and considerable speculation has been evoked.

1. Effects on the cardiovascular system:

Goldblatt (52,53) and von Euler (55,56) recognized the prolonged lowering of blood pressure that follows injection of prostaglandin-containing extracts. On a weight basis, the prostaglandins are as active as epinephrine, norepinephrine, acetylcholine and oxytocin in their pharmacodynamic effects. The E-compounds are active on both vascular and nonvascular smooth muscle, whereas the F-compounds act only on nonvascular smooth muscle. Intravenous injections of prostaglandin E have less effect than intra-arterial injections. This may be due to the rapid inactivation of the compound by the lung in the case of intravenous administration.

Intravenous infusion of PGE-1 at a rate of 0.2 to 0.7 µg./kg./min. in man caused an increase in heart rate, vasodilatation of the skin and fall in systemic arterial blood pressure (116). There was no marked change of pressure in the right ventricle, although there was a tendency to a rise in pulmonary artery pressure during the infusion. The cardiac output decreased about 20%. Symptoms were marked, with headache, a feeling of constriction in the pharynx and a pressure sensation in the thorax as the most disturbing sensations.

Infusion of PGE-1 intravenously and intra-arterially in man at rates too low to alter the blood pressure (0.1-0.2 µg./kg./min.) produced tachycardia. Simultaneous intravenous infusion of norepinephrine and PGE-1

(both at 0.2 µg./kg./min.) caused a blood pressure increase that was 50% less than that seen with infusion of norepinephrine alone. It also abolished the bradycardia usually induced by norepinephrine infusion (117, 118).

After injection of prostaglandin E-1 into the anaesthetized rabbit, there was a decreased pressor response to subsequent injections of epinephrine, norepinephrine, angiotensin II and vasopressin (119). Both blood pressure and blood flow were altered by this nonspecific effect of PGE-1. Similar antagonism of responses to catecholamines was observed when other depressor substances such as bradykinin were used instead of PGE-1. There are two alternative explanations of this effect. It is possible that PGE-1 blocks a biochemical sequence of events initiated by vasoconstrictor substances but has no vascular effects in the absence of these substances (120). If this is the actual mechanism, the block must be somewhere on the final common path for the action of vasopressin, the catecholamines and angiotensin. On the other hand, such an antagonism might be expected to occur from the algebraic summation of effects when a pressor and depressor substance are injected either simultaneously or within a short time of one another.

Intra-arterial injections of prostaglandin increased blood flow in the isolated hind limbs of frogs (56) and cats (121). PGE-1 had no effect on the contractile force of the isolated frog heart (56).

Unlike other vasodilator substances (such as bradykinin, histamine and acetylcholine), prostaglandin E-1 neither evoked release of epinephrine from the adrenal medulla nor produced a marked increase in capillary permeability. It did not produce any sensation of itch or pain when applied to exposed blister bases (122).

2. Effect on gastrointestinal smooth muscle:

Among the principal biological actions of the prostaglandins are its stimulation of most smooth muscle organs *in vitro* and *in vivo* (123). There are exceptions to this generalization and they will be stated. The F-compounds and E-compounds have approximately equal activity in stimulating intestinal smooth muscle *in vitro*. Isolated rabbit jejunum and duodenum respond to 0.25 ng. of PGF-2 per ml. (124) and this sensitive preparation has therefore been widely used for the bioassay of prostaglandin.

3. Effects on the reproductive system:

Prostaglandin may be important in human fertility. It has been demonstrated that prostaglandins, known to be in high concentration in human seminal plasma, are absorbed via the vaginal mucosa, significantly affecting blood levels (as evidenced by a blood pressure reduction) and having detectable effects on the motility of the uterus and Fallopian tubes (125,126). Prostaglandin E decreased the tone of rabbit oviducts *in vivo*, whereas PGF increased the tone (121). Human Fallopian tubes *in vitro* were contracted by prostaglandin E-1 at their uterine ends but they were relaxed elsewhere, like the uterus itself (127). The action of the prostaglandins on the oviduct is direct. It is mediated neither through the central nervous system nor by changes in blood pressure (128). In contrast with the large amount of prostaglandin in human semen, the amount in the semen of rabbits and sheep is too small to have any significant effect upon oviduct smooth muscle even if complete and rapid vaginal absorption were to occur (128). This finding, however, does not exclude a direct effect on the smooth muscle of the cervix uteri in these species.

Isolated myometrial strips from non-pregnant women were inhibited by the E-compounds (129). This inhibition was greatest at the time of ovulation (130). These findings, combined with the earlier observation that the effects of the prostaglandins on the smooth muscle of the male ejaculatory mechanism promoted emptying of the seminal vesicles (55,56), point strongly to a physiological role for the prostaglandins in fertility control.

4. Effects on the respiratory tract:

Pharmacological studies have shown that the E-compounds and PGF-1 relax tracheal muscle in vitro and, except in the cat, decrease lung "resistance to inflation" in vivo. In the anaesthetized cat, PGE-1 increased the lung "resistance to inflation" (131). Prostaglandin F-2α is the prostaglandin which occurs most commonly in the lungs. It had only one-thirtieth the activity of PGE-1 on isolated cat tracheal muscle (132). This suggests that PGF-2 might be stored as a less active precursor of PGE-2, a compound of more immediate physiological importance (133).

5. Effects on the central nervous system:

The prostaglandins have striking central nervous system effects. Injections of the E-compounds (in doses greater than 3 µg./kg.) directly into the ventricles of unanaesthetized cats produced sedation, stupor and catatonia after a latent period of 20 minutes or more. The stupor lasted for several hours and the cats showed diminished activity for up to 48 hours. Injections of PGF-2 in equally high doses produced no effect. With intravenous injection of PGE-1 in doses greater than 20 µg./kg., only slight sedation was observed. In chicks, which are believed to lack a blood-brain barrier, intravenous injection of prostaglandins

(10-400 µg./kg.) caused respiratory depression, profound sedation, loss of normal posture and, in the higher doses, loss of the righting reflex (134). Intravenous injections of prostaglandin F-2 increased neuromuscular tone of unanaesthetized chicks, acting centrally on the spinal cord. It potentiated the spinal reflexes, resembling strychnine in action and potency (135).

6. Effects on fatty acid metabolism:

Prostaglandins, which can be synthesized from essential fatty acids, may also play a role in the control of fatty acid release. With rat epididymal fat pads in vitro, prostaglandin E inhibited the release of glycerol and free fatty acids, and inhibited the stimulation of this release by epinephrine, norepinephrine and glucagon (136,137). In man, infusions of PGE-1 at rates too low to alter blood pressure (0.1-0.2 µg./kg./min.) increased plasma free fatty acids and plasma glycerol concentrations, suggesting increased lipolysis in adipose tissue (117). This was a surprising discovery since simultaneous infusions of PGE-1 and norepinephrine (both at 0.2 µg./kg./min.) slightly reduced the rise in free fatty acid and plasma glycerol concentrations seen with infusions of norepinephrine alone. This latter finding was more in keeping with previous observations during infusions of norepinephrine and PGE-1 in dogs (43). All the E-compounds block the catecholamine-induced release of free fatty acids with equal potency, whereas the F-compounds are at least 10 times less active in this regard.

7. Effect on antidiuretic hormone activity:

Prostaglandin E-1 reversibly counteracts the permeability response of the toad bladder to vasopressin and theophylline, but not to cyclic-AMP

(adenosine-3',5'-monophosphate). Prostaglandin E-1 alone has no effect on this permeability to water flow in the direction of an osmotic gradient. It has been postulated that prostaglandin E might serve as a regulator of antidiuretic hormone action (138).

F. EXTRACTION AND PURIFICATION:

Prostaglandins may be removed from homogenized tissues (such as sheep prostate and vesicular glands) (90,91) or semen (94,95) by extraction with 80% ethanol. After stirring and sedimentation, often overnight, the material is centrifuged, re-extracted and recentrifuged. The combined supernants are reduced to a small volume by evaporation in vacuo. The crude residue is extracted with ether and the aqueous phase is acidified to pH 3.5 with HCl and re-extracted with ether. The aqueous phases are discarded. The combined ether phases are extracted six times with half-volume amounts of 0.2 M sodium phosphate buffer (pH 8). The ether phases are discarded. The combined buffer phases are acidified to pH 3 with HCl. They are again extracted with ether (3 times) and the buffer phases are discarded. The combined ether phases are washed with small amounts of water to remove the free chloride ions. The ether phases are evaporated to dryness in vacuo, leaving a dark brown paste.

Purification of the tissue extracts (91) may be initiated by five or ten-stage counter-current distribution between equal volumes of ether and 0.5 M sodium phosphate buffer (pH 6.4).

The biologically active material (as determined by its stimulation of isolated rabbit duodenal strip) is further purified by reversed phase partition chromatography in the following system: 50% (v/v) methanol: water (moving phase) and 50% (v/v) isoöctanol:chloroform as the station-

ary phase on 4.5 g. of hydrophobic Supercel (silane treated kieselguhr) (90,91).

The active material can then be purified further by a second reversed phase partition chromatography in 50% (v/v) ethylene chloride: heptane and 60% acetic acid (stationary phase on hydrophobic Supercel) (91).

As an alternative to the second reversed phase partition chromatography, the material can be purified by silicic acid column chromatography and gradient elution with ethyl acetate:benzene mixtures (139). Final purification can take place by repeated crystallization from ethyl acetate/light petroleum until a melting point of 102° to 103°C. is reached (for prostaglandin F) (90) or from ethyl acetate/heptane until a melting point of 115°-117°C. is reached (for prostaglandin E) (91). More recently, final purification has been carried out by thin-layer chromatography on activated silica gel G:silver nitrate (30:1) using such systems as ethyl acetate:methanol:water (160:25:100), benzene:dioxane (50:40) or ethyl acetate:acetic acid:methanol:trimethylpentane:water (110:30:35:10:100) (140). The recovery rate with thin-layer chromatography of the methyl esters of the prostaglandins is in the order of 90-95% as judged by isotope methods using the tritium-labeled methyl ester of PGE-1 (95).

A method for the quantitative determination of the prostaglandins without bioassay was developed by Bygdeman and Samuelsson in 1964 (141). It involves fractionation of the prostaglandins into PGE and PGF compounds. The F-compounds are determined by gas chromatography as methyl esters and trimethylsilyl ether derivatives. The E-compounds are separated by thin-layer chromatography and determined by treating the eluates with NaOH and measuring the developing ultraviolet absorption at 278 mμ.

INVESTIGATIVE SECTION

I - INTRODUCTION.

This investigation was begun following the report by Hickler et al (82) of their extraction and initial characterization of a vasodepressor lipid from the renal medulla. This substance was impure and its identity had therefore not yet been established, although in its physicochemical and biological characteristics it seemed to be closely related to the prostaglandins. It was reported to differ from prostaglandin E-1 in thin-layer chromatographic mobility (83).

We felt challenged to attempt to purify the renomedullary vasodepressor lipid, and having done that, to attempt to establish its identity. Because Page et al (63,64,65,66) had shown the angiotensin-destroying effect of renal extracts, we performed several preliminary experiments to see if vasodepressor lipid had any effect on "renin-activity" determination or angiotensin II *in vitro*.

II - MATERIALS AND METHODS.

A. EXTRACTION:

1. Materials:

(a) Chloroform, Mallinckrodt Analytical reagent. This was washed 5 times with equal volumes of distilled water to remove the 0.75% ethanol added to it as stabilizer. It was then dried over anhydrous calcium chloride for at least 4 hours and distilled immediately before use.

(b) Methanol, "Baker Analyzed" reagent. For extraction, the methanol was used directly from newly-opened bottles.

(c) Rabbit renal medullas, "quick-frozen", purchased from Pel-Freez Biologicals, Inc., Rogers, Arkansas, U.S.A.

(d) Rotary flash-evaporators: Rinco and Büchi "Rotavapor".

(e) Magnetic stirrer ("Magnestir", Labline, Inc., Chicago) and 2" polyethylene-coated bar magnet.

(f) Glass flasks: Erlenmeyer, 100 ml. conical and 1000 ml. round.

(g) "Waring" blender.

2. Method:

The crude extract of renal tissue is obtained using the method illustrated in Figure 2. This method is based on that reported by Hickler et al (82), with minor modifications which improve the yield of active material.

Some of the source tissue has been kidney removed surgically from patients with renovascular hypertension, or beef and pork kidney obtained at meat packing plants immediately after the animals were killed. Since there was usually a delay before the tissue could be extracted, it was frozen with dry-ice and kept in a freezer at -20°C. At this temperature, there was no decrease in yield of vasodepressor activity with tissue storage up to several months. The medulla was separated from cortex by gross dissection with scissors and a scalpel. This was most easily done on chilled or lightly frozen tissue. Sometimes a thin rim of cortical tissue was left adherent to the medulla to ensure that the outer renal medulla (adjacent to the vascular arcade) would be extracted.

Most of the extractions were carried out on the rabbit renal medullas obtained commercially. This was done mostly for convenience, but also to duplicate the conditions reported by Hickler et al (82) more exactly. Extractions have been done on tissue amounts ranging from 1 g. to 500 g.

with similar yields of vasoactive material per gram of source tissue.

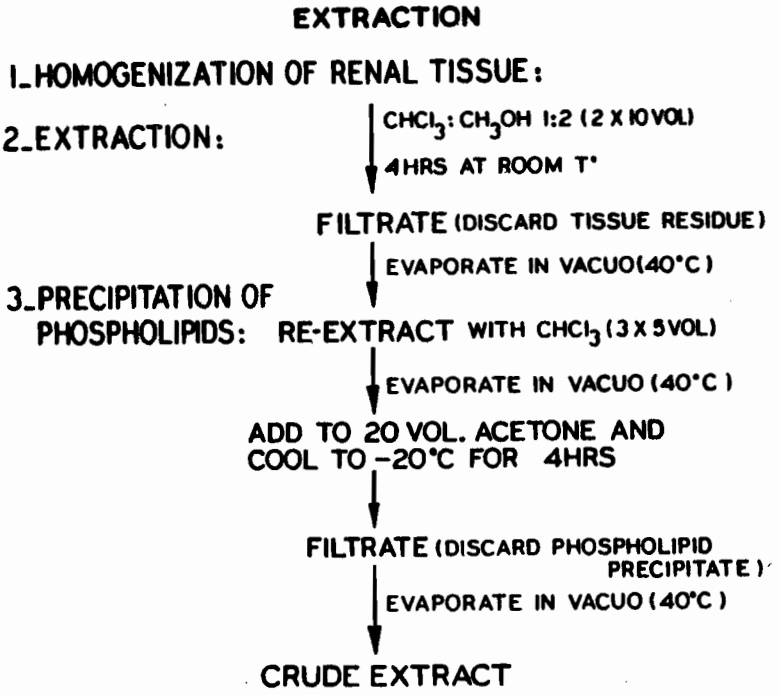


Figure 2

Extraction of vasodepressor lipid from renal medullary tissue.

The source tissue is homogenized (by blender) in 10 volumes of a chloroform:methanol mixture (1:2 v/v). Extraction is carried out in this mixture for 4 hours at room temperature in an Erlenmeyer flask of such a capacity that the extraction mixture just fills it. The flask is covered to exclude air. A magnetic stirrer ensures constant mixing during extraction. The tissue residue is removed by filtration. The residue is re-extracted for 4 hours under the same conditions. After the second filtration, the tissue residue is discarded. The combined filtrates are flash-evaporated at 40°C.

To remove the vasopressor phospholipids from the residue, it is extracted 3 times with 5 volumes of chloroform (which dissolves more of the active lipid than did the petroleum ether formerly used in this step) (84).

The combined chloroform extracts are reduced to one-tenth volume by evaporation in vacuo at 40°C. and the remaining material is poured into 20 volumes of acetone. The flasks containing the acetone extract are placed in a freezer where the acetone is cooled to -20°C. for at least 4 hours (and frequently, overnight). The phospholipids precipitate in the cold acetone and are removed by filtration. The precipitate is washed with 10 volumes of cold acetone (-20°C.) and the combined acetone filtrates are evaporated in vacuo at 40°C. This yields a crude extract weighing about 35 mg. per g. of source kidney tissue.

B. BIOASSAY:

1. Materials:

(a) Rats: 100-150 g., male, albino; Sprague-Dawley strain.

(b) Mercury manometer: A capillary tube replaces the original manometer. A light rod within the tube floats on the surface of the mercury. A pen mounted on the top of the rod writes directly on the kymograph paper.

(c) Polyethylene tubing: P.E. 10 "Intramedic", Clay-Adams Co. Inc.

(d) Disposable 1 ml. tuberculin syringes, "Tomac", with 27 G, ½" needles.

(e) Rat board and clips, Brookline Surgical Specialties, Brookline, Mass., U.S.A.

(f) Pentobarbital Sodium, U.S.P., 60 mg. per ml. "Abbott".

(g) 20% ethanol. Redistilled ethanol with distilled water added to make a 20% solution (v/v).

(h) Tyrode solution: Each litre contains water and 8 g. NaCl, 250 mg. KCl 150 mg. $CaCl_2$, 10 mg. $MgCl_2$, 0.9 g. dextrose, 50 mg. NaH_2PO_4 and 1 g. $NaHCO_3$.

2. Method:

(a) Preparation of the rats for vasodepressor assay:

The rats were nephrectomized under ether anaesthesia 18 hours before use to provide a more stable blood pressure baseline and a smoother, more prolonged anaesthesia during bioassay.

For use in bioassay, rats were anaesthetized with intraperitoneal pentobarbital (6 mg. initially, and then additional 0.6 mg. doses as needed to maintain deep anaesthesia and a stable blood pressure baseline throughout the bioassay period). A triangle of skin on the ventral aspect of the neck was removed, the neck muscles were retracted and the trachea was exposed. A tracheotomy was performed. The jugular veins were dissected free and P.E. 10 polyethylene cannulas were tied into the veins. A carotid artery was dissected free and clamped proximally. It was then tied distally and cut half way through, between the clamp and the ligature. Into the opening was inserted a shortened 21 G needle which was connected to the mercury reservoir of the manometer by a heparin-saline filled polyethylene tube. The needle was tied in place and the clamp was removed, transmitting the carotid blood pressure to the mercury manometer. The float pen recorded the pressure directly on the paper of the kymograph drum.

Vagotomy and pentolinium treatment were not used, since neither was found to increase the sensitivity of the preparation (although administration of pentolinium did produce a slight prolongation of blood pressure depression).

(b) Bioassay media:

The bioassay medium used was 20% ethanol. The amounts injected varied from 0.01 to 0.08 ml. The larger doses of 20% ethanol gave slight

(up to 5 mm.) initial pressor responses. These responses did not mask the vasodepressor effect of active lipid. The lipid solutions were usually sufficiently concentrated so that injections of 0.04 ml. gave vasodepressor responses which approached the maximum obtainable. Even with dilute solutions of vasodepressor lipid, the pressor response caused by the ethanol was too brief to interfere with interpretation of the sustained blood pressure lowering effect of the lipid. Because it was a pressor response, the ethanol effect did not, of course, lead to false-positive interpretations. Other media were tested, but many had interfering intrinsic vasodepressor activity. Dilute serum albumin and various salt solutions were satisfactory media, but they carried with them the disadvantage that if the active lipid was to be recovered for further purification it had to be extracted with organic solvents. On the other hand, 20% ethanol could be removed by evaporation, making it the bioassay medium of choice during the development of the purification procedure.

(c) Standardization:

Rats were found to vary considerably in their sensitivity to vasodepressor lipid. Consequently, it was necessary to standardize the bioassay preparation by injection of vasodepressor lipid of known potency (as determined by previous bioassay). In the early part of the investigation, the exact relationship of vasodepressor lipid to the prostaglandins was not known. Prostaglandin E-1 was therefore not used as a bioassay standard, although, in retrospect, this would have been a good idea. Instead, large batches of lipid were extracted, purified as much as the stage of development of the technique would allow, bioassayed and divided into aliquots with known, equal vasodepressor activity. These "standards" were kept in the freezer at -20°C. for use in bioassay comparison with

subsequent unknowns.

Vasoactive lipid tended to adhere to the walls of bioassay syringes, so that if an active unknown were assayed, the syringe had to be washed many times with organic solvents to prevent contamination of subsequent unknowns. We therefore adopted the use of disposable 1 cc. tuberculin syringes. Each syringe contained the material from only one particular fraction or thin-layer chromatographic area, and it was then discarded. Similarly, vasoactive lipid adhered to the walls of the polyethylene cannulas. Washing with 20% ethanol until there was no further vasodepressor response and, rarely, re-insertion of a clean cannula were required to circumvent this interference.

For bioassay, one of the "standard" aliquots of vasoactive lipid, and each of the unknowns was dissolved in 1 ml. of 20% ethanol and each was taken up into a disposable tuberculin syringe. The effect of 20% ethanol itself was ascertained by intravenous injections of 0.01, 0.04 and 0.08 ml. The sensitivity of the rat was determined by injection of the "standard" lipid solution in volumes of 0.01, 0.02, 0.04 and 0.08 ml. (corresponding to the lipid derived from 0.1, 0.2, 0.4 and 0.8 g. of rabbit renal medulla). Unknowns were then injected into the cannula entering the other jugular vein. Comparisons with the standard were not elaborately carried out because for most of the bioassays it was sufficient to determine the presence or absence of vasodepressor activity in any particular unknown, this being the sole criterion for retaining or discarding that fraction. Now that our "pure" vasodepressor lipid has been tentatively identified as prostaglandin E-1, quantitative four-point bioassays can be carried out using accurately measured quantities of prostaglandin E-1 as the standard. During the experiments reported here,

however, only semi-quantitative estimations of vasoactivity were made.

(d) Dose-response relationships:

Typical bioassay curves and a graphical representation of the dose-response relationships of renomedullary vasodepressor lipid are illustrated in Figure 3.

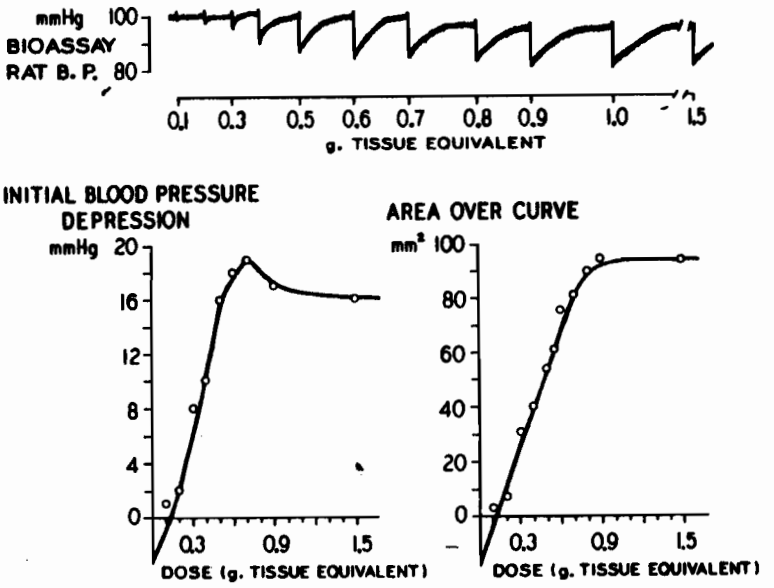


Figure 3

Dose-response relationships of renomedullary vasodepressor lipid.

The typical bioassay curve consists of the slight, brief initial ethanol pressor response followed immediately by a sharp fall in blood pressure, with a gradual return to the baseline over the next five to twelve minutes.

If one uses the magnitude of the initial blood pressure fall as the criterion of response, a maximum is reached at a dose corresponding to the lipid derived from 0.7 g. of rabbit renal medulla. If, on the other hand, one uses the area over the curve (measured by planimetry) as the response criterion, a plateau is reached at a slightly higher dose. The

difference between the two may be partly related to the slow progressive fall in blood pressure baseline seen with repeated injections of active lipid.

(e) Nonvascular smooth muscle bioassay:

Dr. L.S. Wolfe, Director of the Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, performed the bioassay on rat gastric fundus smooth muscle: The well-fed 250 g. albino rat was rendered unconscious by a sharp blow to the base of the skull. The stomach was removed and the animal was killed. A strip of fundal smooth muscle was dissected free and it was suspended in a constant temperature bath in oxygenated Tyrode solution. Contractions of the smooth muscle strip were recorded directly on a smoked-drum kymograph, the muscle strip being anchored below and attached above to the free end of the pen lever. The activity of the muscle was standardized with injections of accurately measured quantities of crystalline prostaglandin E-1 dissolved in Tyrode solution. The unknowns, vasodepressor lipid and acid-treated vasodepressor lipid, were assayed by dissolving the material in Tyrode solution and injecting small amounts (0.05 to 0.2 ml) into the medium surrounding the muscle. This smooth muscle assay preparation was extremely sensitive, responding vigorously to nanogram quantities of prostaglandin E-1.

C. COLUMN CHROMATOGRAPHY*.

1. Materials:

(a) Chloroform: Mallinckrodt Analytical reagent. Washed 5 times

* The terms "adsorption" and "partition" are used to designate the first and second chromatography columns respectively. It is probable, however, that on the first column a mixed phenomenon (both adsorption and partition) takes place. During elution with the chloroform-methanol mixture there is an exothermic partial adsorption of the methanol by the silica gel. By the same token, adsorption may play a role in the second or "partition" column chromatography, a phenomenon known to occur with partition chromatography on paper.

with distilled water and distilled prior to use, as described previously.

(b) Methanol: "Baker Analyzed" reagent grade. Redistilled before use.

(c) Glass chromatography columns. Internal diameter: 1 cm. Height: 21 cm. Reservoir capacity: 200 ml.

(d) Silica gel (Grace-Davison, mesh 100-200) was activated by heating to 120°C. for 1 hour. It was then stored in stoppered Florence flasks.

(e) Pyrex wool filtering fibre, Corning Glass Works, Corning, N.Y., U.S.A.

2. Method:

(a) Adsorption chromatography:

(i) Column preparation:

A small plug of pyrex wool was tamped down to the bottom of the glass column where it would prevent leakage of silica gel from the column during subsequent elutions.

Seven grams of activated silica gel was mixed into 50 ml. of redistilled chloroform and the resulting slurry was poured into the glass column. During percolation of the chloroform down through the column, the silica gel was constantly tamped with a glass stirring rod. The surface of the chloroform was never allowed to fall below the level of the tamped silica gel. After all the gel had settled, another small pyrex wool plug was tamped into place atop the column. The silica gel was then washed with 50 ml. of redistilled chloroform.

(ii) Development of the elution technique:

The "crude extract" from the extraction procedure was dissolved in 1 ml. of redistilled chloroform and added to the top of the silica gel column. A series of elution experiments was then performed to determine

the optimal composition of the chloroform-methanol elution mixture.

It had been reported that if the lipid-charged column were washed with 50 ml. of 100% redistilled chloroform and then eluted with 50 ml. of chloroform-methanol (4:1 v/v) almost all the vasoactivity would be recovered in the latter eluting mixture (82,87). We confirmed this experimentally.

Another column was prepared as described and the crude extract was applied. The column was washed with 50 ml. of 100% redistilled chloroform. It was then eluted with 25 ml. of 5% methanol: 95% chloroform (v/v). No vasoactivity was present in this fraction. It was then eluted with 25 ml. of 10% methanol:90% chloroform and all the vasoactivity was recovered in this fraction, none occurring in subsequent 25 ml. eluents of increasing methanol content including 15% methanol, 20% methanol, 30% methanol, 40% methanol, 50% methanol, 75% methanol and, finally, 100% methanol. This experiment was repeated three times and each time the vasodepressor activity was recovered in the 10% methanol:90% chloroform fraction.

It was next determined that if the column were washed with 50 ml. of 100% chloroform and then eluted with 10% methanol:90% chloroform, all the vasodepressor activity would be present in the second 10 ml. aliquot of the latter eluate. This was confirmed by repeating the procedure three times.

The next step was to determine the exact percentage of methanol in successive mixtures of methanol:chloroform that would elute the vasodepressor lipid. This percentage was estimated to be between 5% and 10%, based on the previous results. The column was eluted with 50 ml. of 5% methanol:95% chloroform (v/v), followed by 50 ml. amounts of methanol-chloroform, each time increasing the proportion of methanol by 1%. All

the vasodepressor activity came off the column in the 6% methanol:94% chloroform. This was repeated, with the 6% methanol:94% chloroform being collected in 5 ml. aliquots, which were flash-evaporated and bioassayed separately. The third, fourth and fifth aliquots contained all the vasodepressor activity. This was subsequently rechecked and reconfirmed three times.

Based on these experiments, a standard procedure was adopted (Figure 4).

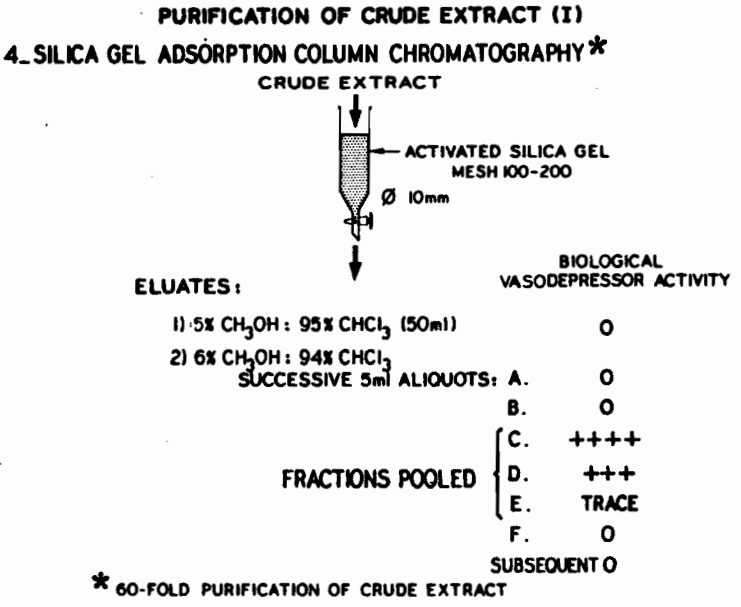


Figure 4

The "crude extract" is dissolved in 1 ml. of redistilled chloroform and applied to the top of the column. The column is washed with 50 ml. of 5% methanol:95% chloroform (v/v). It is then eluted with 6% methanol: 94% chloroform (v/v). The third, fourth and fifth 5 ml. aliquots of this latter eluate are collected, pooled and flash-evaporated.

(b) Partition chromatography:

(i) Column preparation:

The glass column used for partition chromatography is the same as that used for the first chromatography. A plug of pyrex wool is tamped into the bottom of the column as described.

Four ml. of methanol is added to seven grams of silica gel in a small beaker, where the silica gel exothermically adsorbs the methanol. This mixture is stirred vigorously with a spatula until the excess methanol evaporates, the mixture cools and a "dry" powder results. The powder, silica gel with its strongly adsorbed methanol, is mixed into 50 ml. of redistilled chloroform. The slurry is poured into the glass column and the chloroform is allowed to run through. The silica gel is constantly tamped with a glass rod. When the gel has settled and a pyrex wool plug has been tamped into place atop the column, it then receives the pooled active lipid fractions from the first column dissolved in 1 ml. of redistilled chloroform.

(ii) Development of the elution technique:

With this column, purification takes place primarily by partition between the adsorbed methanol and the eluting chloroform-methanol mixtures of increasing polarity. The silica gel acts as a support for the adsorbed methanol.

The elution technique was developed by a series of experiments similar in many respects to those described for the first column.

After the pooled active fractions from the first column had been applied to the partition column, the latter was washed with 100 ml. of redistilled chloroform. It was then eluted with 50 ml. amounts of methanol-chloroform mixtures, starting with 1% methanol:99% chloroform, and increasing the proportion of methanol by 1% with each successive eluent. Bioassay revealed that most of the vasodepressor activity came off the column in the 5% methanol:95% chloroform, with a trace of vasodepressor

activity in the 6% methanol:94% chloroform. The experiment was repeated and these results were confirmed.

During the next experiment, the column was eluted first with 50 ml. of 3% methanol:97% chloroform (v/v). This was followed by elution with 6% methanol:94% chloroform (v/v) and 5 ml. aliquots of this eluate were collected separately for bioassay. The fourth, fifth and sixth 5 ml. aliquots contained all the vasodepressor activity. This finding was confirmed in each of three identical subsequent experiments. This was then adopted as the technique to be used on all subsequent purifications. It is illustrated in Figure 5.

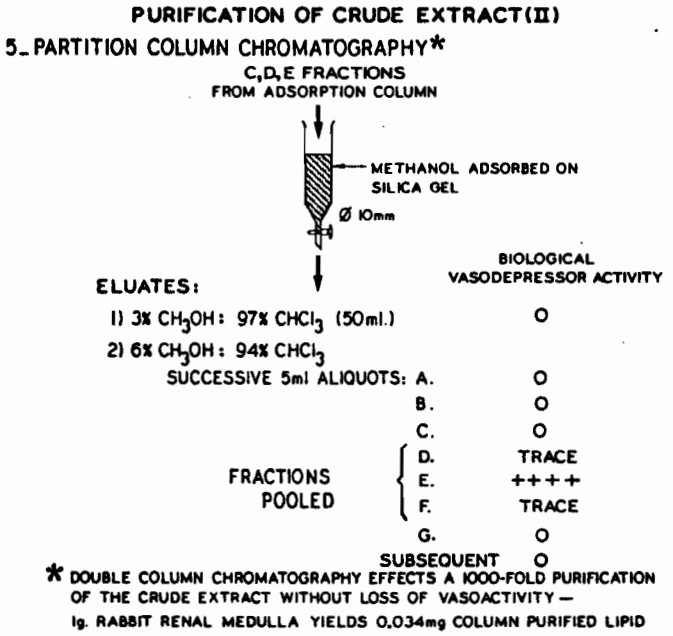


Figure 5

Only traces of vasodepressor activity are found in the fourth and sixth 5 ml. aliquots, with most of the activity occurring in the fifth aliquot. These three aliquots are pooled, evaporated in vacuo at 40°C. and stored in a freezer at -20°C. for further purification by thin-layer

chromatography.

D. THIN-LAYER CHROMATOGRAPHY:

1. Materials:

(a) Eastman "Chromagram" sheets, type K 301 R, Distillation Products Industries, Rochester, New York, U.S.A. These were 20 x 20 cm. sheets of inert polyethylene terephthalate, coated with activated silica gel, polyvinyl alcohol as a binder and lead-manganese-activated calcium silicate as a fluorescent indicator. The sheets were stored in a dessicator over anhydrous calcium chloride in sealed packages as supplied commercially. Uniform activity of the silica gel was observed during the phase of investigation in which these sheets were used.

(b) Disposable glass capillary pipettes.

(c) Elution tubes: L-shaped capillary tubes, the upper ends of which were fused to test tubes containing the eluent.

(d) Acetic acid, glacial, U.S.P., "Shawinigan", the MacArthur Chemical Co., Montreal.

(e) Organic solvents. All the following solvents were "Baker Analyzed" reagent grade and redistilled before use:

(i) Benzene

(ii) 1,4-dioxane

(iii) Acetone

(iv) Methanol

(v) 2,2,4-trimethylpentane (isoöctane)

(vi) Iso-propyl alcohol (2 propanol)

(vii) Ethyl acetate

(f) Chloroform, Mallinckrodt Analytical reagent, washed and redistilled as described previously.

(g) Conventional rectangular glass thin-layer chromatography jars, lined with filter paper. The lids were sealed with silicone grease.

(h) 10% phosphomolybdic acid, prepared by dissolving phosphomolybdic acid ("Baker Analyzed" reagent) in redistilled ethanol.

(i) Ultraviolet light, "Chromato-Vue", Ultra-violet Products, Inc., San Gabriel, California, U.S.A. This unit contains ultraviolet lights with maximum emissions at 254 and 366 mμ.

2. Systems:

The developing systems used were:

(a)	Benzene	100
	Dioxane	70
	Acetic acid	2
(b)	Acetone	100%
(c)	Trimethylpentane	125
	Iso-propyl alcohol	35
	Acetic acid	1
(d)	Chloroform	150
	Methanol	10
	Acetic acid	1
(e)	Ethyl acetate	110
	Acetic acid	30
	Methanol	35
	Trimethylpentane	10
	Water	100

Systems (a) and (e) were adopted from Green and Samuelsson (140). System (e) was allowed to equilibrate for 2 hours and the less polar phase was used. System (c) was adopted from Eneroth (142). System (d) was adopted from Skipski et al (143). When systems (a), (c), and (d) were used, the proportions of solvents were changed from those reported to bring the vasoactive spot into the mid-range of Rf values. System (e) was used in the proportions reported (140).

3. Method:

Lipid for chromatography was dissolved in 1 ml. of chloroform and spotted on the "chromagram" sheets with disposable glass capillary pipettes. The spots were dried in a stream of cold air and the sheets were placed in the chromatography jars containing the appropriate developing systems.

The chromatograms were developed by the ascending technique to a height of 12-15 cm. (45-90 minutes) at room temperature. To locate the lipid spots for bioassay, the plates were air dried and viewed under ultraviolet light with a maximum emission at 254 mμ. Thin strips of the chromatogram were cut out and exposed to iodine vapour or sprayed with 10% phosphomolybdic acid in 95% ethanol and heated with a hot air blower.

When the spots were located, they were cut out of the chromatogram sheets with scissors, suspended from a platinum wire at the capillary outlet of the eluting tubes, and eluted by a continuous descending flow of chloroform:methanol (1:1 v/v). Approximately 50 ml. of the eluting mixture was used for each spot. The solvent was collected in conical glass flasks as it dripped off the areas being eluted. The solvent was removed by evaporation in vacuo at 40°C. and the remaining lipid (compound or mixture of compounds) was dissolved in 20% ethanol for rat bioassay.

E. HIGH VOLTAGE STARCH GEL ELECTROPHORESIS:

1. Materials:

(a) High voltage power supply, NJE Corporation, Kenilworth, New Jersey, U.S.A.

(b) E-C pressure-plate electrophoresis cell (EC 451),

E-C Apparatus Corporation, Philadelphia, Pa., U.S.A.

This plexiglass unit has nonwettable migration surfaces and platinum electrodes. The migration path is "direct-contact" cooled by water at 0-5°C. continuously pumped through the upper and lower contact plates. The 2500 ml. volume electrode compartments contain anticonvection sponge baffles. The compartments are connected by a leveling tube. An electrical safety switch is closed automatically when the upper pressure plate is clamped into place.

(c) E-C starch tray: 12" long; for molding the starch block. E-C Apparatus Corporation, Philadelphia, Pa., U.S.A.

(d) Starch gel (see preparation below).

(e) Buffer (pH 8). Composed of 0.1 M LiOH-H_2O (4.196 g./L.) and 0.38 M boric acid.

(f) 95% ethanol, redistilled.

(g) "Whatman No. 2" filter paper, W. & R. Balston, Ltd., England.

(h) Ultraviolet light, "Chromato-Vue", Ultra-violet Products, Inc., San Gabriel, California, U.S.A.

(i) 10% phosphomolybdic acid in redistilled ethanol.

2. Method:

Electrophoresis was carried out on a 14% starch gel prepared with 50% distilled water, 5% 0.02 M LiOH (0.8392 g./L.); 0.076 M boric acid (4.698 g./L.) and 45% 0.0033 M citric acid (0.6346 g./L.): 0.016 M T.H.A.M. (tris-hydroxymethyl-aminomethane 1.9386 g./L.). The gel was prepared by heating in a double boiler with constant magnetic stirring. The gel was "de-gassed" under reduced pressure before and after heating. It was poured into the plexiglass starch tray to a depth of 2 mm. and allowed to gel with cooling.

The lipids to be run were dissolved in small amounts of chloroform and spotted on strips of filter paper 2 mm. wide and 2 cm. long. After drying in a stream of cold air, the paper strips were inserted, on edge, into a transverse incision in the gel. This origin or "starting line" was located 5 cm. from the center of the starch block, towards the cathode.

Buffer-soaked cloths were led from the buffer compartments to the gel, overlapping the gel by 2 cm. at each end. The gel and cloths were covered with Saran Wrap. The upper pressure plate was clamped into place, closing the safety switch. The cold water pump was activated.

A direct current potential of 1000 volts was applied to the two buffer compartments. Voltage constancy was maintained by frequent adjustment of the power output by the monitoring investigator or technician.

After electrophoresis runs of 2 hours, the gel was viewed under ultraviolet light with a maximum emission at 366 mμ. Two other detection methods were employed: (a) The gel was covered with "Whatman No. 2" filter paper moistened with distilled water. This was covered with Saran Wrap and the lipid was allowed to diffuse up into the paper overnight. The paper was then marked to identify the gel position, air dried, and sprayed with 10% phosphomolybdic acid in 95% ethanol. Heating with a hot air blower rendered the lipid spots visible. (b) The gel was divided into two equal parts longitudinally (to separate the gel areas bearing the compounds under comparison). Each part was cut transversely at 1 cm. intervals and the strips were placed in individually numbered beakers containing 95% ethanol. Each gel strip was minced with a small spatula. Elution of the gel was allowed to procede overnight at 4°C. The contents of each beaker were filtered separately and the ethanol filtrate was

evaporated *in vacuo* at 40°C. Each fraction was dissolved in 1 ml. of 20% ethanol for rat bioassay.

In later experiments, the areas which fluoresced under ultraviolet light (366 mμ) and reacted with phosphomolybdic acid were marked and cut out. These areas were eluted with 95% ethanol, as described above. The remaining gel areas were combined, eluted and bioassayed to confirm the absence of vasodepressor activity where no lipid was detectable by other means.

F. RENIN ACTIVITY:

1. Materials:

(a) Dowex resin 50W-X2, 100-200 mesh: "Baker Analyzed" reagent. Before use, 500 g. of resin was washed with 2 litres of 4 N NaOH. The sodium salt of the resin was then washed with 1 litre of water, 1 litre of 2 N HCl and again with 2 litres of water. Finally the resin cake was washed with 0.2 N ammonium acetate solution, pH 2, until the pH of the eluate reached 6.

(b) Angiotensin standard for bioassay: Valine-5 angiotensin II, aspartic β-amide, Synthetic, Ciba Company preparation 19990-A. A solution of 1 μg./ml. in 20% aqueous ethanol, kept at 4°C., was used as standard solution in all bioassays. The solution was prepared every two weeks.

(c) Constant temperature shaking bath, Lab-Line Instruments, Inc., Chicago, Illinois, U.S.A.

(d) Flash-evaporators, Rinco Instrument Co. Inc., Greenville, Ill., U.S.A.

(e) "Freeze-Dryer", Thermovac Industries Corp., Brooklyn, New York, U.S.A.

(f) Vacuum pumps: model 75, Precision Scientific Co., Chicago; model 1403, "Duo-Seal", Welch Scientific Co., Skokie, Illinois, U.S.A.

(g) Rat blood pressure bioassay preparation as described previously.

(h) Siliconized 25 ml. Erlenmeyer flasks.

(i) Glass chromatography columns, as described previously.

(j) Microsyringes, No. 263-M, The L.S. Starrett Co., Athol, Mass., U.S.A. These syringes, which accurately deliver volumes as small as 0.0001 ml., were used in the bioassay of angiotensin.

2. Method:

In these experiments, renin-activity was determined by the method of Boucher et al (144) with the recently reported modifications (145).

(a) Effect of vasodepressor lipid (VDL) on angiotensin liberation in vitro.

In the first experiment, 4 ml. of moist Dowex 50W-X2 resin (ammonium form) was added to four 10 ml. aliquots of pooled dog plasma which had been adjusted to pH 5.5 with 1 N HCl. The first flask (A) had nothing further added to it. Flasks B, C, and D each had 1 hog unit of renin added. To flask C was added 1 ml. of 95% ethanol. To flask D was added vasodepressor lipid, derived from 100 g. of porcine renal medulla, partially purified by double column chromatography, and dissolved in 1 ml. of 95% ethanol.

The 4 siliconized Erlenmeyer flasks were sealed and incubated for 3 hours in a constant temperature shaking bath at 37°C. Under these conditions, as angiotensin was generated it was absorbed by the Dowex 50W-X2 (NH_4^+) resin, preventing destruction by the angiotensinases present in plasma. The resin had no effect on the reaction involving renin-substrate (angiotensinogen) and renin.

Following incubation, the flask contents were transferred into glass columns containing 1 ml. of the Dowex 50W-X2 (NH_4^+) resin. The columns were washed with 15 ml. of ammonium acetate (pH 6.0), 20 ml. of 10% acetic acid (v/v) and 15 ml. of distilled water. These washings were discarded. The angiotensin was eluted from the columns with 15 ml. of 0.1 N diethylamine followed by 15 ml. of 0.2 N ammonium hydroxide. These eluates were collected in a flask containing a small amount of concentrated acetic acid and a color indicator. This was evaporated in vacuo at 40°C. for 45 minutes. Thereafter, sublimation was carried out under high vacuum at 40°C. with 5 washes of 80% aqueous ethanol and one wash of acid-alcohol (1 N HCl:95% ethanol, 1:1 v/v). After sublimation, the dry residue was dissolved in 1 ml. of 20% ethanol. This was bioassayed in the rat preparation previously described. A four-point bioassay comparison was carried out against a standard of synthetic valine-5 angiotensin II amide (Ciba) in 20% aqueous ethanol. (The accuracy of this assay is in the order of 90% when repeated measurements are made).

(b) Effect of vasodepressor lipid (VDL) and prostaglandin E-1 (PGE-1) on "renin-activity" determination.

In the second experiment, a normal male subject was fed a diet low in sodium (10 mEq. Na, 90 mEq. K) for 3 days to increase the peripheral plasma renin-activity. Peripheral venous blood was withdrawn through a cooling coil into a bottle containing 10 ml. EDTA as anticoagulant (144). The plasma was separated at 4°C. by centrifugation in a high speed refrigerated centrifuge and decanted. Six 10 ml. aliquots of plasma, which had been adjusted to pH 5.5 with 1 N HCl and filtered through glass wool, were placed in siliconized incubation flasks and to each was added 4 ml. of moist Dowex 50W-X2 (NH_4^+) resin. The first two flasks were incubated

in a shaking bath at 37°C. for 3 and 2 hours respectively. To the next two flasks was added 100 g. tissue equivalent of partially purified (by double column chromatography) rabbit renomedullary vasodepressor lipid. These aliquots were then incubated as described for 3 and 2 hours respectively. The last two flasks each received 1 mg. crystalline prostaglandin E-1 (kindly supplied by Dr. J. E. Pike, The Upjohn Co., Kalamazoo, Michigan). They were incubated as described. Following incubation, the tube contents were poured into columns containing 1 ml. of Dowex 50W-X2 (NH_4+) resin, washed, eluted, evaporated, sublimated and bioassayed as described above.

(c) Effect of vasodepressor lipid (VDL) on angiotensin II in vitro:

Two 25 ml. Erlenmeyer flasks were siliconized and to each was added 100 ng. of standard valine-5 angiotensin II aspartic β-amide in 20% aqueous ethanol. Vasodepressor lipid derived from 25 g. of rabbit renal medulla, dissolved in 0.5 ml. of 20% aqueous ethanol, was added to the second flask. The contents of both flasks were made up to 10 ml. by the addition of 20% ethanol. The flasks were left at room temperature for 5 hours with occasional shaking. At the end of that time, 4 ml. of moist Dowex 50W-X2 (NH_4+) resin was added to each flask and the flasks were shaken for 10 minutes. The flask contents were then poured into glass columns containing 1 ml. of Dowex 50W-X2 (NH_4+) resin. The columns were then washed and eluted as described above. The eluates containing the angiotensin II were evaporated, sublimated and bioassayed.

III - RESULTS.

A. PURIFICATION OF VASODEPRESSOR LIPID:

1. Column chromatography:

The crude extract of renal medullary tissue is purified approximately 1000-fold without detectable loss of vasoactivity when submitted to chromatography on the two columns (Figures 4 and 5). Identical aliquots of crude extract, each derived from 10 g. of rabbit renal medulla, were bioassayed. One was in the crude form and the other had been partially purified by chromatography on the two columns. Equivalent tissue amounts of the two forms of the lipid produced identical vasodepressor responses in the bioassay rat preparation. On two occasions the extract was accurately weighed (on an Oertling Balance, model 141, "Oertling", London, England, which is accurate to 3×10^{-7} g.), before and after double column chromatography. When 0.3652 g. of crude extract was applied to the first column, all the vasoactivity was recovered from the second column in material weighing 0.3375 mg., representing a 1082-fold purification. Similarly, when 0.3554 g. of crude extract was applied to the first column, the vasoactivity was recovered from the second column in lipid weighing 0.3404 mg., a 1044-fold purification.

When the columns were assessed separately by weighing the active lipid before and after chromatography, the first column proved to be capable of a 60-fold purification of the vasodepressor lipid. The second column purified the crude extract about 40-fold, but it concentrated the partially purified lipid from the first column only 18-fold. It is the use of these columns in series that effects the 1000-fold purification of the vasodepressor lipid.

2. Thin-layer chromatography:

The initial thin-layer chromatographic purification of the pooled active fractions (D, E and F) from the second column was carried out on

Eastman "chromagram" sheets in system (a) (benzene 100:dioxane 70: acetic acid 2). The results of this chromatography are illustrated in Figure 6.

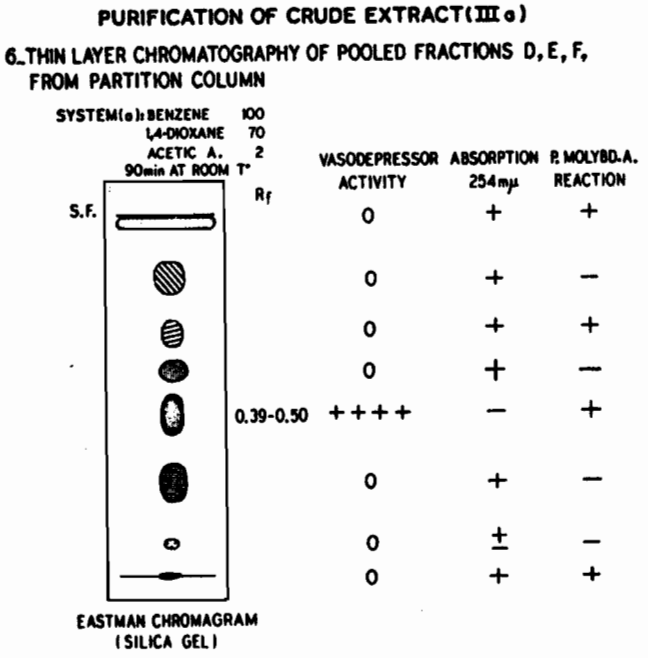


Figure 6.

In this system, the vasodepressor activity was confined to a single spot with an Rf value of 0.45. This spot was not visible when the chromatogram was viewed under ultraviolet light with a maximum emission at 254 mμ., but it was rendered visible on exposure to iodine vapour and when the chromatogram was sprayed with 10% phosphomolybdic acid in 95% ethanol and then heated with a hot air blower.

The final purification was effected by eluting the active lipid from the system (a) chromatogram, applying it to another Eastman "chromagram" sheet and developing the chromatogram in system (d) consisting of chloroform 150:methanol 10:acetic acid 1 (Figure 7).

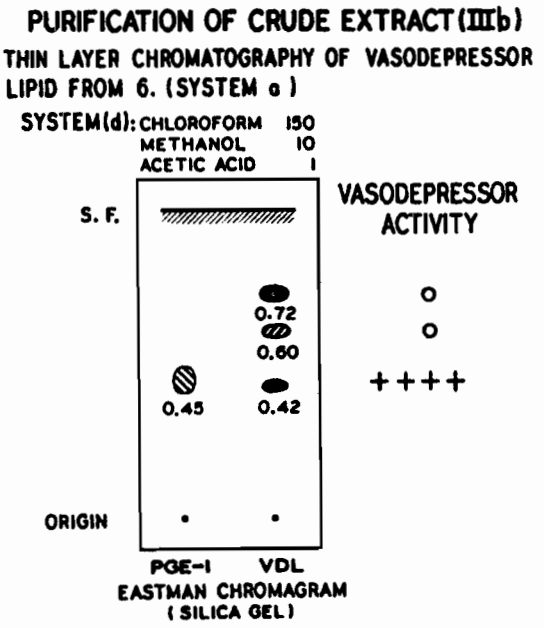


Figure 7.

"Pure" renomedullary vasodepressor lipid was recovered from the spot with an Rf value of 0.42. This was not visible when viewed under ultraviolet light (maximum emissions 254 mμ and 366 mμ), but it was made visible by exposure to iodine vapour and by the phosphomolybdic acid reaction.

As will be referred to again later, the "pure" vasodepressor lipid spot coincides with that of a crystalline prostaglandin E-1 standard in this system.

Subsequent chromatography of the lipid from this spot on Eastman "chromagram" sheets in systems (c) (trimethylpentane 125:iso-propyl alcohol 35:acetic acid 1) and (e) (ethyl acetate 110:acetic acid 30:methanol 35:trimethylpentane 10:water 100) effected no further purification, the lipid moving as a single spot.

3. High voltage starch gel electrophoresis.

"Pure" vasodepressor lipid (from the second thin-layer chromato-

graphic purification) moves as a single line during high voltage starch gel electrophoresis. This line was detectable by viewing the starch gel block under ultraviolet light with a maximum emission at 366 mμ., and by the two other detection methods (described in the "Method" subsection on page 46) involving the phosphomolybdic acid reaction and vasodepressor bioassay.

Inability to separate "pure" vasodepressor lipid into two or more lines by virtue of differing electrophoretic mobility enhances the conclusion that the lipid is relatively pure following the second thin-layer chromatography.

B. COMPARISON WITH PROSTAGLANDIN E-1*:

1. Thin-layer chromatography:

When "pure" vasodepressor lipid is compared with crystalline prostaglandin E-1 in chromatographic systems (a),(b),(c),(d), and (e) on Eastman "chromagram" sheets they have the same mobility, or such small differences in Rf values as to make the lipids inseparable.

Systems (a), (b) (Figure 8), (c) (Figure 9), and (d) (Figure 7) will not separate prostaglandin E-1 from prostaglandin E-2 or prostaglandin E-3. In these systems, therefore, prostaglandin E-1 standard serves as a prototype for the E-compounds.

* Crystalline prostaglandin E-1, kindly supplied by Dr. J.E. Pike of the Department of Chemistry, the Upjohn Company, Kalamazoo, Michigan, U.S.A.

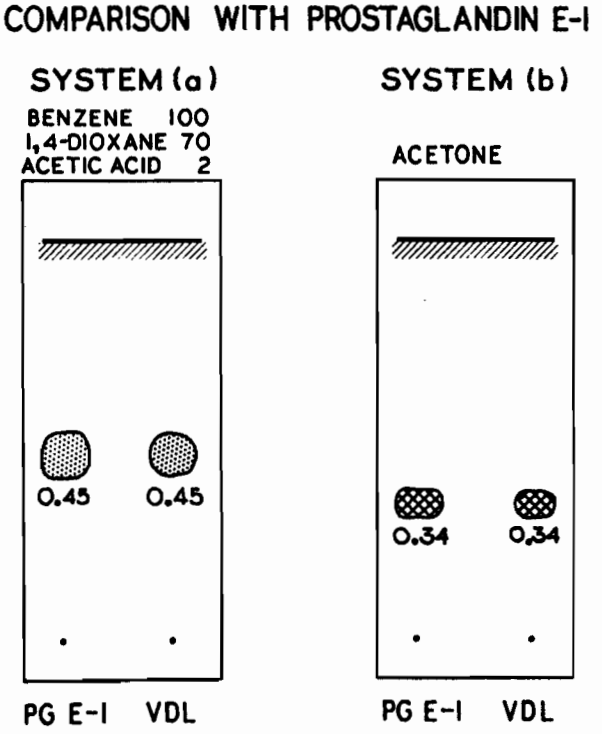


Figure 8.

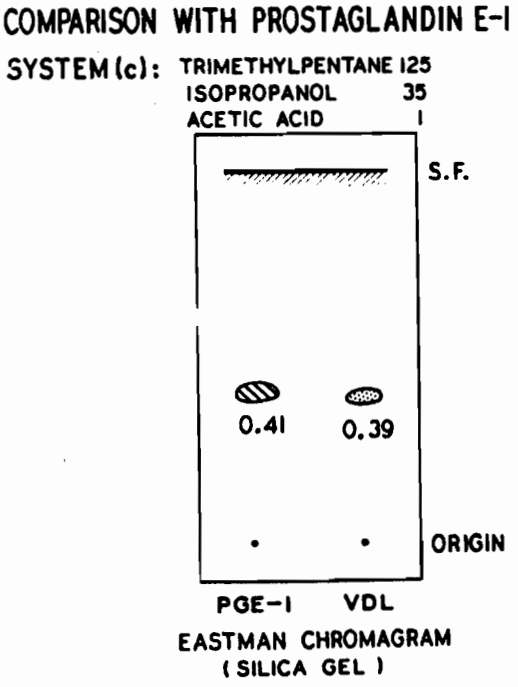


Figure 9.

System (e), however, will separate the E-compounds (140). The reported Rf values for these compounds in this system are: PGE-1 = 0.80; PGE-2 = 0.70; and PGE-3 = 0.35. It is significant that in this system "pure" vasodepressor lipid has the same mobility as crystalline prostaglandin E-1 (Figure 10).

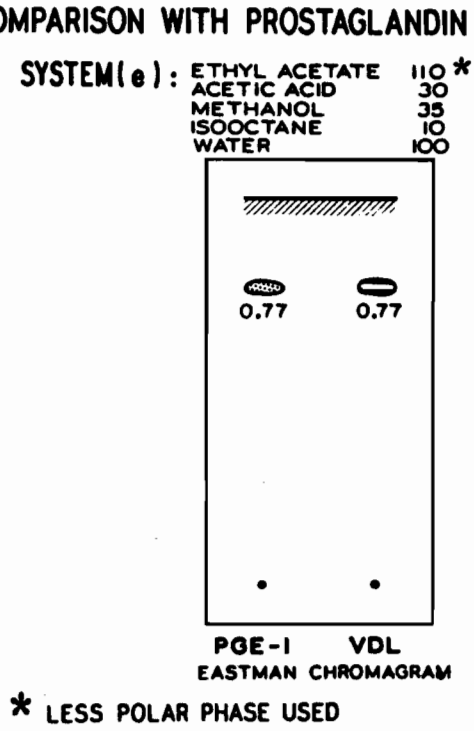


Figure 10.

2. High voltage starch gel electrophoresis.

When "pure" vasodepressor lipid and crystalline prostaglandin E-1 standard are subjected to high voltage starch gel electrophoresis they migrate at the same rate. This is illustrated in Figure 11.

Migrating toward the anode, ahead of the vasodepressor lipid and the prostaglandin E-1, is a line of altered gel translucency, fluorescence under ultraviolet light (maximum emission at 366 mμ.), and slightly positive phosphomolybdic acid reaction. No vasoactivity is found in

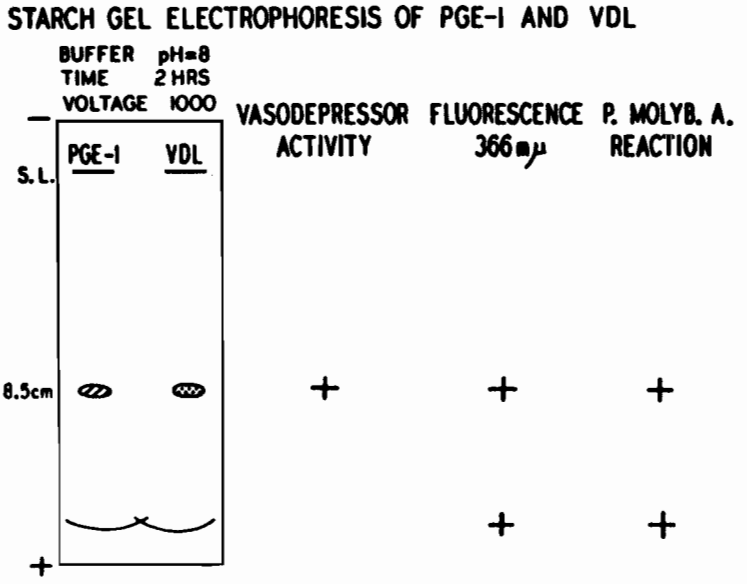


Figure 11.

the gel at this location. This boundary is probably caused by replacement of citrate ions in the gel by borate ions from the buffer (146).

Both prostaglandin E-1 and vasodepressor lipid fluoresce under ultraviolet light (366 mμ) when they are in the starch gel. This contrasts with their lack of fluorescence on Eastman "chromagram" sheets when the chromatograms are viewed under ultraviolet light at 254 mμ. and 366 mμ. The reason for this difference in behaviour is not known. The ions in the gel may play some role in this.

C. RELATIONSHIP TO THE RENIN-ANGIOTENSIN SYSTEM:

1. Effect of vasodepressor lipid (VDL) on angiotensin liberation in vitro;

The effect of the presence of renomedullary vasodepressor lipid in the incubation medium during renin-induced liberation of angiotensin

from angiotensinogen is outlined in Table II:

TABLE II

Contents of Incubation Flask (Incubated 3 hrs. at 37°C)					Angiotensin liberated
Flask	10 ml. Dog Plasma 4 ml. Dowex 50W-X2 (NH_4^+)	Renin 1 hog u.	1 ml. 95% ethanol	Vasodepressor lipid (100 g. equiv.)	(nanograms)
A	+	0	0	0	200
B	+	+	0	0	1237
C	+	+	+	0	1300
D	+	+	+	+	1250

As the results of the experiment demonstrate, vasodepressor lipid has no significant effect on the in vitro liberation of angiotensin from dog plasma by added renin. The differences in the values for B, C and D are within the range of bioassay experimental error (±10%).

2. Effect of vasodepressor lipid and prostaglandin E-1 on "renin-activity" determination.

The effect of the addition of 100 g. tissue equivalent of vasodepressor lipid and 1 mg. of crystalline prostaglandin E-1 to the media during incubation of the plasma of a subject fed a low salt diet for 3 days prior to sampling is outlined in Table III.

The results of this experiment show no significant effect of vasodepressor lipid or prostaglandin E-1 on the incubation phase of the determination of human renin-activity by the method of Boucher et al (144).

TABLE III

Subject: J.H., male; [Low salt diet (10 mEq. Na, 90 mEq. K) 3 days].
Hematocrit 43
Volume of blood withdrawn 200 ml.

Flask	Flask contents	Incubation time (min.)	Angiotensin content (ng.)	"Renin-activity" ng./L./min. (averaged)
a	10 ml. plasma 4 ml. Dowex 50W-X2 (NH_4^+)	180	125	84
b	"	120	100	
c	10 ml. plasma 4 ml. Dowex 50W-X2 (NH_4^+) 100 g. equiv. VDL	180	137.5	88
d	"	120	100	
e	10 ml. plasma + 4 ml. Dowex 50W-X2 (NH_4^+) + 1 mg. PGE-1	180	137.5	82
f	"	120	87.5	

3. Effect of vasodepressor lipid on angiotensin II <u>in vitro</u>.

The effect of semi-purified vasodepressor lipid on standard valine-5 angiotensin II aspartic β-amide is outlined in Table IV:

TABLE IV

Flask	Flask contents	Angiotensin II content (after 5 hours at room T.°)
a	100 ng. standard angiotensin II	87.5 ng.
b	100 ng. standard angiotensin II + 25 g. equiv. VDL	87.5 ng.

These results demonstrate no angiotensin-destroying effect of semi-

purified rabbit renomedullary vasodepressor lipid.

D. RELATIONSHIP TO "MEDULLIN".

When Lee et al (88) reported the extraction and purification of a nonvascular smooth muscle stimulating lipid ("compound 1") and two potent vasodepressor lipids ("compound 2" and "medullin") from rabbit renal medulla, we had just isolated our vasodepressor lipid as a single spot on activated silica gel thin-layer chromatography in the same system (benzene:dioxane:acetic acid). Our failure to find "compound 1" was not surprising since it was reported to have "weak to absent vasodepressor effects" (88) and we had been discarding fractions with absent vasodepressor activity.

Why were we finding only one vasodepressor compound? There seemed to be two possibilities. On one hand, our extraction technique might fail to remove "medullin" from renal tissue. On the other hand, "medullin" might be an artifact resulting from some chemical change in "compound 2". We had no "medullin", so we could not investigate the first possibility.

As described in our Review of the Literature (page 16), there is a step in the extraction procedure reported by Lee et al (88) where the vasoactive materials are dissolved in 0.005 M Na_2HPO_4, acidified to pH 1-2 and extracted three times with benzene, chloroform or diethyl ether.

We investigated this step by dissolving "pure" vasodepressor lipid or crystalline prostaglandin E-1 in 100 ml. of 0.005 M Na_2HPO_4, acidifying the solution to pH 1.5 with 1 N HCl and leaving the acidified solution at room temperature for periods varying from 1 to 24 hours. The solution was then extracted three times with equal volumes of chloroform. The chloroform phases were flash-evaporated at 40°C. The chloroform-ex-

tracted lipid was dissolved in 1 ml. of chloroform, and spotted on Eastman "chromagram" sheets. The chromatograms were developed in systems (a) (benzene 100:dioxane 70:acetic acid 2) and (c) (trimethylpentane 125:isopropanol 35:acetic acid 1). The results are displayed in Figures 12 and 13.

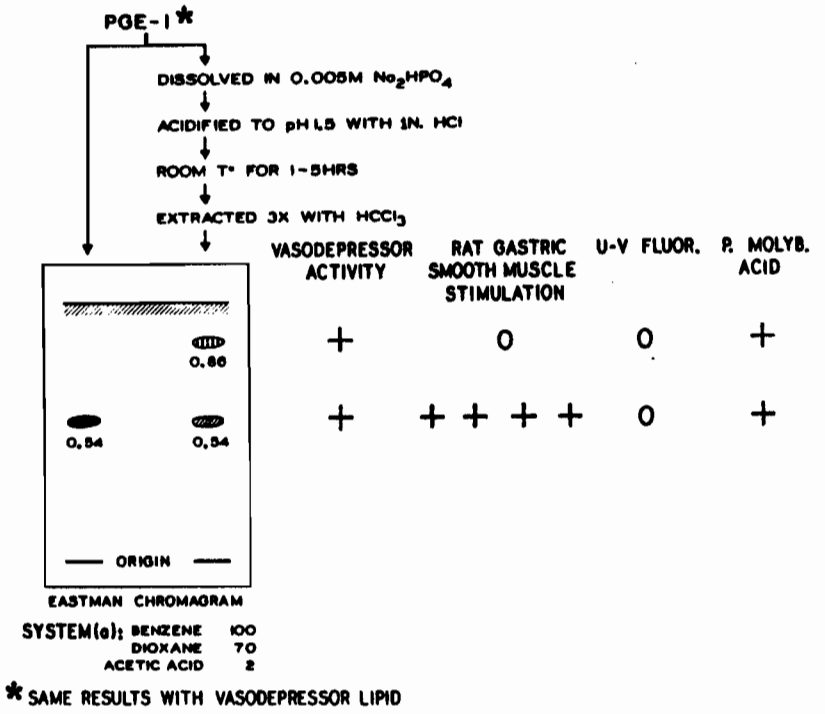


Figure 12.

Thin-layer chromatography of acid-treated VDL or PGE-1 [system (a)].

When the lipids are treated this way, there appears a new chromatographic spot with approximately one and one-half times the thin-layer chromatographic mobility of crystalline prostaglandin E-1 or "pure" vasodepressor lipid in these systems. This new compound resembles "medullin" in other ways also: it retains the vasodepressor activity of prostaglandin E-1 or "pure" vasodepressor lipid, but shows complete loss of ability to stimulate nonvascular smooth muscle. (This latter assay on rat

gastric fundal smooth muscle was done by Dr. L.S. Wolfe, Director of the Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute).

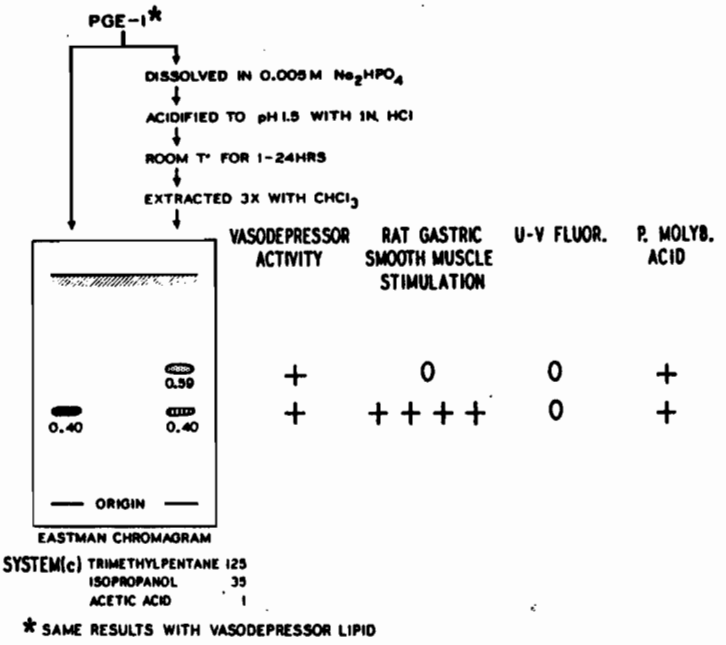


Figure 13.

Thin-layer chromatography of acid-treated VDL or PGE-1 [system (c)].

E. RELATIONSHIP TO PROSTAGLANDIN E_1-217 (Upjohn).

A new prostaglandin derivative has recently been isolated by thin-layer chromatography from material produced by the enzymatic cyclization of homo-γ-linolenic acid by homogenates of sheep vesicular glands (147). This procedure yielded prostaglandin E-1, prostaglandin F-1α and this major product, less polar than prostaglandin E-1, now named "prostaglandin E_1-217".

Since "medullin" and PGE_1-217 were both less polar than prostaglandin E-1 (and therefore less polar than "pure" vasodepressor lipid), this new derivative was of great interest to us.

We compared vasodepressor lipid (or prostaglandin E-1) with acid-

treated vasodepressor lipid (or prostaglandin E-1) and prostaglandin E_1-217* by thin-layer chromatography on Eastman "chromagram" sheets in systems (a), (c) and (d). The results are shown in Figure 14.

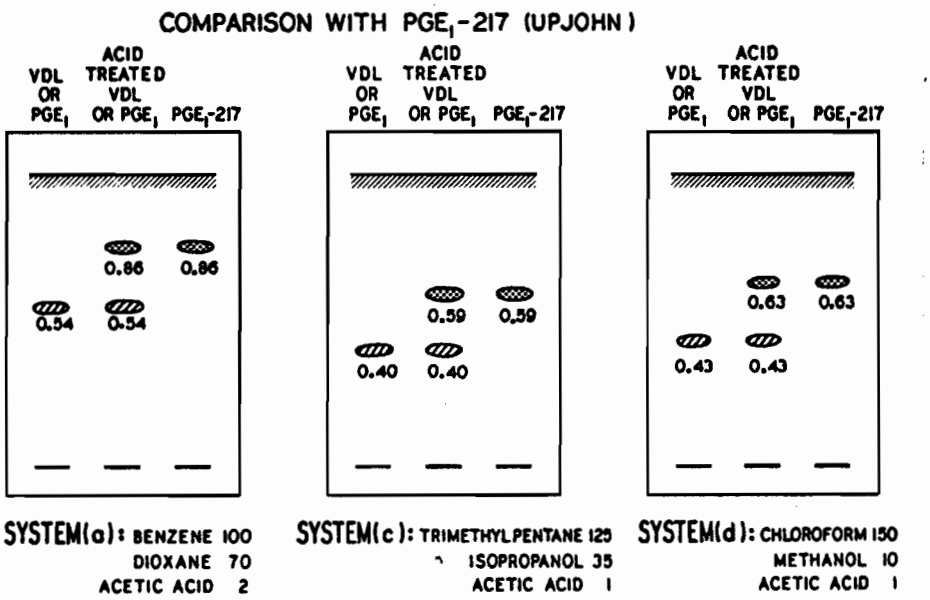


Figure 14.

Prostaglandin E_1-217 (Upjohn) and the compound created by the acid treatment of VDL or PGE-1 have identical thin-layer chromatographic mobility on Eastman "chromagram" (silica gel) sheets in the three systems used. Like the thin-layer mobility of "medullin", this mobility was approximately one and one-half times that of "pure" vasodepressor lipid (or prostaglandin E-1).

There are other points of similarity among prostaglandin E_1-217, "medullin" and the compound produced by acid-treatment of "pure" vasodepressor lipid. On guinea pig ileum and rabbit duodenum, PGE_1-217 had 1/100 th. the nonvascular smooth muscle stimulating activity of PGE-1

* Dr. J.E. Pike of the Department of Chemistry, the Upjohn Company, Kalamazoo, Michigan, U.S.A., very kindly gave us 5 mg. of crystalline prostaglandin E_1-217.

but it had vasodepressor activity at least equal to that of PGE-1 (147). "Medullin" was reported to be more unsaturated and to have fewer hydroxyl groups than prostaglandin E-1 (88). Figure 15 demonstrates that this is also true of prostaglandin E_1-217.

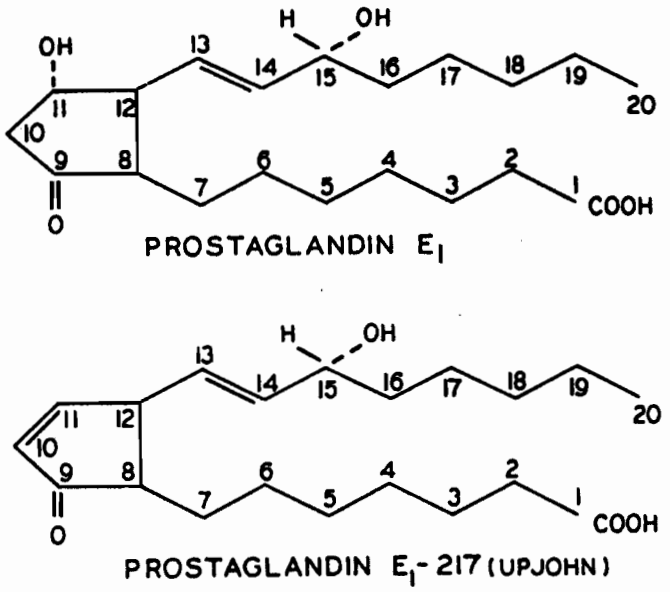


Figure 15.

Structures of PGE_1 and PGE_1-217 (Upjohn). After Daniels, E.G. et al. (147).

In comparison with prostaglandin E-1, PGE_1-217 (Upjohn) has an additional double bond (Δ 10) and it lacks a hydroxyl group on carbon-11 (147).

Treatment of prostaglandin E-1 with 90% acetic acid (acetic acid: water 9:1 v/v) for 18 hours at 60°C. produced a compound which was identical with PGE_1-217 by infrared spectroscopy and thin-layer chromatography (147). Although the pH was lower (less than 1), the temperature was higher and the time of treatment was longer than those we had used in

most of our experiments with acidification of solutions of "pure" vasodepressor lipid, this report tends to confirm our impression that "medullin" might be an artifact resulting from the low pH used at one point in the reported extraction procedure of Lee et al (88).

F. HUMAN VASODEPRESSOR LIPID:

It is possible to extract vasodepressor lipid from human kidney tissue. Figure 16 demonstrates an interesting preliminary study involving the extraction of vasodepressor lipid from a kidney removed surgically in the treatment of renovascular hypertension.

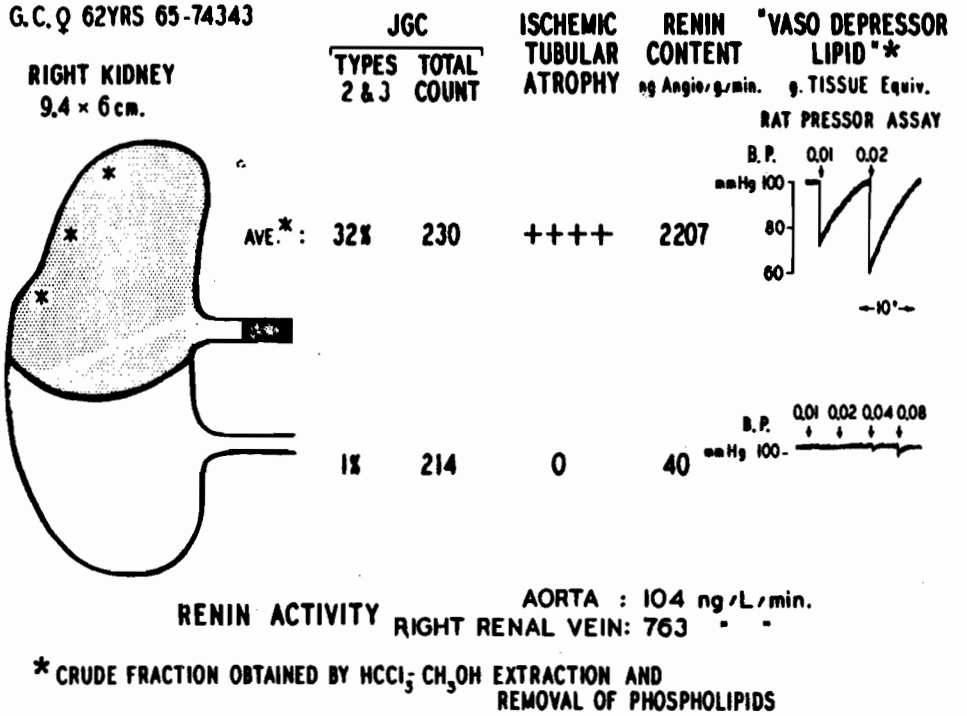


Figure 16.

This 62 year old hypertensive woman had two arteries to the right kidney. The upper principal artery was completely thrombosed. Peri-

pheral venous renin-activity was elevated at 54 ng./L./min. (normal: 0-24 ng./L./min. with a mean of 9.5 ± S.D. 7.6 ng./L./min.). In plasma taken during operation, the right renal venous renin-activity was 763 ng./L./min., which is greater than the normal range (0 to 115 ng./L./min.) for renal venous renin-activity, and greater than the renin-activity in aortic plasma taken at the same time (104 ng./L./min.). The ischemic upper segment of the kidney yielded abnormally high values for those parameters important in renovascular hypertension: juxtaglomerular cell count (normal: 192 to 221 per 25 juxtaglomerular bodies) and granularity of types II and III (normal: 1 - 3.5%). The renin content of the ischemic upper segment was markedly (55 times) greater than that in the lower segment.

Separate, but identical, extractions of ischemic and normal medulla showed that tissue concentration of vasodepressor lipid in the ischemic part was at least 50 times greater than that of the non-ischemic portion.

The initial vasodepressor assay was done using crude extracts. When the purification technique had been established, these crude extracts were purified. After double column chromatography the bioassay results were the same as those observed with crude extracts. This human vasodepressor lipid behaved the same way as rabbit renomedullary vasodepressor lipid on thin-layer chromatography in systems (a) and (d), the purification systems. There was insufficient lipid to continue with thin-layer chromatography in the other systems.

IV - DISCUSSION.

Review articles by Horton (133) and Bergstrom and Samuelsson (115) in 1965 clearly stated that the prostaglandins were of widespread

occurrence, and not restricted merely to the male accessory genital glands and their secretions. Neither review article, however, reported the discovery of the prostaglandins in extracts of renal tissue. Hickler et al (82) expressed the belief that renomedullary vasodepressor lipid was closely related to the prostaglandins, but they reported (83) that it could be differentiated from prostaglandin E-1 by thin-layer chromatography. Lee et al (88) tentatively identified "compound 2" as prostaglandin E-1. Our work is therefore amongst the first to describe the extraction from renomedullary tissue of a compound that is very probably prostaglandin E-1. It seems likely that our "pure" vasodepressor lipid is the same as "compound 2" of Lee et al (88). It seems probable, also, that we do not extract "medullin" from renomedullary tissue because that compound is an artifact produced by the acidification of a 0.005 M Na_2HPO_4 solution of "compound 2" during the extraction process of Lee et al (88), a step that does not take place in our method.

The technique that we have chosen and reported here is relatively simple. Because the initial purification steps utilize small columns, the method is best suited to the processing of relatively small amounts of renal tissue. It does have the advantage that organic solvents are used throughout and a relatively neutral pH is maintained, tending to minimize chemical alteration of the lipids being handled.

The procedure allows the vasodepressor lipid to be brought to a high degree of purity. The lipid, as it is obtained from the second thin-layer purification, cannot be purified further in the two other thin-layer systems and high voltage starch gel electrophoresis. Had time permitted, we could have accepted the very kind offer of Drs. L. S. Wolfe and C. Pace-Asciak of the Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, to assess the purity of our material

by gas chromatography. Perhaps others will be able to utilize this technique in following up this or similar work.

"Pure" vasodepressor lipid is inseparable from prostaglandin E-1 on thin-layer chromatography in the five systems reported and by high voltage starch gel electrophoresis. Here again, it should be possible in the future to confirm this probable identity by such procedures as gas chromatography, infrared spectroscopy and mass spectroscopy of the methyl ester derivative of "pure" vasodepressor lipid. This will depend upon the investigator having access to the necessary equipment in his laboratory or being able to accept generous offers of help such as we have had.

The artifact created by the acidification to pH 1.5 of a 0.005 M Na_2HPO_4 solution of "pure" vasodepressor lipid (or prostaglandin E-1) has the same mobility as PGE_1-217 on Eastman "chromagram" thin-layer chromatography sheets in systems (a), (c), and (d). The artifact, "medullin" and PGE_1-217 are similar in that each is more unsaturated and less polar than prostaglandin E-1. They also share a reduced ability to stimulate nonvascular smooth muscle and a retained vasodepressor potency. This is suggestive, although not conclusive, evidence that "medullin" is an artifact. If it is an artifact, our method of extraction and purification gains in acceptability, because it does not yield "medullin".

Our general approach to the recognition of the active substance in renomedullary extract deserves comment, favorable and unfavorable. Rat blood pressure bioassay has some difficulties, which have been described in this thesis. With this technique, it is important to ensure standardization of rat sensitivity, to select the bioassay medium appropriate to the particular purpose of the bioassay, and to use such convenient equipment as disposable syringes. Beyond this, however, lies the decision

to use a vasodepressor response as the criterion for retaining any particular fraction or compound. As we outlined in the original experimental protocol, this was to be our criterion because we were dealing with a "vasodepressor" lipid. This has led to the retention of fractions which probably contain prostaglandin E-1. It has also meant, however, that the F-compounds were discarded, since their vasodepressor properties are relatively weak or absent. It is reasonable to suggest that future workers in this field should use nonvascular smooth muscle bioassay preparations, as well as blood pressure assay preparations, so that the F-compounds might also be retained and measured. This is important because there is evidence to suggest that the F-compounds are the main storage forms and precursors of the E-compounds. If this is so, then the tissue content of the F-compounds is a valuable measurement to make, even when assessing some particular function of the E-compounds.

Horton (133) has drawn interesting analogies between the prostaglandins and the adrenal hormones. While only a few adrenal steroids are normally secreted, many other steroids have been isolated from the adrenal cortex. Many of these are precursors in the biosynthetic pathways of the secreted steroids. Similarly, the F-compounds are the predominant tissue storage form of the prostaglandins, but the E-compounds have greater physiological significance. Generally speaking, the E-compounds (analogously to epinephrine) have more potent inhibitory actions but both the E-compounds and the F-compounds (or both epinephrine and norepinephrine) are very active on tissues that are stimulated. In both cases a more potent inhibitor (prostaglandin E and epinephrine) is formed in the tissues from a less potent inhibitor (prostaglandin F and norepinephrine). The stimulatory and inhibitory effects of the prostaglandins, like those of the catecholamines may each have a physiological im-

portance at different sites.

The effects of prostaglandin E-1 on blood pressure have been outlined in the Review of the Literature. Prior injection of prostaglandin E-1 reduces the pressor responses to subsequent injections of angiotensin II (82, 119), the catecholamines and vasopressin (119). Holmes et al (119) raised the possibility that prostaglandin E-1 might interfere with some biochemical step common to the pressor activities of these different agents. This hypothesis might be carried further. The enzymatic conversion of adenosine triphosphate to cyclic AMP (adenosine-3',5'-monophosphate) by adenyl cyclase is enhanced by vasopressin (148) and the catecholamines (149). Prostaglandin E-1 opposes this enhancement by vasopressin (138). Perhaps it is this conversion of adenosine triphosphate to cyclic AMP, or some closely allied reaction, that is the biochemical "common pathway" affected by prostaglandin E-1.

The results reported in this thesis shed some light on the mechanism of action of vasodepressor lipid on blood pressure, even if in a negative way. All the bioassay rats used had been nephrectomized 18 hours before use, so it is clear that normal levels of renin and angiotensin are not required for manifestation of the vasodepressor effects of vasodepressor lipid. Similarly, our preliminary experiments showed no effect of vasodepressor lipid on the liberation or destruction of angiotensin in vitro. We still do not know whether vasodepressor lipid has any local effect on the renal production or release of renin, or on the renal tubular action of angiotensin. Finally, in this regard, we do not know whether renomedullary vasodepressor lipid has any effect on blood pressure under physiological conditions. We do not know if this compound is released in amounts sufficient to alter blood pressure. We do not know whether it acts locally to regulate renal blood flow. Much remains to be eluci-

dated.

Is this compound, or is any of the prostaglandins a hormone? Prostaglandin E-1 inhibits the tone of vascular, respiratory and reproductive smooth muscle, and in large doses it depresses central nervous activities. Whether it circulates in sufficient concentrations to cause any of these effects under physiological conditions is not known.

Perhaps prostaglandins act locally as intracellular coenzymes. Horton has suggested that the conversion of an F-compound to an E-compound might provide two hydrogen atoms for a hydrogenase system (133).

With the knowledge that kidney medulla probably contains prostaglandin E-1, and that prostaglandin E-1 reversibly counteracts the permeability response of toad bladder to vasopressin (138), it will be fascinating to learn whether this effect takes place also at the renal sites of action of antidiuretic hormone, the distal convoluted tubules and collecting ducts. Although this has not been reported yet, to my knowledge, no doubt some investigator, somewhere, is observing the effects on urine concentration and flow produced by injection of prostaglandin E-1 into the renal artery of a hydropenic experimental animal.

Further experiments in human subjects will shed more light on the interesting relationship between the prostaglandin content of human seminal fluid and the control of human fertility.

It is not possible, on the basis of present knowledge, to explain the simultaneous occurrence of greater medullary vasodepressor lipid content and cortical renin content in ischemic renal tissue as compared to non-ischemic renal tissue. We do not know if this is a coincidental finding or if a cause-effect relationship exists. Obviously, this finding requires confirmation. It may evoke interesting investigative work

into the common effects of altered tissue perfusion and oxygen supply, metabolism of the prostaglandins and the production and release of renin.

There is one interesting pertinent generalization that can be made at present: all the organs from which the prostaglandins have been isolated have been shown to respond to the prostaglandins. This response has been mediated by various cells in addition to vascular smooth muscle cells (which, of course, also occur in these organs and respond to the E-compounds). The thymus is the one organ which is at present an exception to this generalization. Whether the kidney may be included in this generalization will depend upon the results of the work suggested above, involving the distal convoluted tubules, collecting ducts and juxtaglomerular bodies.

V - SUMMARY.

Extraction of renomedullary tissue by the method of Hickler et al (82,84) yields a substance with potent, sustained vasodepressor activity when given by intravenous injection to anaesthetized, nephrectomized rats. This substance can be extracted from renomedullary tissue of many species, including man.

The renomedullary vasodepressor lipid may be brought to a high degree of purity by double column chromatography followed by thin-layer chromatography in two systems. The "pure" vasodepressor lipid thus obtained cannot be purified further in two other thin-layer chromatographic systems or by high voltage starch gel electrophoresis.

When "pure" renomedullary vasodepressor lipid is compared with crystalline prostaglandin E-1 by thin-layer chromatography on Eastman "chromagram" sheets (activated silica gel) in five systems and by high voltage starch gel electrophoresis, the two are indistinguishable. They

share potent vasodepressor and nonvascular smooth muscle stimulating properties as well.

During the course of this work, Lee et al (88) reported that extraction of renomedullary tissue, by a different method, yielded two potent vasodepressor lipids (not a single lipid as we had found). One of these, called "compound 2", was similar to our "pure" vasodepressor lipid in biological activity and thin-layer chromatographic mobility. Like our compound, it was tentatively identified as prostaglandin E-1. The other potent vasodepressor lipid of Lee et al was called "medullin". It was less polar and more unsaturated than prostaglandin E-1. It had a markedly reduced ability to stimulate nonvascular smooth muscle.

We have shown experimentally that there is strong evidence to suggest that "medullin" of Lee et al might be an artifact produced by acidification to pH 1-2 of a 0.005 M Na_2HPO_4 solution of "compound 2" (or, in our terminology, "pure" vasodepressor lipid). We have shown further that such an artifact and a compound called prostaglandin E_1-217 (151) have the same thin-layer chromatographic mobility on Eastman "chromagram" sheets in three systems. The artifact and PGE_1-217 (Upjohn) retain the vasodepressor potency of prostaglandin E-1, but lack its ability to stimulate nonvascular smooth muscle. The structure of PGE_1-217 (Upjohn) is known (147)and it differs from that of prostaglandin E-1 in the same way that "medullin" is reported to differ from prostaglandin E-1 (88), lacking a hydroxyl group (on carbon-11) and having an additional (Δ10) double bond.

In preliminary experiments we found no relationship between vasodepressor lipid and the in vitro reactions associated with the liberation and destruction of angiotensin.

The observation that ischemic human renal medulla contains more

vasodepressor lipid than non-ischemic medullary tissue is fascinating, but it requires confirmation.

Finally, it must be conceded that the tentative identification of renomedullary vasodepressor lipid as prostaglandin E-1 sheds very little light on the question of renal or renomedullary antihypertensive action, In fact, it raises more questions than it answers, because it links the renomedullary factor with a group of compounds which are found in various tissues and secretions of several species and which have multiple biological effects. The relative importance of the kidney as a source tissue, or as a site of metabolism of these compounds remains to be elucidated. The relationship between the renal content of a compound of this nature and the control of blood pressure is equally elusive.

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