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THE NATURE AND ACTION OF A HYPOTENSIVE AGENT FROM EUCALYPTUS ROBUSTA.

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THE NATURE AND ACTION OF A HYPOTENSIVE AGENT FROM *EUCALYPTUS ROBUSTA*

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By

George Wesley Read

Dissertation Committee:

James F. Lenney, Chairman
Windsor C. Cutting
Shao-Chia Chou
Shoji Shibata
Terence O. Moore

ABSTRACT

Extracts of some species of Eucalyptus have been found to be hypotensive in rats, guinea pigs, cats, and dogs. Of the 29 species of this genus available for assay, Eucalyptus robusta contained the highest concentration of the hypotensive principle. Active and inactive species were randomly distributed throughout the sections and subsections of the genus, indicating that activity is not associated with any particular group of the eucalypts. The active principle occurs in highest concentration in the leaves but is also found in the phloem, roots, and to a lesser extent in the xylem. Little or no activity is present in the bark.

The hypotensive factor is soluble in water, lower alcohols and acetone but insoluble in chloroform or ether. Addition of chloroform or ether to the alcoholic extracts precipitated a more purified fraction. Attempts to purify the active component further by adsorption, steam distillation, gel filtration, heavy metal ion precipitation, liquid-liquid extraction, and dialysis met with little success.

The molecular weight of the active principle was found to be approximately 3000, using Craig's dialysis method. The active component's molecular weight, solubility characteristics, precipitability by heavy metals (especially ferric chloride), base lability, strong adsorptive tendencies especially to Sephadex), non-volatility, ease of oxidation, and

ineffectiveness orally indicate that it is probably a specific tannin.

Five mg/kg of the partially purified material depressed the blood pressure of rats to less than 50% of its original value for approximately 15 minutes. Larger doses lowered the blood pressure to 30% of normal and for periods greater than several hours. The active agent was effective intravenously and intraperitoneally but ineffective orally. The absence of an effect when it was given to the vascularly isolated heads of cross-circulated animals indicated that the agent's action was peripheral.

Prior treatment with hexamethonium, reserpine, phenoxybenzamine, pronethalol, and atropine had little or no effect on the action of the Eucalyptus factor. The antihistaminics tripellenamine and diphenhydramine, however, did attenuate the depressor effect of the extracts. When the active principle was given first, it had little or no effect on the response to subsequently administered phenylephrine, angiotensin, isoproterenol, or methacholine. However, there was an interference with the response to histamine. These results suggest that the hypotensive agent acts on the histamine pathway.

Depletion of the histamine stores with the drug 48/80 blocked the action of the hypotensive agent, as did a one-week pretreatment with the agent itself. These results, along with the blockade by antihistamines and the

interference with the response to histamine, indicate that the active principle is a histamine liberator.

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INTRODUCTION

Background. About half of the drugs used today are of natural origin (Gosselin, 1962) and it is logical to anticipate that additional valuable drugs will be discovered in plants and animals. Therefore, in the Department of Pharmacology of the University of Hawaii a natural products program has been developed which is directed toward this end.

In this program, the natural products to be tested are extracted and tested for activity in four separate assays: 1) antiviral, 2) anticancer, 3) antifertility, and 4) "classical" (for effects on cardiovascular, respiratory, nervous, and smooth muscle systems). Extracts showing promise are studied further in regard to mode of action and chemical structure of the active principle.

It was under these conditions that the hypotensive activity to be discussed in this dissertation was found in a fungus (and later traced to Eucalyptus) in July 1966. At that time some 206 plant and animal extracts had been processed and 29 showed moderate to strong hypotensive activity in rats (blood pressure depression greater than 20 mm Hg and longer than two minutes) with little effect on the other systems (especially EKG, respiration, and EEG). Of the 29 extracts possessing potential antihypertensive agents, the fungal extract and two others emerged as having very strong actions which lasted several hours.

The need for antihypertensives. More people die of cardiovascular disease than from any other cause. In 1964, the last year for which figures were available (Surgeon General's Report, 1966), cardiovascular conditions killed 54% of the people who died in the United States. A total of 1,798,051 people died in 1964, therefore presumably 970,000 died of cardiovascular conditions. Of the top 15 causes of death, five were cardiovascular categories. Not all deaths in the cardiovascular category are due to hypertension; however, most of them involve hypertension either directly or indirectly.

The control of hypertension is also desirable to increase the usefulness of people afflicted with this disease. The National Health Education Committee (1966) estimates that 15,258,000 people have hypertensive heart disease. They also estimate that 132 million working days are lost each year because of heart disease of all types. This latter figure is equivalent to 540,000 man years or approximately 2.5 billion dollars.

Hypotensive agents have other uses besides therapeutic ones. Numerous agents for which the mechanisms of action are known are used in the laboratory in the study of other less understood drugs. Examples of this are the various blocking agents such as antihistaminics, adrenolytics and parasympatholytics. These agents are used as tools to block specific pharmacological pathways so that when a new drug's action is blocked, one of its mechanisms is illuminated.

When enough of a new drug's mechanisms are known, it too can serve as a tool for the study of other agents. In these ways then, this hypotensive agent might be useful.

The eucalypts. The genus Eucalyptus is one of the 80 genera of the Myrtaceae family which contains many plants known for their fruits and spices (rose apple, guava, cloves, allspice, oil of bay rum, tea tree, myrtle, pimento, and Eucalyptus oil; Lawrence, 1951). The genus Eucalyptus itself has over 600 species ranging in height from the small, shrubby, multi-stemmed mallees at two meters to the tallest hardwoods in the world at 138 meters (Penfold and Willis, 1961). Eucalyptus robusta, commonly called "swamp mahogany," grows naturally on water-logged soils and is used in the lumber industry and as an ornamental shade tree.

Penfold and Willis (1961) have reviewed the industrial and medicinal uses of the eucalypts in Hawaii and internationally. In Hawaii, there are over 70 species of Eucalyptus. It is not known who introduced them but it is thought that they were planted in about the middle of the last century (in 1907, H. L. Lyon made the first report of groves of them occurring on Kauai, Oahu, Maui, and Hawaii; Eucalyptus robusta predominated). Subsequently, the government and logging interests have planted several million trees and lumber is being harvested from groves 35 years old.

In Australia and New Zealand, the native habitat of the eucalypts, the group has been exploited most. The essential or volatile oil industry began in 1788 at Port Jackson (now

Sydney) and has grown to its present volume of 900 metric tons per year. As a crude product, it was initially exported to England and used for "cholicky complaints." More recently, only the purified products are used medicinally while the crude distillates are used industrially as solvents, disinfectants, and deodorants. The phenolic, aldehydic, and ketonic constituents of the oil (especially cuminal, phellandral, and cryptone) possess germicidal properties and are used in inhalants, liniments, soaps, gargles, sprays, lozenges, etc. The terpenes are blended into turpentines and the methanol and acetic acid are marketed as such. The cineole (eucalyptol) is used as an inhalant, solvent and cleaning agent. The piperitone is used as a precursor in the synthesis of thymol and menthol. In 1967, Lysenko reported on the use of azulene intramuscularly for burns. A few species yielding geraniol, eudesmol, citronellal, and citral are used in the perfume industry.

Aside from the volatile oils, the eucalypts produce other substances of industrial and medicinal value. Paper, fiberboard, cellulose, fuel, charcoal, and lumber are among the more common products. Tannins, present in most of the species, are extracted from the wood and marketed for their astringent and antiseptic as well as their tanning properties. Myrtan, another extract, is used in the feed waters of boilers to reduce scale formation. Rutin, from a water extract of the leaves, enjoyed a broad and lengthy acceptance for its vitamin P-like properties. It seemed to reduce

capillary fragility and was used in the prevention of vascular accidents. However, the Food and Drug Administration has ruled that flavonoids are not effective in man "for any condition" and has initiated action to have rutin and other similar agents removed from the market (Anon., 1968).

In other work, Bolliger (1953) has refuted the idea that Eucalyptus leaves contain adrenal hormone-like substances. John (1922) working with diabetics, showed that extracts of Eucalyptus leaves were of no value in the treatment of this disease. Degtyareva (1962) isolated a crystalline phenolic substance from Eucalyptus leaves that has antibiotic activity, and Satwalekar et al. (1957) found citriodorol to be an antibacterial agent. In 1931, Labo proposed the use of sanosine in tuberculosis. Chisholm (1926) received a patent on the use of dried, macerated, and cured Eucalyptus leaves as a "tobacco substitute with therapeutic properties."

Several compounds reported to occur in the eucalypts have been found in other investigations to have hypotensive activity. These are not responsible for the hypotensive activity described in the present work, and are discussed in Section III. Organic substances described in the literature as occurring in the genus are listed in the Appendix.

The distribution of the Eucalyptus factor. Although the hypotensive activity was originally found in a fungus, subsequent collections revealed that the fungus grew only on decaying Eucalyptus logs suggesting that the latter might be

the source of the activity. To test this possibility, samples of the logs were collected and assayed. When activity was found in the eucalypts, Miss Gwen Naguwa, a high school Summer Science Apprentice under the direction of the author, assayed extracts of all of the species of Eucalyptus available in the Harold L. Lyon Arboretum of the University of Hawaii.

In addition to determining the species which is the best source of the hypotensive agent, knowledge of the distribution of an active substance within a genus may 1) lead to its identification if it parallels the distribution of a known constituent, and 2) contribute to the taxonomy of the genus (Penfold and Morrison, 1961). It is now common to group organisms according to their chemical composition to substantiate existing morphological arrangements or indicate alternative relationships (Hillis, 1966, 1967a, 1967b, 1967c, 1967d).

To determine the best starting material for the purification of the hypotensive agent, the tissues of the species possessing the highest level of activity were assayed. Once the preferred species and tissue had been selected, it was possible to proceed with the purification, identification, and pharmacology of the hypotensive agent.

METHODS

Collection. Specimens for assay and identification were obtained at the original site of collection on Oahu,

Hawaii: the Eucalyptus grove at the first ford, after the bridge, on the Manoa Falls Trail. The fungi were collected whole and vacuum dried at 50°C.

The Eucalyptus specimens were collected as separate tissues, except for the roots and leaves, which were collected intact. Bark, phloem, sapwood, and heartwood were easily distinguished as there were sharp changes in texture and color between these tissues. The bark and phloem could be peeled off separately, while the sapwood and heartwood had to be chiseled apart. As with the fungi, the Eucalyptus tissues were vacuum dried at 50°C.

Identification. Preserved specimens or living organisms in the field were identified by authorities on taxonomy. The fungus was identified by Dr. Gladys Baker of the Botany Department of the University of Hawaii. For the Eucalyptus, Dr. Albert C. Smith, also of the Botany Department, and Dr. Peter van Royen of Bishop Museum were consulted.

Extraction. The dried tissue was ground to a fine powder in a Waring blender and distilled water was added to a weighed amount of powder. The suspension was stirred for an hour at room temperature; it was then filtered and the filtrate was assayed.

Assay. Large (400-550 gram) Wistar derived (MW-3) male rats were anesthetized with pentobarbital sodium (Abbott) (50 mg/kg) and connected to a six channel Grass model 7 polygraph. Skin leads were used to obtain the electrocardiogram (lead I) and electroencephalogram (parietal skin leads).

The heart rate was determined with a tachograph preamplifier driven by the output of the electrocardiogram channel. Arterial pressure and intragastric pressure were measured with Statham P23dc liquid pressure transducers connected to the animal with cannulae to the femoral artery and stomach (via the esophagus) respectively. Respiration was measured with a Grass PT5A low pressure air transducer connected to the trachea with a cannula. The latter was equipped with a small opening so that air could be exchanged with the atmosphere yet a measurable pressure change would develop across the orifice during breathing. Heparin sodium (Nutritional Biochemicals) (10 mg/kg) and the extracts and drugs were given intravenously through a cannula inserted into the femoral vein.

In order to measure the activities of the various extracts or fractions, it was necessary to define a unit of activity and to determine a dose-response curve. One unit was defined as the activity contained in a specific water extract made from 10 mg of dried and ground phloem. Twenty-four animals were employed. Mean blood pressures were noted just before the administration of the extract, and again five minutes later. (The mean pressures were mathematically determined by adding one third of the pulse pressure to the diastolic pressure, or were electrically derived by damping the driver amplifier with the frequency response control.) Finally, the depressed blood pressure reading was expressed as a percent of the initial level and the dose-response curve constructed.

Statistics. To indicate the significance of the points on the dose-response curve, standard errors were calculated. These values appear as vertical bars on the graphs and in parentheses in the tables. Differences between experimental means were evaluated using the Students' t test. A P value less than 0.05 was considered to be significant and is indicated by a superscript ^{s}.

RESULTS

Dose-response curve. Figure 1 shows a typical "classical" polygraph record, run at a slow chart speed. In this record a Eucalyptus extract was injected and the depressor response is seen to be prompt and extended. There was little effect on other systems except for the intragastric pressure, which was also depressed. Lower doses were hypotensive for several minutes. Higher doses acted for the duration of the experiments--several hours.

Figure 2 shows the dose-response curve, plotting the actual systolic and diastolic pressures. Figure 3 is a dose-response curve based on mean arterial pressures and showing the depressed response as a percent of initial. This figure was used to determine the activity present in fractions of unknown potency. The linear portion of the curve extends from about 4-19 units per kilogram body weight. Doses below three units per kilogram body weight cannot be detected.

Taxonomic distribution of the hypotensive activity. Table I shows that the fungus, identified as Xylaria

Fig. 1. A "classical" record at slow speed, showing the typical response to a Eucalyptus robusta extract.

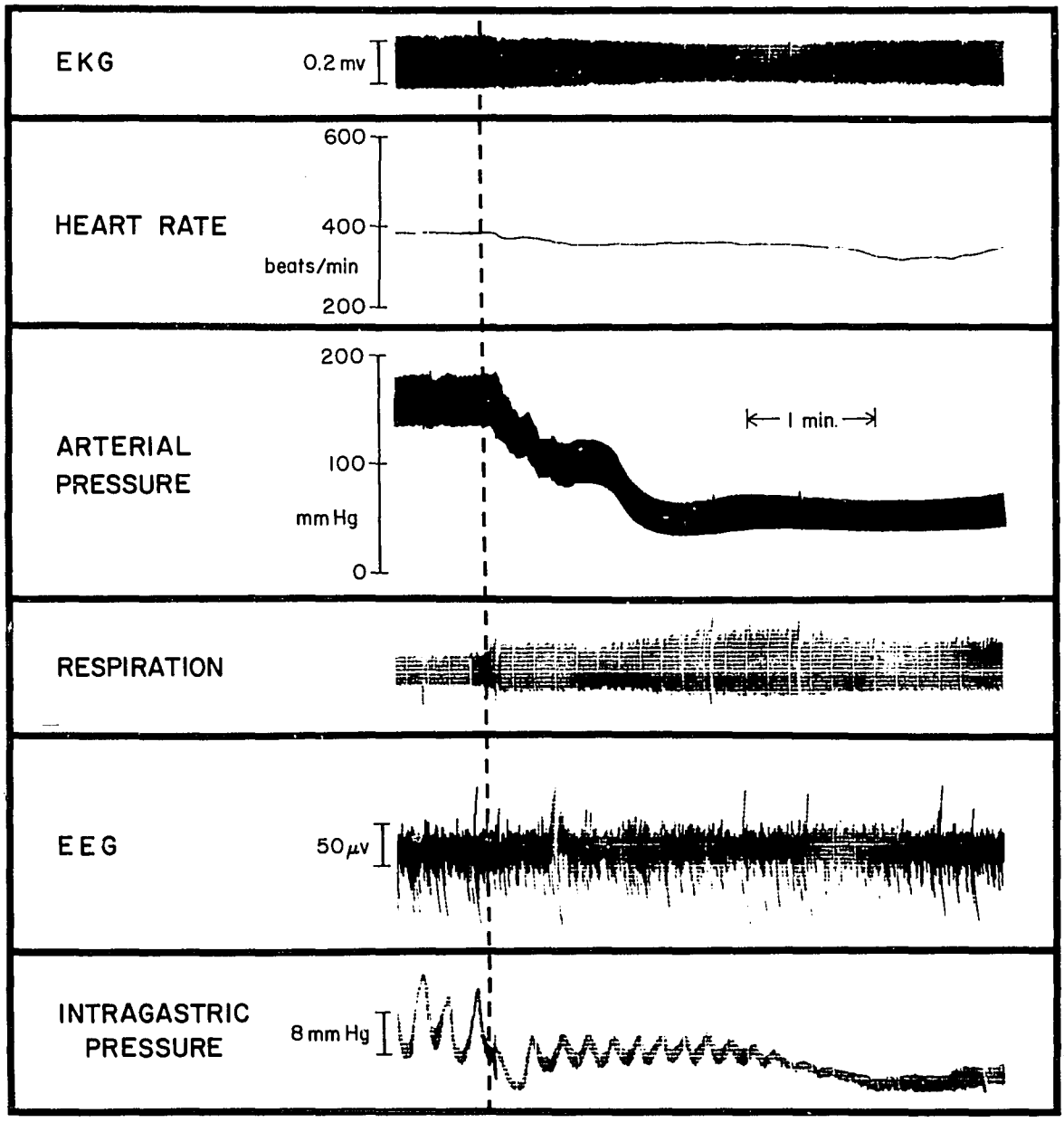

EKG
0.2 mv
HEART RATE
600
400
beats/min
200
ARTERIAL PRESSURE
200
100
mm Hg
0
← 1 min. →
RESPIRATION
EEG
50 μv
INTRAGASTRIC PRESSURE
8 mm Hg

Fig. 2. Dose-response curve showing the effect of the Eucalyptus factor on the systolic and diastolic pressures of rats.

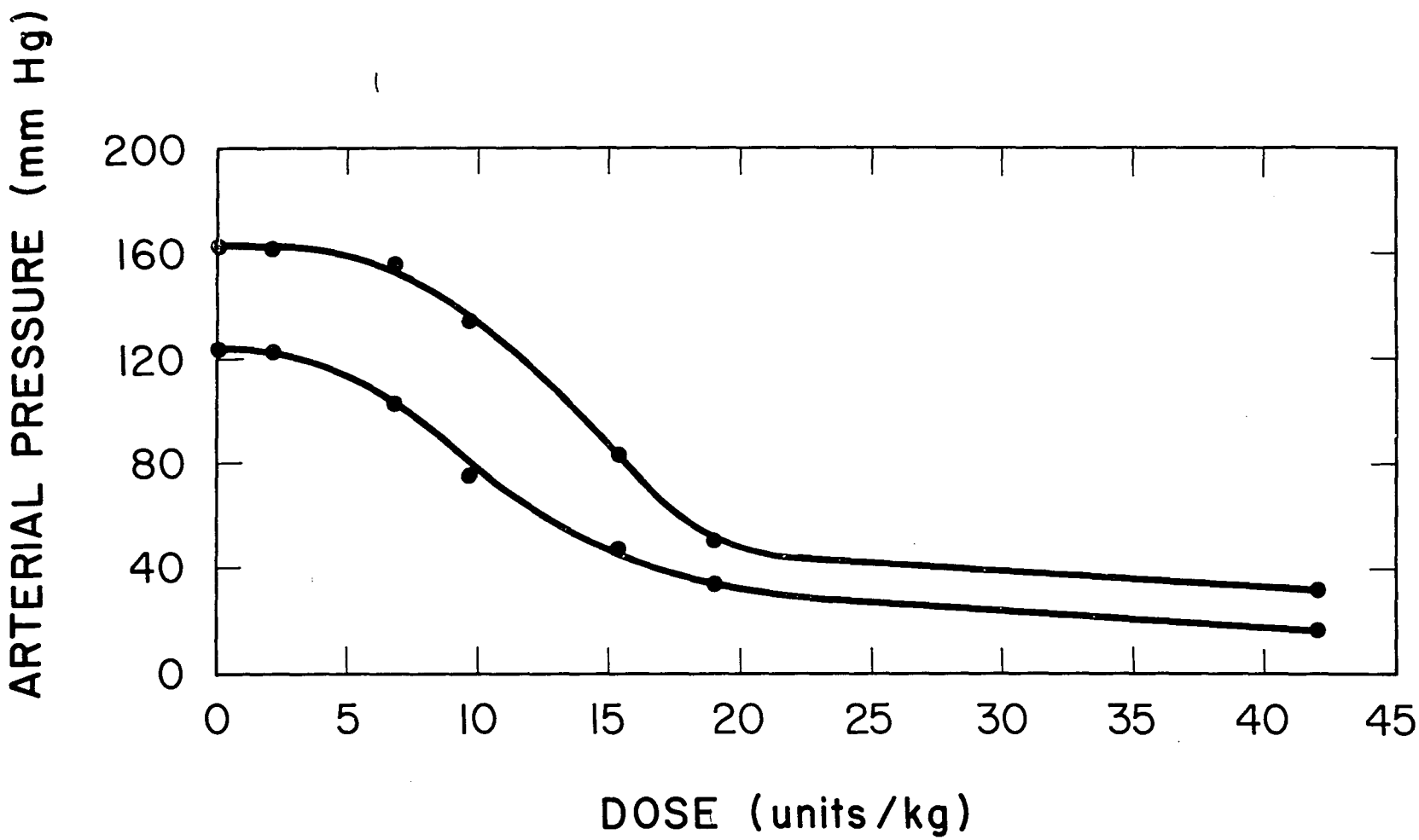
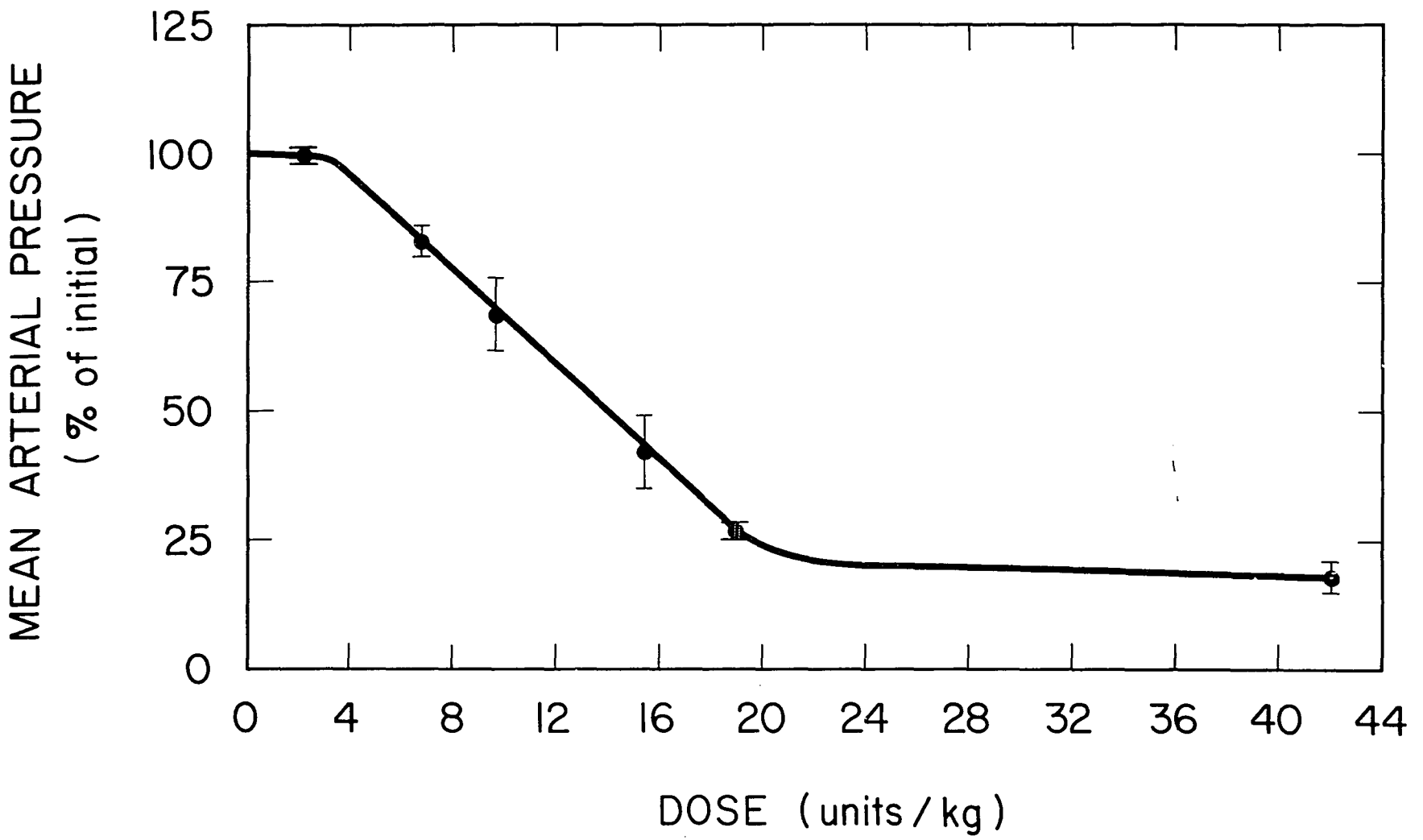

ARTERIAL PRESSURE (mm Hg)
200
160
120
80
40
0
0
5
10
15
20
25
30
35
40
45
DOSE (units/kg)

Fig. 3. Dose-response curve showing the relation between the amount of the Eucalyptus factor administered and the resulting decrease in mean blood pressure.


MEAN ARTERIAL PRESSURE
(% of initial)
125
100
75
50
25
0
0
4
8
12
16
20
24
28
32
36
40
44
DOSE (units / kg)

polymorpha, was not as good a source of the activity as the tree on which it grew. The tree, identified as Eucalyptus robusta, is compared to other species of Eucalyptus in Table II. Here it can be seen that of the 29 species tested, E. robusta gave the most potent phloem extracts, and that in about half of the species, activity could not be detected at all. In Table III, active and inactive species are compared according to their currently accepted classification, and it can be seen that the distribution of the activity is in no way related to the classification.

Distribution of the hypotensive activity in E. robusta. Within the species E. robusta, the leaves are the best source of activity (Table I), with the adult leaves appearing to be slightly more active than the juvenile leaves. (The members of this genus develop two kinds of leaves, a roundish juvenile type and a sickle-shaped adult type; the two types are entirely different leaves and not stages of development of the same leaf.)

DISCUSSION

It is not known at this time whether the hypotensive factor in Xylaria polymorpha is different from that in Eucalyptus but it is probably that the former, in its saprophytic existence on the latter, absorbs the water-soluble factor along with the nutrients that it takes up. It should be possible to resolve this problem either by extracting Xylaria polymorpha grown on inactive species or by comparing

TABLE I. HYPOTENSIVE ACTIVITIES OF AQUEOUS EXTRACTS OF VARIOUS TISSUES

Plant	Tissue	Mass Extracted (grams)	Volume (ml)	Soluble Solids (mg/ml)	Number Of Trials	Extract Activity (u/ml)	Tissue Activity (u/gm)	Specific Activity (u/mg solids)
Xylaria polymorpha	whole plant	100.0	60	56.9	4	31	19	0.5
Eucalyptus robusta	bark	5.4	54	0.5	3	<3	--	--
	sap wood	20.3	45	23.9	4	14	31	0.6
	heart wood	18.6	93	6.6	3	7	33	1.1
	phloem	10.0	100	15.6	28	10	100	0.6
	whole root	8.5	42	17.3	3	22	109	1.3
	juvenile leaves	9.2	92	19.4	4	21	210	1.1
	adult leaves	15.2	150	21.2	4	27	266	1.3

TABLE II. HYPOTENSIVE ACTIVITIES OF EXTRACTS OF 29 SPECIES OF EUCALYPTUS. EACH RAT WAS GIVEN THE WATER EXTRACT OF 0.1 GM OF PHLOEM.

E. Species	Number of Trials	Avg. of mean pressures Initial	Avg. of mean pressures 5 min. after treatment	Treated as % of Normal	Tissue Activity (u/gm)
robusta	2	141	40	28	94
melliodora	2	128	68	53	66
cornuta	2	129	85	66	52
beyeri	2	121	82	68	50
deglupta	2	163	114	70	48
dealbata	2	148	107	72	46
microcorys	2	150	108	72	46
citriodora	3	127	99	78	40
paniculata	3	143	111	78	40
umbra	4	141	114	81	36
crebra	2	145	130	90	27
albens	3	142	132	93	24
teretecornis	2	127	122	96	20
botryoides	2	136	133	98	18
deanei	2	134	132	98	18
ovata	2	124	122	98	18
maculata	2	127	126	99	<15
pilularis	2	151	150	99	<15
piperita	2	152	151	99	<15
propinqua	2	136	135	99	<15
colossea	2	129	129	100	<15
exerta	2	143	143	100	<15
longifolia	2	139	139	100	<15
resinifera	2	138	139	101	<15
sideroxylon	2	138	139	101	<15
acmenoides	3	123	126	102	<15
gomphcephla	3	123	126	102	<15
punctata	2	137	141	103	<15
smithii	2	118	122	103	<15

TABLE III. TAXONOMIC* DISTRIBUTION OF THE TEN MOST ACTIVE AND THE TEN LEAST ACTIVE EUCALYPTUS SPECIES POSSESSING HYPOTENSIVE ACTIVITY

Section	Subsection	Ten Most Active Species	Ten Least Active Species
Macrantherae I	Cordiformes Ovoidae Longiores	citriodora robusta cornuta	diversicolor longifolia propinqua punctata resinifera gomphcephla
	Truncatae Subtruncatae Orbiculares Ovulares Tereticornes	dealbata	exserta smithii
Macrantherae II			
Renantheroidae			
Renantherae I	Cordatae Papilionantherae		
Renantherae II		umbra microcorys deglupta	acmenoides
Porantheroidae I			
Porantheroidae II	Normales Attenuatae Oblongae Elongatae		
Terminales		beyeri paniculata melliodora	sideroxylon
Graciles			
Micrantherae			
Platyantherae			

*Because the genus Eucalyptus is so large, it is subdivided into Sections, Subsections, and Series; this latter subdivision was not included here, for simplification (there are over 50 series groupings).

the characteristics of the active agents from each source.

No other genus of the family Myrtaceae was assayed for activity and so it is not known if the activity is found outside the genus. Because of the random distribution of the activity within the genus (Table III), the hypotensive factor will probably not prove useful in chemical taxonomy.

The absence of activity in the bark may be due to the leaching action of the heavy rains prevalent where it was collected. The leaves, which possess the activity in highest concentration, may either concentrate it or are the site of synthesis from which it is transported to all the other tissues. Because of its water-soluble nature and ubiquitous distribution within the tissues of E. robusta, it is likely that all the tissues contain the same hypotensive agent.

Hillis (1966, 1967a, 1967b, 1967c, and 1967d) has analyzed most of the Eucalyptus species for 22 different polyphenols. It was thought that by comparing the quantities of these compounds in the five most active species and the five least active ones, it might be possible to identify the active substance or possibly a metabolic product or precursor. Table IV shows this comparison and it can be seen that none of the compounds is more prevalent in the active species than in the inactive species.

This appears to be the first report of a hypotensive action for Eucalyptus extracts.

TABLE IV. POLYPHENOLS IN THE LEAVES OF ACTIVE AND INACTIVE SPECIES

	Active species of Eucalyptus					Inactive species of Eucalyptus				
Polyphenol	rob	mel	cor	deg	dea	sid	acm	gom	pun	smi
leucodelphinidins	0	0	0	0	0	1	0	0	0	3
leucocyanidins	0	1	0	1	0	2	3	0	0	2
leucopelargonidins	0	1	0	0	0	0	0	0	0	0
myricetin	1	0	0	t	0	0	0	1	0	2
quercetin	4	5	3	5	5	4	5	3	2	2
kaempferol	1	1	0	2	0	0	0	0	0	0
ellagic acid	5	5	5	5	5	5	5	5	5	5
gallic acid	3	4	4	5	5	3	5	5	5	5
gentisic acid	1	2	3	1	2	2	2	3	3	2
caffeic acid	1	0	1	1	1	1	0	0	1	0
p-coumaric acid	0	0	0	0	2	0	0	0	2	0
sinapic acid	0	0	1	0	1	0	0	0	2	0
ferulic acid	0	1	1	0	1	0	0	0	0	0
macrantherin	2	2	1	2	3	1	0	0	2	1
renantherin	0	0	0	0	0	0	3	0	0	0
taxifolin	0	0	0	0	0	0	0	0	0	0
aromadendrin	0	1	0	t	t	0	1	0	1	0
astringin	0	0	0	0	0	0	0	0	0	0
rhapontin	0	0	0	0	0	0	0	0	0	0
piceid	0	0	0	0	0	0	0	0	0	0
chlorogenic acid	3	2	1	1	1	1	0	2	3	0
p-coumarylquinic acid	t	2	1	1	4	1	0	t	1	0

Numbers represent relative amounts after two dimensional chromatography: 0 = none, t = trace, 5 = most. (See Hillis, 1966, 1967a, 1967b, 1967c, and 1967d).

rob - robusta, mel - melliodora, cor - cornuta, deg - deglupta, dea - dealbata, sid - sideroxylon, acm - acmenoides, gom - gomphcephla, pun - punctata, smi - smithii

II. STUDIES ON THE PURIFICATION AND CHEMICAL NATURE OF THE *EUCALYPTUS* FACTOR

INTRODUCTION

Purification of a crude extract is essential for accurate studies of the chemistry and pharmacology of an active constituent. Chemically, contaminants can obscure the characteristics of the active component by complexing with it to change it or by reacting with test reagents to give false indications. Pharmacologically, contaminants can alter the effects of a constituent by acting synergistically or antagonistically to it.

Ordinarily one purifies a mixture by taking advantage of several characteristics of the substance which differ substantially from those of most of the contaminants. Thus, in a series of dichotomous steps, most of the unwanted materials might be removed from the mixture leaving a pure (or purified) solution from which the desired substance might be crystallized. Some chemical families are more amenable to purification than others and apparently a few have not yet been purified extensively. The tannins are an example of this latter group (Haslam, 1966; Somers, 1966). As will be shown in the discussion, the properties of the active substance resemble those of the tannins and this may explain why the purification of the active substance has been difficult.

The tannins are a poorly understood family of compounds that occur in plants. Haslam (1966) has reviewed their uses

and noted that they have been used since 1500 BC for the curing of hides. More recently, tannins have also been employed in the production of inks and plastics, the preservation of fish nets, in oil well drilling, as mordants in dyeing, in the removal of boiler scale, and as drugs. The tanning process is thought to cause the proteins (especially collagen) to change their amorphous regions so that the hide hardens and shrinks and therefore becomes more stable to water, heat, bacteria, and abrasion. The therapeutic uses of tannins (Sollmann, 1957) also relate to their actions on proteins and therefore include astringent, antiseptic and styptic applications. In addition, the tannins readily complex with metals and therefore are useful as antidotes.

Chemically (Haslam, 1966; Robinson, 1963), the tannins are polyphenols which usually fall in a molecular weight range of 500 to 3000 (although Somers, 1966, has shown that the upper limit probably extends to 50,000). They are classified into two groups, the hydrolyzable tannins and the condensed tannins, depending on their reactions under hydrolytic condition. The hydrolyzable tannins are polyesters that yield, upon hydrolysis, a sugar and a phenol carboxylic acid. The latter is usually ellagic acid or gallic acid, thus permitting a further subdivision of the hydrolyzable tannins into ellagitannins or gallotannins. However, there may be other phenol carboxylic acids in the molecule and there may also be stilbenes or flavonoids present. The sugar is usually glucose but can also be any of several others.

The condensed tannins do not hydrolyze with acid but instead may polymerize or are not materially affected (as is probably the case with the larger molecular weight tannins). Those tannins which polymerize yield the phlobaphens.

Very little can be said about the fine structure of tannins as they have not yet been separated into individual pure substances. Until they can be separated and studied individually, the arrangement of their components cannot be easily determined.

The function of the tannins within plants is also not understood. One theory for their presence suggests that the tannins act to protect plants from invading microorganisms. Sommers and Harrison (1967) discuss this in regard to apricot black heart disease. They point out that apricot trees which become infected with Verticillium albo-atrum spontaneously recover after about six months. During this time, the tannin concentration in the infected portion of the plant has increased five-fold. They propose that the antibiotic activity of tannins is due to their ability to inhibit enzymes. Enzymes which have been found to be inhibited by tannins are beta-glucosidase, proteinase, pectinase, cellulase, catalase, peroxidase, alcohol dehydrogenase, lactate dehydrogenase, and acetyl-cholinesterase (Booth and Bell, 1968; Goldstein and Swain, 1965; Herz, 1968). Actually, enzyme inhibition could be expected for a substance which nonspecifically binds to proteins. Marchal, Thomas and

Massignon (1949), however, studied a group of plant pathogens and showed that they were not particularly susceptible to tannins. Thus the structure and function of tannins is still obscure.

Hypotensive activity has been reported for several constituents of the tannic acid molecule, as well as for some extracts rich in tannic acid itself. Comparison of the Eucalyptus factor (EF) to these substances and the other hypotensive agents found in the eucalypts (mentioned in Section I) will be made at the end of this section, after some of the characteristics of EF are described.

METHODS

Soxhlet extraction. Eight to twenty grams of dried and finely ground phloem or leaves were placed in a Soxhlet extractor and extracted with methanol, ethanol, 2-propanol, 2-methyl-2-propanol, acetone, 2-butanone, ethyl acetate, or chloroform. The minimal duration of extraction was 24 hours; this was extended if it was apparent that color was still being extracted at the end of this period. When completed, the extract was dried at 50°C in vacuo and the residue dissolved in water and assayed. Aqueous solutions of the ethanolic extracts were used in the other procedures described below.

Liquid-liquid extraction. Aqueous solutions of EF were placed in a liquid-liquid extractor and then ether, ethyl acetate, or 2-butanol were used to extract the activity.

When the extraction was terminated, the organic solvent extracts were dried *in vacuo* at 50°C and the residue redissolved in water for assay.

Precipitation. Ferric chloride (1%), lead acetate (5%), sodium chloride (20%), or gelatin (10%) solutions (Robinson, 1963) with EF were prepared. The precipitates that formed after 24 hours in the cold were removed by centrifugation. The gelatin supernatant was assayed directly while the other supernatants were further treated to remove the toxic precipitants. The lead acetate supernatant had carbon dioxide bubbled through it to precipitate the excess lead which was then removed by centrifugation. The sodium chloride supernatant was dried, mixed with acetone, centrifuged, and the salt-free supernatant dried and dissolved in water. The ferric chloride supernatant was neutralized with sodium hydroxide and the precipitated iron removed by centrifugation. These fractions were then assayed.

In other experiments, ethanolic or butanolic extracts of EF were exposed to various concentrations of ether or chloroform. The precipitates that formed after standing 24 hours in the cold were collected by centrifugation and dissolved in water for assay.

Dialysis. A modification of Craig's technique (1957) was used. A length of dialysis tubing (#27/100, Union Carbide, 22 mm in diameter) was pulled over a section of glass tubing (12 mm in diameter and 85 mm long) onto which rubber washers had previously been fitted so as to leave a uniform

space between the glass tubing and the dialysis tubing. Nine ml of an aqueous solution of EF were then injected through one of the rubber washers into the concentric space between the dialysis bag and the glass tubing; the excess tubing at each end was then tucked inside the glass tubing and held in place with snugly fitting corks. This dialysis assembly was then rotated in 900 ml of distilled water for periods of one, two, four or six hours (a separate run for each time period) and the contents of the dialysis bags were then assayed. In a few instances, the dialysate was also assayed after concentration to the starting volume at 50°C in vacuo.

Solutions of substances of known molecular weight were dialyzed in the same manner and the rate of dialysis was followed spectrophotometrically so that the half-time for their dialysis could be determined.

Gel filtration. Columns of Sephadex G-10 (five runs), G-25 (one run), G-50 (one run), and G-75 (one run) were prepared by mixing the bed materials with distilled water or buffer solutions and packing them into columns 4 cm x 50 cm, 8 cm x 60 cm, or 1.5 cm x 108 cm. Aqueous solutions of EF ranging from 2 to 25 ml were layered onto the beds and then eluted with distilled water, buffer solutions with an ionic strength between 0.02 and 1M, or acetone. The water eluates and eluates of low molarity were assayed without further treatment, or were concentrated back to the volume of the extract applied at the top of the column. The eluates containing acetone or high salt concentrations were further

treated before assay. The acetone eluate was completely dried *in vacuo* at 50°C and then redissolved in a volume of water equal to the starting volume. The fractions developed with the eluants of high salt concentration (usually ammonium bicarbonate) were dried *in vacuo* at 50°C until all of the water had evaporated and the ammonium bicarbonate had sublimed. The residue was then redissolved in a volume of water equal to the starting volume, and assayed.

Adsorption. Column and batch runs using three types of charcoal (Darco, Norit A, or MCB "activated") and aluminum oxide (Merck) were tried. Various ratios (W:V) of adsorbent to extract were used ranging from 0.2:1 to 1:1. With batch treatment, the adsorbent was removed by centrifugation. Acetone and methanol were used as eluants and were removed from the eluates by drying them at 50°C *in vacuo*. In all cases, the various fractions were restored to their initial volumes for assay.

Steam distillation. A solution of EF was steam distilled for two hours. The distillate and the mother liquor were restored to the initial volume of the solution before assay.

pH stability. Samples of the extract were titrated to various pH values with dilute HCl or NaOH and kept at room temperature overnight. The following day the pH was adjusted to 7 and the sample assayed.

Stability to enzymes. Crystalline pepsin (Worthington), crystalline trypsin (Calbiochem), crystalline chymotrypsin

(Calbiochem), pronase (Calbiochem), crystalline ribonuclease (Worthington), crystalline deoxyribonuclease (Worthington), polyphenoloxidase (Worthington), and crystalline tannase (courtesy of Dr. H. Yamada, Kyoto) were incubated with EF. The enzymes were used at concentrations between 60 and 300 ug per ml and were incubated over night at room temperature. A pH of 7 was used for all of the incubations except the pepsin - pH 3.2, polyphenoloxidase - pH 6.5, and tannase - pH 5.5.

Assay and statistics. The procedures described in the previous section under "Assay" and "Statistics" were used unchanged in this section.

RESULTS

Soxhlet extractions. In Table V it can be seen that EF in phloem was more soluble in polar solvents such as water, methanol, ethanol, 2-propanol, 2-methyl-2-propanol, or acetone than in the relatively nonpolar ethyl acetate or chloroform. In terms of total activity extracted per gram of tissue, ethanol was the best solvent. However, when considering the ratio of activity to contaminants, 2-methyl-2-propanol was the best, although it extracted only about 82% of the EF that ethanol extracted. 2-Butanone, a solvent only slightly less polar than acetone, did not appear to extract any EF. It is more likely though that at least some EF was extracted but that it was oxidized by peroxide(s) present in the solvent. The presence of an oxidizing agent(s) was shown by

TABLE V. ACTIVITIES OF 24 HOUR SOXHLET EXTRACTS OF DRIED AND GROUND PHLOEM USING VARIOUS SOLVENTS

Solvent	Mass Extracted (grams)	Final Volume (ml)	Soluble Solids (mg/ml)	Total Solids Extracted (mg)	Animal Trials	Activity (u/ml)	Total Activity (units)	Phloem Tissue Activity (u/gm)	Specific Activity (u/mg)
Water	8.0	40	34.4	1380	5	17.0	680	85	0.5
Methanol	8.0	40	40.0	1600	5	30.7	1230	154	0.8
Ethanol	8.0	40	38.2	1530	5	32.8	1310	164	0.9
2-Propanol	8.0	50	24.5	1230	5	18.1	905	113	0.7
2-methyl-2-propanol	8.0	40	27.4	1100	5	27.0	1080	135	1.0
Acetone	8.8	44	20.2	890	6	15.6	690	86	0.8
2-Butanone	8.0	40	--	--	7	0.0*	0	0	--
Ethyl Acetate	8.0	40	--	--	5	7.7	310	39	--
Chloroform	9.5	48	--	--	2	0.0	0	0	--

*The 2-Butanone gave a positive test for peroxide(s) (a yellow color with KI) while all of the other solvents were negative.

the development of a yellow color upon the addition of a KI solution to the solvent alone. None of the other solvents gave this reaction. Water, the most polar of the solvents used, extracted only about half as much EF as methanol or ethanol. It is possible that EF is not as soluble in water as it is in alcohols but it is more probable that water does not penetrate into the tissue as well. This is indicated by the fact that when the alcoholic extracts were dried, all of the solids quickly dissolved in small quantities of water. The cold water extraction technique described in the previous section removed 100 units per gram of tissue in several hours while the hot water (Soxhlet) technique was able to extract only 85 units per gram in 24 hours. This apparent contradiction may be a result of a slow inactivation at 100°C.

In order to determine the length of time necessary to obtain most of the EF, extracts were removed from the Soxhlet apparatus at various time intervals. Fig. 4 shows that most of the EF is extracted in the first 24 hours.

Liquid-liquid extraction. Table VI contains the results of the liquid-liquid extraction experiments. It can be seen that the distribution coefficient of EF between water and ether, or water and ethyl acetate, is very much in favor of the water; five days of extraction of a water solution with ether or ethyl acetate removed very little EF. 2-Butanol, however, was able to compete with the water for EF as two

Fig. 4. Effect of extraction time on the amount of activity removed from phloem by ethanol in a Soxhlet extractor.

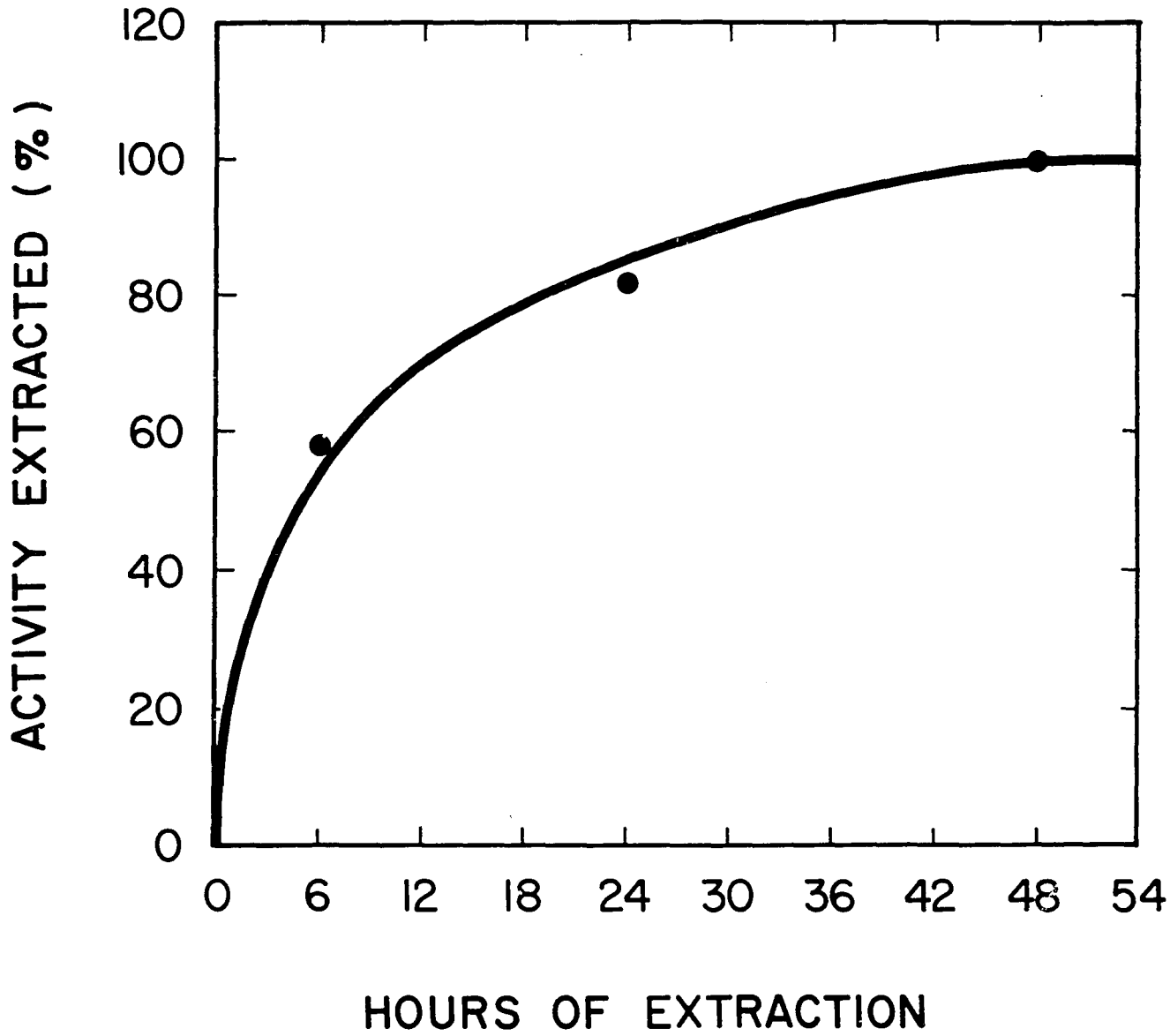

ACTIVITY EXTRACTED (%)
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
54
HOURS OF EXTRACTION

TABLE VI. LIQUID - LIQUID EXTRACTION FROM AQUEOUS SOLUTIONS CONTAINING THE EUCALYPTUS FACTOR

	Aqueous solution		Organic solvent extract						
Solvent	Volume (ml)	Specific activity (u/mg)	Days refluxing	Reflux rate (estimated) (ml/min)	Final volume (ml)	Solids extracted (mg/ml)	Number of trials	Activity (u/ml)	Specific activity (u/mg)
Ether	23	0.8	5	5	23	2.2	3	0.0	0.0
Ethyl acetate	37	0.5	2	3	37	7.9	3	0.0	0.0
	25	1.0	5	3	25	14.8	4	1.3	0.1
2-Butanol	23	0.8	2	1	20	10.3	2	10.7	1.0

days of extraction produced a solution of higher specific activity than the starting solution.

Precipitation. The EF remaining in solution after exposure to ferric chloride, lead acetate, sodium chloride, or gelatin is shown in Table VII. It can be seen that all of these substances precipitate or alter EF. The ferric chloride and gelatin supernatants had lost all of their activity while the lead acetate and sodium chloride supernatants still possessed a small amount of activity.

Precipitation of EF with relatively nonpolar organic solvents is shown in Figures 5 and 6. In Figure 5 it can be seen that the activity is precipitated from an ethanolic extract as the ether level is raised above 50%. At about 95% ether, precipitation is complete. Because of the high concentrations of ether that were necessary and because of its explosive nature, it was decided to do the initial extraction with a less polar solvent (2-methyl-2-propanol) and then to precipitate the activity with chloroform. In Figure 6 it can be seen that the activity was entirely precipitated from a 2-methyl-2-propanol solution when the chloroform concentration reached 90%. As will be shown later, organic solvent precipitation at these levels does not precipitate all of the solids, inasmuch as the specific activity increases after such a procedure.

Dialysis. The activity remaining in the dialysis bag after one, two, four, and six hours is shown in Figure 7. The time necessary to dialyze half the activity was about

TABLE VII. EFFECT OF VARIOUS CHEMICAL TREATMENTS ON EXTRACTS CONTAINING THE EUCALYPTUS FACTOR

Treatment	Number of animals	Dose (ml)	Initial Volume (ml)	Initial Activity (units/ml)	Final Volume (ml)	Final Activity (units/ml)
Ferric chloride precipitation:						
supernatant	3	0.3	5	8.7	5	0.0
Lead acetate precipitation:						
supernatant	3	0.5	10	22.8	10	5.1
Sodium chloride precipitation:						
supernatant	3	0.5	10	14.0	10	3.6
Gelatin precipitation:						
supernatant	4	1.0	5	9.3	13	0.0
Charcoal adsorption:						
all supernatants and eluates	9	1.0	10	22.2	10	2.4
Alumina adsorption:						
all supernatants and eluates	7	0.5	10	27.0	10	2.9
Gel filtration:						
all eluates	28	0.5	10	34.4	10	5.8
Steam distillation:						
distillate	3	0.5	15	17.0	15	0.0
mother liquor	3	0.5	15	17.0	15	13.2
Enzymatic treatment:						
pepsin	3	0.3	5	8.7	5	10.0
trypsin and chymotrypsin	3	0.3	5	8.7	5	8.1
pronase (crude)	3	0.3	5	8.7	5	9.1
ribonuclease	3	0.2	5	16.0[a]	5	20.3[a]
deoxyribonuclease	3	0.2	5	16.0[a]	5	17.9[a]
polyphenol oxidase (crude)	3	0.3	3	8.7	3	9.8
tannase	4	0.3	3	8.7	3	9.1

[a]Untreated extracts had a pH of 3.2, while the treated ones were at pH 7.0.

Fig. 5. Effect of ether concentration on the precipitation of the Eucalyptus factor from ethanol.

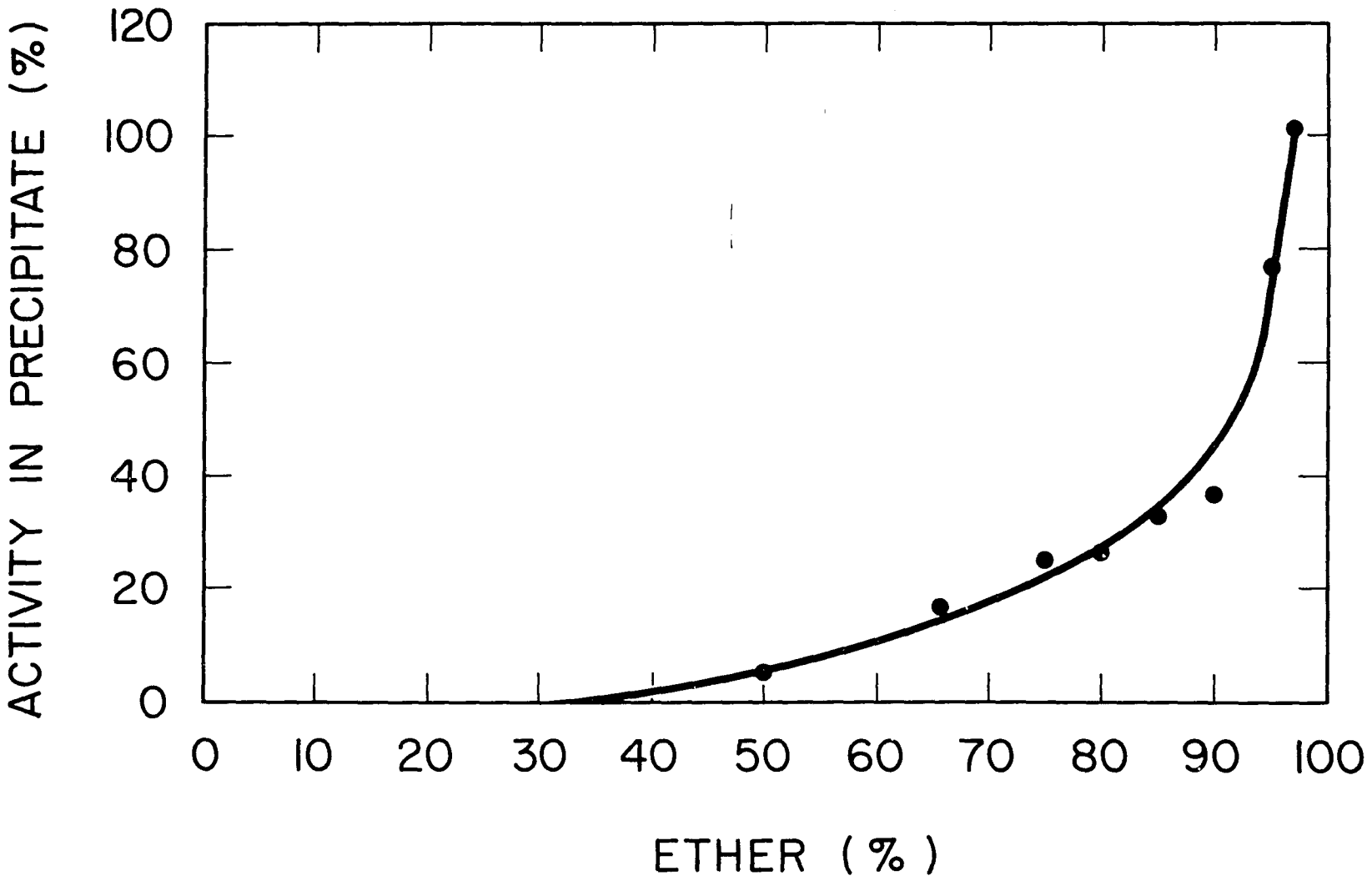

ACTIVITY IN PRECIPITATE (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
ETHER (%)

Fig. 6. Effect of chloroform concentration on the precipitation of the Eucalyptus factor from tertiary butanol.

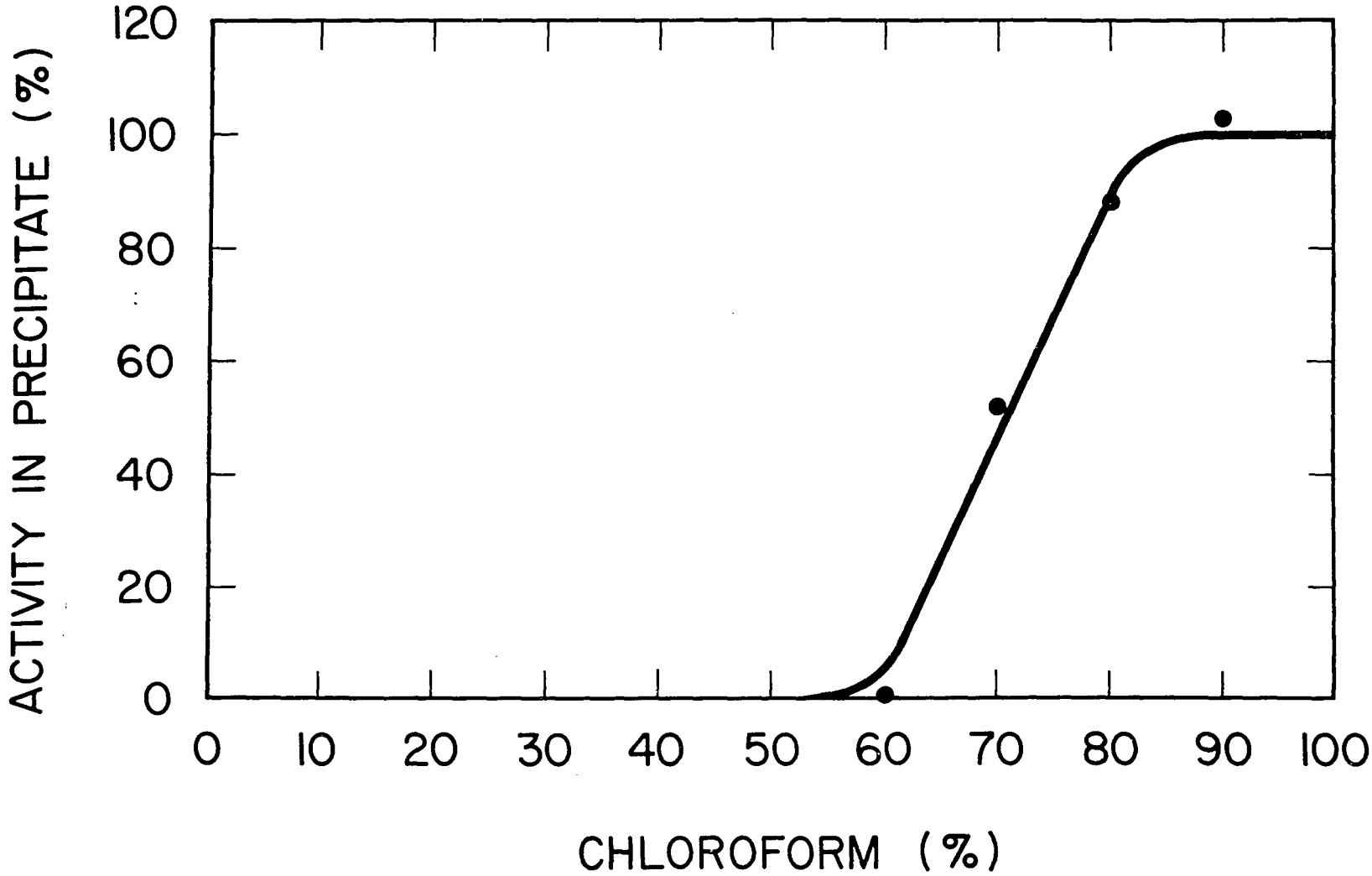
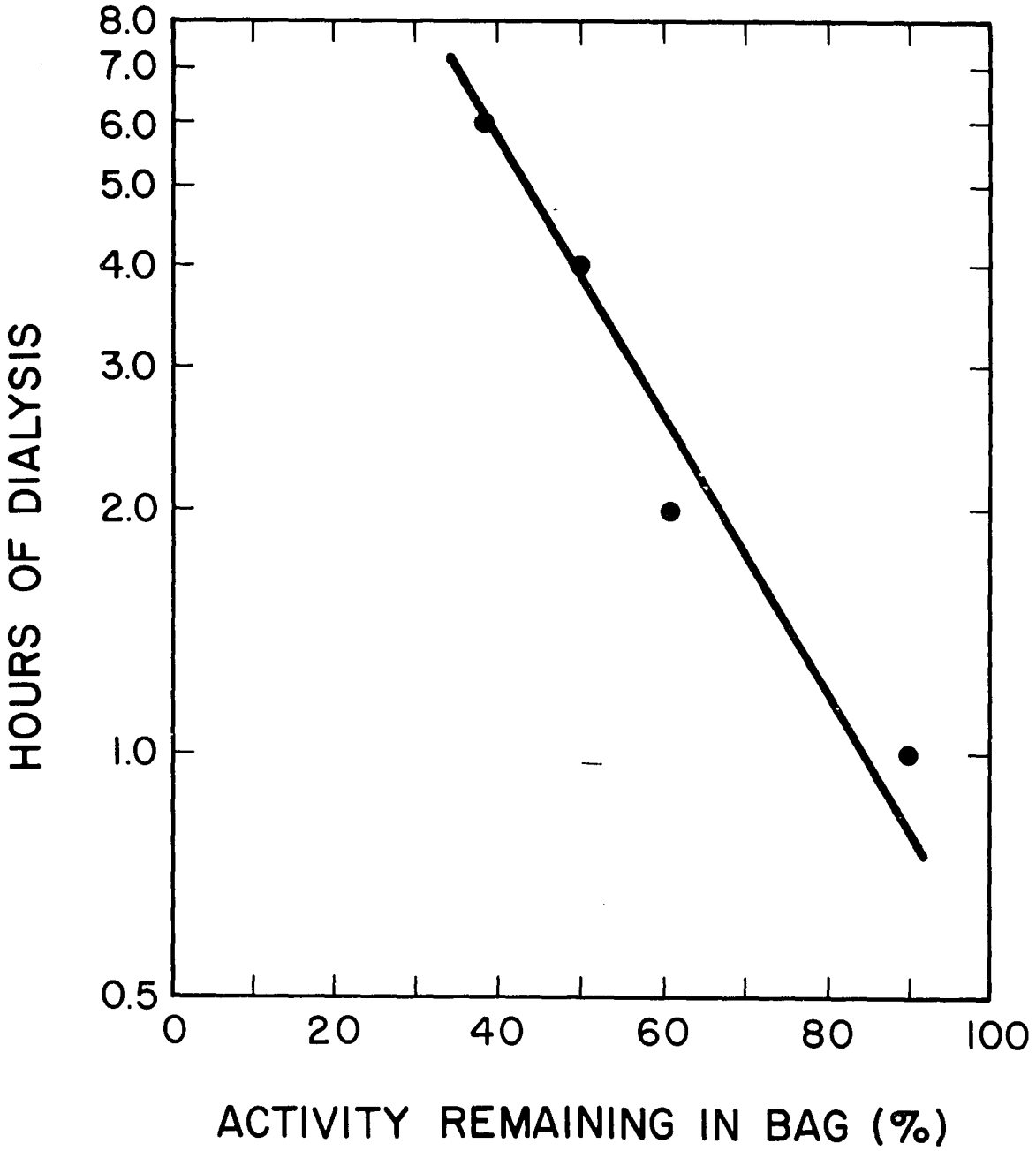

ACTIVITY IN PRECIPITATE (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
CHLOROFORM (%)

Fig. 7. Effect of dialysis duration on the activity of the bag contents.

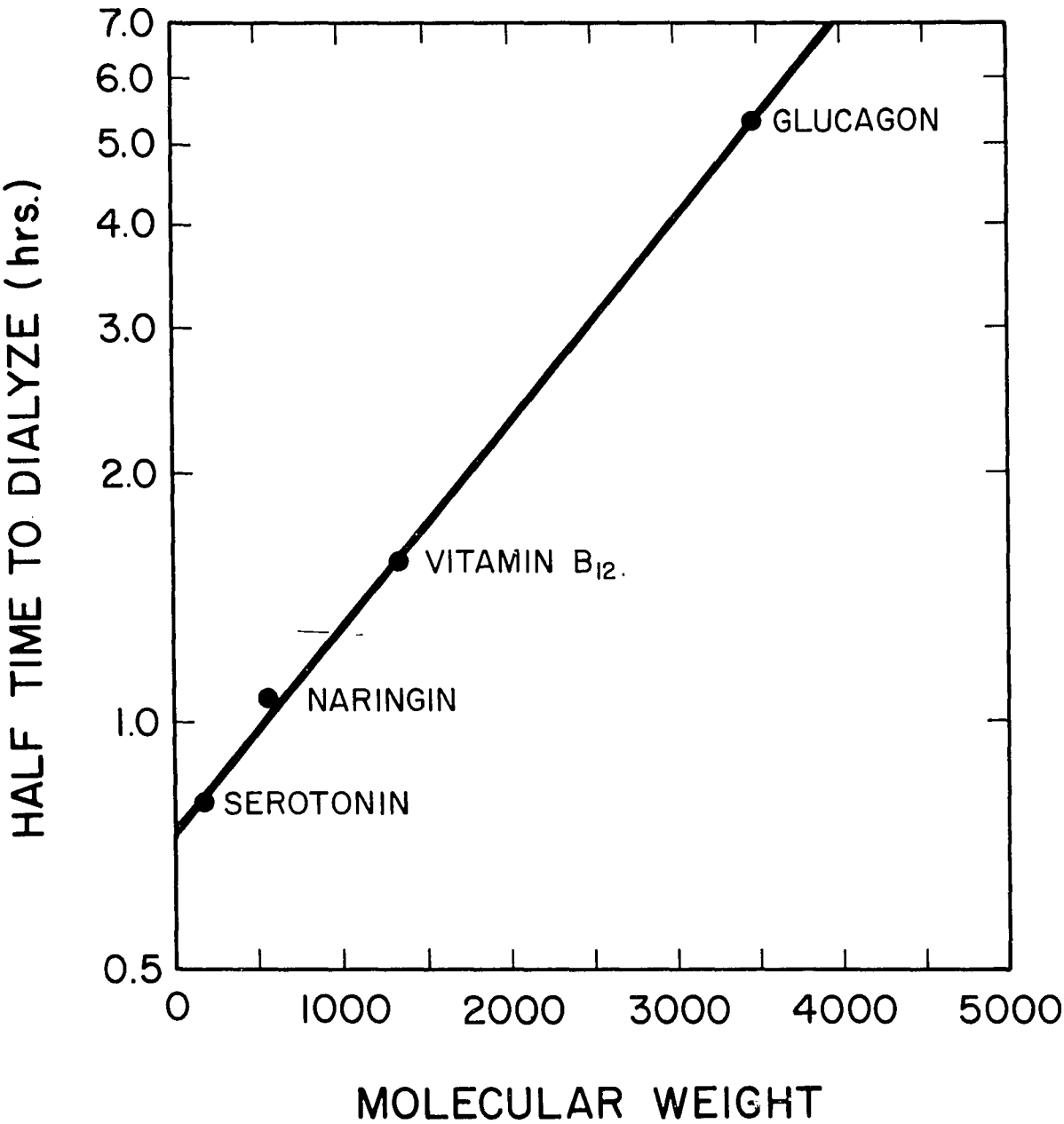

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.5
HOURS OF DIALYSIS
0
20
40
60
80
100
ACTIVITY REMAINING IN BAG (%)

3.9 hours. Similar curves for serotonin, naringin, vitamin B_{12} and glucagon yielded times of 0.80, 1.05, 1.55 and 5.35 hours respectively for the dialysis of half of their initial concentrations (1.0 mg/ml). When these half-times for dialysis were plotted against molecular weights, the straight line in Figure 8 was obtained. Using this plot and the half-time for dialysis of the activity, it can be seen that EF should have a molecular weight of about 2,900-3,000.

There are several factors which could cause this molecular weight determination to be in error. If EF were in reality a family of compounds with different molecular weights, then this determination would be an average value. If EF were adsorbed onto cellophane, as is the case with certain tannins (Haslam, 1966), then it would have a molecular weight greater than the derived value. Finally, inactivation might appear to be a potential source of error. However, the latter two possibilities were ruled out because in several instances the dialysate was tested, and nearly all of the total activity was accounted for.

Gel filtration. This technique for purification failed because the activity invariably was lost, probably as a result of strong adsorption on Sephadex. Elution with water, buffer solutions or organic solvents did not liberate the activity. Table VII includes the results of the gel filtration experiments. The possibility exists that the EF was inactivated by gel filtration instead of being adsorbed.

Fig. 8. Relation between molecular weight and dialysis half time.


7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.5
HALF TIME TO DIALYZE (hrs.)
GLUCAGON
VITAMIN B_{12}.
NARINGIN
SEROTONIN
0
1000
2000
3000
4000
5000
MOLECULAR WEIGHT

This seems unlikely, however, as gel filtration is known to be a very mild procedure.

Adsorption. Charcoal and alumina affected EF in the same way that gel filtration did. As shown in Table VII, about 90% of the activity was adsorbed and could not be eluted by water or organic solvents.

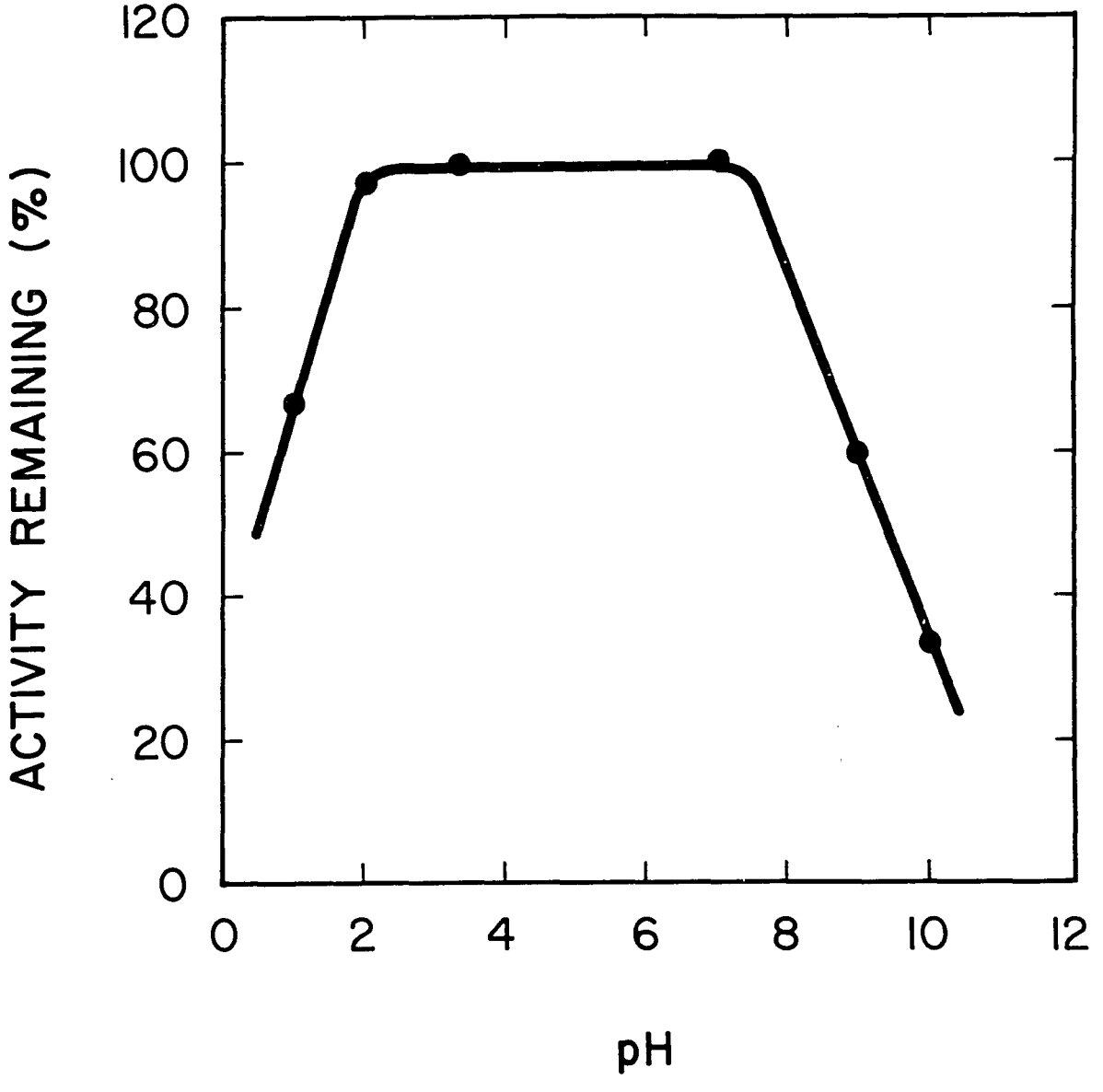
Steam distillation. An attempt to steam distill EF was not successful; the results in Table VII show that no activity distilled out of the extract. The fact that nearly 80% of the activity remained in the mother liquor after two hours of boiling provides a measure of the heat stability of EF.

pH stability. EF was stable over the pH range 2.0-7.5 (Figure 9) but beyond these limits it lost activity when incubated at 27° for 24 hours. No attempt was made to keep oxygen out of the system and so it is possible that oxidation could have occurred, in addition to hydrolysis or other destructive effects.

Stability to enzymes. Table VII contains the results of the incubation of EF with various enzymes. Pronase, pepsin, trypsin, chymotrypsin, ribonuclease, deoxyribonuclease, polyphenol oxidase and tannase did not destroy any activity.

Constituents of the eucalypts and tannins that might be the hypotensive agent. The pure components of the eucalypts and tannins that have been reported to be hypotensive in action were tested to determine whether any of these was EF. These compounds were assayed in the same concentration range

Fig. 9. Residual activities of extracts exposed to various pH values for 24 hours at 27°.


ACTIVITY REMAINING (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
pH

and in the same manner as EF. In Table VIII it can be seen that none of the pure substances was as active as EF. These data alone suggest that none of the known hypotensive compounds is the same as EF; however, other supporting evidence will be brought out in the discussion.

The high potency of EF is especially significant when its purity is considered. Chromatography has revealed the presence of many substances in the extracts (at least seven different spots in UV light and Pauly's reagent after one-dimensional paper chromatography and a minimum of ten UV absorbing peaks from column gel filtration) which suggests that EF is probably present in fairly low concentration.

DISCUSSION

Purification. Partial purification of EF has been achieved using selective Soxhlet extraction, organic solvent precipitation, and liquid-liquid extraction. Because extraction of phloem with any of the lower alcohols (methanol, ethanol, propanol, or tertiary-butanol) in a Soxhlet extractor was much more efficient than the cold water extraction procedure used previously (water at room temperature removed only 100 units of activity per gram of phloem whereas the Soxhlet-alcohol technique was able to remove as much as 164 units per gram), it was decided to use this technique as an initial step.

To remove the less-polar substances taken up by the alcohol in the Soxhlet extractor, dilution of the alcoholic

TABLE VIII. COMPARISON OF THE PROPERTIES OF AN E. ROBUSTA EXTRACT AND OTHER REPORTED HYPOTENSIVE AGENTS THAT MIGHT BE PRESENT IN E. ROBUSTA

Drug	Dose (mg/kg)	Number of Trials	Mean Pressure (mmHg) Normal	Treated	%	Molecular Weight	Chloroform or Ether Soluble	Reported I.V. Dose (mg/kg)	Ref.
Eucalyptus extract	5.2	3	140	81	58	2950	no	-	-
cis-Cinnamic acid	not obtainable					148	yes	70^{c}	2
m-Digallic acid	not obtainable					322	yes	27^{g}	1
Ellagic acid	10.0	3	138	128	93	302	slight	1.5^{R}	3
Eucalyptol (emulsion)	7.3	3	139	122	88	154	yes	$1,700^{R}$(oral)	4
(water layer)	1 (ml)	3	123	126	102	"	"	-	-
beta-Eudesmol	not obtainable						yes	10^{c}	5
Protocatechualdehyde	10.0	3	141	141	100	138	yes	19^{r}	6
Protocatechuic acid	9.4	4	141	136	94	154	yes	13^{r}	6
Quercitrin	7.5	3	154	154	100	448	slight	0.5^{d}	7
Rutin	10.3	3	149	144	98	610	slight	100^{r}	8
Tannic acid (Merck)	4	4	148	143	98	-	no	17^{g}	1
	19	3	151	138	91	-	"	"	"
	33	3	141	110	78	-	"	"	"
	50	2	125	died	-	-	"	"	"
Vanillin	10.3	3	151	151	100	152	yes	19^{r}	6

c - cat, d - dog, g - guinea pig, r - rabbit, R - rat.

1: Fiedler and Hilldebrand (1954), 2: Ramos and Ramos (1964), 3: Gautvik and Rugstad (1967), 4: Osol and Farrar, (1955), 5: Arora et al (1967), 6: Marquardt and Koch (1947), 7: Brooker and Eble (1966), 8: Ambrose and DeEds (1949).

extract with relatively non-polar organic solvents (ether or chloroform) was used to precipitate the activity. This organic solvent precipitation removed one-third to one-half of the solids and very little of the activity. Thus a purification of 1.5 - 2.0x was obtained and the active fraction was prepared in a dry state which could be dissolved in water without any further treatment. This was the procedure employed to prepare phloem and leaf extracts for this and the following section (except where noted otherwise).

Liquid-liquid extraction was the only other technique tested that offered any promise. When relatively polar solvents that are immiscible in water were used to extract the activity from water solutions, some purification was achieved. However, the partition coefficient does not appear to be very favorable for this extraction since much of the activity remained in the water. The long period of time required (almost a week) and the relatively small degree of purification achieved indicated that this procedure was not practical. Therefore, liquid-liquid extraction was not routinely used.

The other purification steps tried--gel filtration, adsorption, steam distillation, and specific precipitants--were not successful. The strong adsorption that occurs between EF and such beds as Sephadex, alumina, and charcoal render these materials useless for the purification of EF. If it were possible to elute the activity after the rest of the starting material had washed on through, it would have been possible to effect considerable purification since much

of the solids did not adsorb. Somers (1966) and Somers & Harrison (1967) have reported that some elution can be obtained for tannins adsorbed to Sephadex by eluting with acidic 60% alcohol. These exact conditions were not used, as the references were found after the experimental work was completed, but similar conditions with acetone at neutral pH were unsuccessful.

The precipitants (with the exception of the organic solvents) were of little use because essentially all of the solids were precipitated so that even if the EF could have been redissolved, no change in purity could be effected. Dialysis was not useful because nearly all of the solids were dialyzable.

Work on the purification was discontinued when it became apparent that EF was probably a tannin. As was pointed out earlier, techniques for isolating tannins intact have not yet been perfected.

Chemical nature of the Eucalyptus factor. At the outset, the solubility characteristics limited the number of chemical families to which EF could belong. Although they could not be absolutely eliminated, the proteins, carbohydrates, nucleic acids and lipids were considered as unlikely categories for a substance which is soluble in water, the lower alcohols and acetone, and insoluble in chloroform and ether. The solubility characteristics of the extract, its strong adsorptive characteristics, and the known presence of tannins in the eucalypts suggested that EF might be a tannin.

To establish the validity of this possibility, the following comparisons were made:

1. Solubility. According to Robinson (1963), tannins may be extracted with water, alcohols and acetone. They are, however, insoluble in chloroform and ether (Stecher, 1968).

2. Molecular weight. The tannins range between 500 and 50,000 in molecular weight (Haslam, 1966; Somers, 1966).

3. Precipitation. Tannins complex, and may precipitate, with gelatin, sodium chloride, lead acetate, and ferric chloride (Robinson, 1963).

4. Adsorption. Tannins are strongly adsorbed to Sephadex (Somers, 1966; Lees & Nelson, 1967).

5. Volatility. Tannins are not distillable (Robinson, 1963).

6. Oxidation. Tannins are easily oxidized (Haslam, 1966).

7. Alkaline stability. Tannins are highly labile in alkaline conditions (Robinson, 1963).

It can be seen that all of the characteristics of tannins listed above are shared with EF. This does not prove that EF is a tannin; for this purpose it would have to be obtained in pure form.

An additional series of experiments to determine the chemical species was the enzymatic treatment of the extracts. It was hoped that the activity would remain after incubation with non-tanninolytic enzymes and be destroyed after incubation with tannase or polyphenoloxidase. However, none of the

enzymes diminished the hypotensive activity of EF. In the case of the polyphenol oxidase, it is possible that the phenolic groups are not responsible for activity or that this enzyme does not attack tannins. The retention of activity after incubation with tannase was unexpected. However, certain tannins contain unusual components and it is possible that such a component is active whether free or combined. In addition, it is probable that the particular pure tannase employed does not hydrolyze all tannins; EF could require a different tannase for its breakdown.

Dissimilarity of the Eucalyptus factor to other hypotensive agents of the eucalypts or tannins. None of the constituents of the eucalypts or tannins that have been reported to be hypotensive seems to be identical with EF, using potency as a criterion. In addition, it is possible to compare some of the physical properties of EF with those of the known substances and furnish additional evidence that none of them represents EF.

In Table VIII the molecular weights of most of these substances are listed. With the exception of tannic acid, which is a crude mixture, all of the other compounds can be eliminated from consideration on the basis of molecular weight. EF, with its molecular weight of around 2950, is more than four times as heavy as any of the other hypotensives.

On the basis of solubility in ether and chloroform, again all of the substances except tannic acid can be

eliminated; only tannic acid and EF were insoluble in these solvents.

Tannic acid is a crude preparation that is made from water extracts of plant tissues. The tannins are usually named for their source; thus gallotannin is from galls, quercitannin is from oaks (Quercus), and catechu tannin is from Acacia catechu. The term "tannic acid" is usually reserved for the official (National Formulary) tannin obtained from the galls of various oaks (Osol and Farar, 1955). It is not known whether the activity of commercial tannic acid is due to any of the reported hypotensive components of tannins listed in Table VIII, or to some other factor.

Only four publications were found that associated tannins and blood pressure:

Boquet et al. (1967) reported that aqueous extracts of bark of Combratodendron africanum were hypotensive in rats. The extracts gave positive tests for tannins and saponins. They found the mechanism of action to be cardiac depression, which is not the case with EF (Section IV).

Denikeava et al. (1967) reported that alcoholic (40%) extracts of Dracocephalum nodulosum were hypotensive in dogs. They found the extracts to contain resins, tannins, essential oils, and flavonoids. They felt the hypotensive action was due to the flavonoids, but the identity of the active compound was not studied.

Koroza (1957) reported a hypotensive action for tea leaf extracts. These preparations, being rich in flavonoids,

were marketed in Russia for their vitamin P activity, but also contained tannins. Koroza assumed that the hypotensive effect was due to vitamin P activity. Daily intravenous injections into rabbits took 4-6 days to produce a 15-20 mm Hg drop in blood pressure, in contrast to the prompt (seconds) effect seen with the EF.

Fiedler and Hildebrand (1954) reported a hypotensive action for commercial tannic acid (Merck) in guinea pigs. Suspecting that the activity might be due to constituents of the tannin molecule, they tested digallic and ellagic acids and found them to be hypotensive also. However, it is not known whether the action of the tannic acid preparation was due to the presence of these free components, a true tannin, or a non-tannin contaminant. In contrast, only a feeble hypotensive response was obtained with Merck tannic acid in this author's assay, and Sammartino (1936) has reported that intravenous injections of Merck tannic acid had no effect on the circulation of rabbits.

The extracts of Boquet et al. and of Koroza are clearly different from EF, because they lowered blood pressure by a different mechanism. In none of these papers was the identity of the hypotensive agent studied.

It is concluded that the EF is probably a unique high molecular weight tannin.

III. THE PHARMACOLOGY OF THE HYPOTENSIVE FACTOR

INTRODUCTION

The understanding of a drug's actions and mechanisms is important to the intelligent use of that drug. A new agent's mechanism can be studied by surgically or pharmacologically blocking different steps of the biochemical pathways involved in its action. Resulting changes in the agent's action can indicate at which step of the pathway it acts. In addition, a drug can be given that stimulates at a known step in a pathway; an effect of the new agent on the action of the drug can indicate the site of action of the new agent.

Some sites of action are preferable to others in therapy. A drug which acts at the last step of a pathway is more specific than one which acts early in a pathway, as there will be fewer side reactions affected. Thus the sympatholytic drugs are more satisfactory than the ganglionic blocking agents in the control of high blood pressure; the latter block the parasympathetic nervous system in addition to the sympathetic nervous system.

Ideally, a therapeutic agent should reverse a pathological condition, thereby eliminating it and rendering the patient well again. Unfortunately, many diseases are not well enough understood that pharmacologists can attack the source of the problem. Therefore, many commonly used drugs are not competitive antagonists but instead are physiological antagonists that merely oppose the manifestations of the

pathological condition and do not correct it. An example of this is the control of hypertension. This disease is inadequately understood and is often treated by attenuating the pressor drive of the sympathetic nervous system. A more appropriate treatment would be to restore the pathological lesion to its non-diseased condition. The pharmacological studies to be described in this section indicate that the Eucalyptus factor (EF) is a histamine liberator and therefore does not act to reverse any of the mechanisms of hypertension described below.

Hypertension. Theoretically, an elevated blood pressure can be due to any of many factors, since it is directly related to the cardiac output and the peripheral resistance, each of which is a function of several variables. The cardiac output, for example, is equal to the product of the heart rate and the stroke volume, with the latter being influenced by the blood volume, the peripheral resistance, the tone of the blood vessels, and the condition of the heart. The peripheral resistance is a function of the viscosity of the blood, the smoothness of the vascular endothelium, and the radii of the blood vessels. Because the resistance of a vessel to blood flow is inversely proportional to the fourth power of its radius, while the other factors are first power variables, it can be seen that vessel diameter is probably more important in blood pressure control than any other factor (Burton, 1965).

Historically, two principal types of hypertension have been described: primary (or essential) and secondary. Hypertension of the secondary type is characterized by a lesion not directly associated with the cardiovascular system. Thus tumors of the adrenal medulla or the adrenal cortex can produce excessive levels of pressor catecholamine or aldosterone, which in turn can influence the blood pressure. Hypertension of the primary or essential type, however, is thought to be directly related to lesions of the cardiovascular system. The causes of essential hypertension are not well understood, and many may be found to be secondary in nature when their etiologies are worked out. Essential hypertension can be further subdivided into benign and malignant types, depending on its rate of development. The benign type can take many years to run its course, while the malignant type can be terminal within several months of its onset (Boyd, 1965).

The average person usually develops some hypertension as he ages, so that the distribution of blood pressure within an age group becomes skewed. In older groups, there is no precise line which divides a high average normal blood pressure from a hypertensive blood pressure (Smirk, 1967; Deming, 1968). Operationally, hypertension is defined as a resting blood pressure greater than 135/90 (Scher, 1965). The prognosis for all types of hypertension is unfavorable and the outlook worsens as one progresses from moderate normotension to severe hypertension (Deming, 1968). If

permitted to persist, elevated blood pressures produce changes in the individual which constitute hypertensive disease. Some of the characteristics of this disease are increase in heart size, cardiac asthma, retinal changes, edema, shortness of breath, headache, aneurisms, and arteriosclerosis. Death from hypertension is usually due to cerebral hemorrhage or congestive heart failure (Boyd, 1965).

As was indicated above, the causes of essential hypertension are not well understood. Page (1966) has come to the conclusion that it is a "disease of regulation," and most other workers appear to have accepted this thesis. In this disease the peripheral resistance appears to be abnormal and the cardiac output unchanged (Laurence and Bachrach, 1964). Thus attention is centered on those variables that affect the peripheral resistance: the viscosity of the blood, the quality of the vascular endothelium, and the diameter of the blood vessels.

Although many workers believe there is no single cause of essential hypertension, the kidney is the most commonly implicated organ in etiological theories. The hypertensive actions of this organ are usually described in relation to the renin-angiotensin-aldosterone pathway (Koch, 1965; Woodbury, 1965). In this scheme, the afferent renal arterioles are volume receptors and act to cause the release of renin from the juxtaglomerular cells whenever renal blood flow is reduced (as in arteriosclerosis, atherosclerosis, renal arterial embolism or occlusion, and heart failure). Renin, a

specific proteinase, releases the decapeptide angiotensin I from one of the serum globulins. A lung peptidase then hydrolyses this to the octapeptide angiotensin II, a pressor agent that by weight is six times more active than norepinephrine (De Bono, 1963). In addition to its pressor action, angiotensin II causes the release of aldosterone from the zona glomerulosa of the adrenal cortex. This hormone acts on the renal tubules to promote the reabsorption of sodium ion, and is important in the control of both plasma volume and ion composition. Thus aldosterone and angiotensin work in concert to enlarge the fluid compartments of the body and reduce the diameter of the blood vessels, two changes which elevate the blood pressure.

Although a pathological change in this system is the best explanation for many cases of essential hypertension, by itself it is not enough. Fishman et al. (1968) have assayed normal and hypertensive populations and found that 92% of the individuals with essential hypertension had aldosterone levels within the limits of the normal group. In addition, hexamethonium produces a larger than normal lowering of the blood pressure in hypertensive animals, suggesting a nervous system involvement (Smirk, 1967). Thus the etiology of essential hypertension requires further elucidation.

Current procedures for the treatment of essential hypertension reflect the multifactorial nature of the disease. As mentioned earlier, the ideal therapeutic agent is one

that reverses a pathological condition. Because a means of specific therapy is unknown in essential hypertension, currently used drugs are hypotensive rather than being antihypertensive (Laurence and Bachrach, 1964). It is generally assumed that most drugs that lower blood pressure will delay the development of hypertensive disease. Thus although none of the currently used drugs is ideal, if therapy is initiated soon enough, it is possible to prolong life and to reverse some of the pathological manifestations of essential hypertension (Boyd, 1965).

Histamine. Histamine occurs in nearly every tissue of the body (Feldberg, 1956); some occurs in the liver and intestine and unidentified storage sites, but most of it is associated with mast cells. The discovery of mast cells is attributed to Ehrlich who in 1877 identified two types, the tissue mast cell and the blood mast cell (basophil). Both types possess intracellular granules which contain histamine. Besides histamine, mast cells also store serotonin, heparin, and possibly bradykinin, slow reacting substance, and other agents. Mast cells constitute about 0.2% of the tissue and each cell contains between 7-32 picograms of histamine. Thus some tissues possess as much as 200 micrograms of histamine per gram of tissue (Riley and West, 1966).

The physiological role of histamine is not clear, especially in anaphylaxis and allergy, where the release of histamine appears to be deleterious. Three possible functions are: action as a neurohumor, the stimulation of gastric

secretion, and the local control of the circulation. The neurohumoral role has been subjected to much criticism and its acceptance has waxed and waned over the years. The presence of histamine in the brain (White, 1966) and peripheral nerve endings (von Euler, 1956), and the presence of the enzymes for its synthesis and destruction, have been evidence for this role. Additional support has come from reports like that of Bartlett and Beck (1968) which suggest that antihistamines can block the actions of certain nerves. Some of the opposing arguments cite the presence of histamine in organs after degeneration of the nervous tissue (von Euler, 1956), inability to confirm some of the above results (White, 1966), and the contention that the presence of histamine in the brain and other nervous tissue is attributable to other roles (eg. local control of circulation and the normal metabolism of histidine; White, 1966).

Unlike the controversial neurohumoral role, the role of histamine in gastric secretion is considered to be established (Ivy and Bachrach, 1966; Code, 1956). The evidence for this role is the presence of histamine in high concentration in the gastric mucosa, its extremely strong action there (it is more potent than acetylcholine or the purest fractions of gastrin in causing the release of gastric acid, and it acts at concentrations below those necessary for its other actions), and its concurrent release in the gastric secretions in proportion to the HCl secreted. It is thought that histamine is involved in the final step in the sequence

of reactions leading to the release of gastric acid.

Lewis in 1927 proposed that histamine may act in the local control of the circulation, especially in reactive hyperemia. Previously it had been shown that histamine is a potent depressor substance, although there was much confusion then, as there is now, in regard to its action. This so-called histamine paradox is that histamine causes most smooth muscle to contract, yet lowers blood pressure. Rocha e Silva (1966a) reviewed the subject and concluded that the blood pressure depression caused by histamine is the result of a capillary vasodilatation and an increase in capillary permeability which more than compensate for the pressor effect of the arteriolar constriction. The capillary vasodilatation has been shown to occur *in vitro*, if proper precautions are taken to preserve the normal tone that exists *in vivo* (Dale and Richards, 1918), but the contrasting responses of the capillaries and arteries to histamine is still unexplained.

The increase in capillary permeability also appears to be an established event but it is not clear whether it is passive or active. The argument that the change in permeability is passive is that an increase in hydrostatic pressure in the capillaries will upset the Starling equilibrium and cause a fluid shift toward the tissue. (An increase in capillary hydrostatic pressure would occur with the increase in venous pressure that is seen (Turchetti, 1938; Fleisch and Kuchler, 1939) and is to be expected in the face of a

capillary dilatation.) Evidence that the permeability change is active is that the administration of histamine increases the permeability of the capillaries to large molecular weight dyes (Rocha e Silva, 1966a).

Whatever the cause(s) of the changes in tone and permeability of capillaries following the administration of histamine, the response is prompt and dramatic. Histamine is as potent as acetylcholine or norepinephrine in producing vascular responses (Barnes and Eltherington, 1964). Studies on the participation of histamine in reactive hyperemia, post-exercise hyperemia, cold vasodilatation, and epinephrine vasodilatation have yielded conflicting results. In each of these types of vasodilatation some workers have claimed to be able to detect elevated histamine levels in the venous blood, whereas others have been unsuccessful. Similarly, antihistamine administration has produced equivocal results.

Whelan (1956) has come to the conclusion that histamine does not play a role in the local control of the circulation unless the intrinsic release of histamine, as envisioned by Dale, occurs. Intrinsic release of histamine implies that the histamine releasing cells are in close proximity to the effector cells (in this case the vascular smooth muscle) and that the liberated histamine readily penetrates the effector cells. The absence of a detectable increase in the histamine level of venous blood draining tissues undergoing local vasodilatation could be caused by an enzymatic destruction of the histamine before it can enter the blood.

Originally, it was thought that histamine release was caused only by venoms, toxins, and immunological responses but now it is known to occur with other agents also. Paton (1956) divided the histamine liberators into seven categories: 1) antigens, 2) tissue damaging compounds (venoms, toxins, etc.), 3) proteolytic enzymes, 4) surface active agents (Tween 20, bile salts, etc.), 5) large molecules (egg white, dextran, etc.), 6) monobasic compounds, and 7) dibasic compounds. All histamine liberators do not act in the same way; three or four mechanisms seem to prevail: 1) frank cell damage with rupture of the cell membrane, 2) an involved enzymatic pathway triggered by antigen-antibody complexes, 3) a simpler pathway triggered by the histamine liberators (perhaps a portion of the previous pathway), and possibly 4) an ion exchange between bound histamine and basic liberators.

The histamine in mast cells has been shown to be taken up and stored in much the same way that the neurotransmitters acetylcholine and norepinephrine are. Cabut and Haegermark (1968) have studied mast cells and isolated granules, and shown that histidine is actively transported into the cytoplasm where it is decarboxylated and the resulting histamine stored in the granules. The effect of histamine liberators on granules is not clear. Rat mast cell granules are extruded and disrupted in the presence of compound 48/80 (Mota, 1966) whereas guinea pig mast cell granules swell and fuse (Rothschild, 1966).

Ion exchange and disruptive mechanisms may occur in some instances, but an active process seems to be involved in the case of the more specific liberators such as 48/80 (it has been shown that one molecule of this compound can cause the release of ten molecules of histamine; McIntire, 1956). Uvnas (1961) has postulated that the basic liberators such as 48/80 ultimately activate lytic phospholipases located in the mast cell membrane. (Compound 48/80 is thought to be a small polymer, larger than the dimer or trimer, of p-methoxyphenylethylmethylamine and formaldehyde; De Graw et al., 1966.) These phospholipases increase the permeability of the cell, causing the release of intracellular constituents. Evidence for this mechanism derives from the observations that lecithinase A is able to disrupt mast cells, and the action of 48/80 and of lecithinase A is inhibited by the same agents (Mota, 1966).

Intravenous administration of a histamine liberator such as 48/80 produces a dramatic fall in the blood pressure of as much as 100 mmHg which persists for hours (Dews et al., 1953). The response is usually delayed slightly compared to the hypotensive action of histamine itself, since the liberators must first cause the release of the histamine (Paton, 1956). The initial downward course of the blood pressure may be interrupted by a momentary rise or snoulder due to the release of catecholamine. This biphasic response can be eliminated by adrenalectomy (Rocha e Silva, 1966a). Repeated administration of histamine liberators produces an

attenuated response, in contrast to the undiminished effect of repeated injections of histamine. This tachyphylaxis is attributed to the depletion of the histamine stores in the mast cells (Mota et al., 1956).

METHODS

Assay and statistics. The methods described in Section I under "Assay" and "Statistics" were used unchanged in this section except for the following additions: Guinea pigs (0.53 - 1.0 kg), mongrel cats (0.6 - 4.6 kg), and mongrel dogs (3.7 - 23.5 kg), of either sex, were employed. Intraperitoneal and oral routes of administration were employed, and the following drugs were used: hexamethonium chloride (City Chemical Corp.), reserpine (Robinson), phenoxybenzamine HCl (Smith Kline and French), pronethalol HCl (Ayerst Labs.), atropine sulfate (City Chemical Corp.), tripellenamine HCl (Ciba), diphenhydramine HCl (Parke, Davis and Co.), norepinephrine bitartrate (Winthrop), phenylephrine HCl (Robinson), epinephrine bitartrate (Winthrop), angiotensin amide (Ciba), 48/80 (Burroughs Wellcome), isoproterenol sulfate (Lilly), methacholine chloride (Nutritional Biochemical Corp.), and histamine (City Chemical Corp.). The assistance of Miss Jennifer Wigington in performing some of the assays is gratefully acknowledged.

Cross-circulation. Pairs of 450 - 550 g male albino rats were cannulated so that blood from the femoral arteries of the donor flowed to the carotid arteries of the recipient,

perfused this animal's head, and then returned from the jugular veins of the recipient to the femoral veins of the donor. The blood pressure, EEG, and respiration of the donor and recipient were recorded in the same manner as described under "assay," except that the blood pressure of the donor was measured from a side arm of one of the arterial cannulae, and the blood pressure of the recipient was measured from one of his carotid stubs. The extract was given either intra-arterially into the head of the recipient or intravenously into the body of the donor.

In vitro smooth muscle. Taenia coli from guinea pigs, and aortae from rabbits and rats were cut into strips 2 - 4 mm in width and 40 - 60 mm in length, and stripped of any associated fat. One end was then anchored to the inside bottom of a muscle bath and the other end fastened to a Grass FT03 strain gauge transducer. The muscle strips were maintained in normal Ringer's solution at 37°C, pH 7.0, and aerated with 95% O_2, 5% CO_2. Before use, they were incubated under 1.5 grams tension for one-half hour. Extracts and drugs (guanethidine sulfate - Ciba, MJ1998 HCl - Mead Johnson, reserpine - Robinson, angiotensin amide - Ciba, norepinephrine bitartrate - Winthrop, tannic acid - Merck, and histamine HCl - City Chemical Corp.) were added directly to the muscle-bath medium. Recordings were made with a Grass model 7 polygraph.

RESULTS

Effect of the Eucalyptus factor by other routes and on

other species. In Figure 10 it can be seen that cats, dogs, and guinea pigs, as well as rats, were made hypotensive by EF. Guinea pigs were the most sensitive of these animals; they responded to relatively low doses and were killed by moderate doses (respiratory arrest).

Figure 11 shows that EF is effective intraperitoneally as well as intravenously. The absence of an effect by the oral route suggests that it is either destroyed in the digestive tract or not absorbed. The lability of EF at pH 1 would suggest the former.

Cross-circulation experiments. EF circulating through the head of the recipient had only a feeble and delayed effect on the blood pressure in its body, while the blood pressure of the donor showed the usual fall (Table IX). The relatively weak depressor response in the recipient from this very high dose of EF indicates that the action of EF is probably peripheral rather than central. The mild blood pressure depression in the recipient was possibly due to collateral circulation passing some of the EF into the body of the recipient or to cerebral hypoxia; a result of stagnant circulation from the hypotension in the donor (there was considerable flattening of the EEG of the recipient after the blood pressure of the donor was depressed). The initial blood pressure of the donors was lower than usual, probably because of a decreased peripheral resistance from the addition of the head of the recipient to the circulatory bed of the donor.

Fig. 10. Effect of intravenous administration of the Eucalyptus factor on the mean arterial pressure of rats, cats, dogs, and guinea pigs.

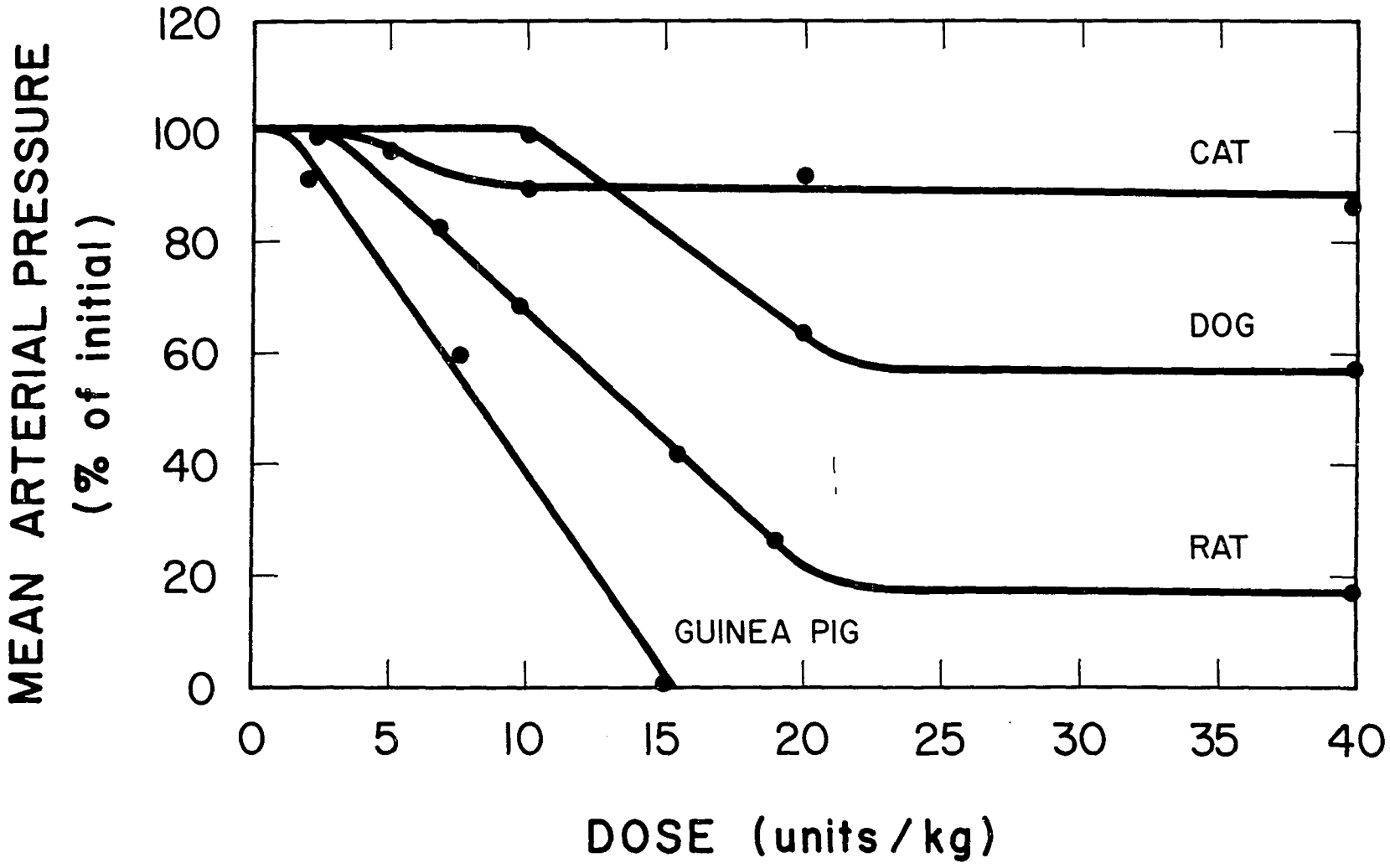

MEAN ARTERIAL PRESSURE
(% of initial)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
CAT
DOG
RAT
GUINEA PIG
DOSE (units / kg)

Fig. 11. Effect of the Eucalyptus factor by intravenous, intraperitoneal, and oral routes on the mean blood pressure of rats. In the oral route experiments the blood pressures were measured at 30 minutes instead of 5 minutes.

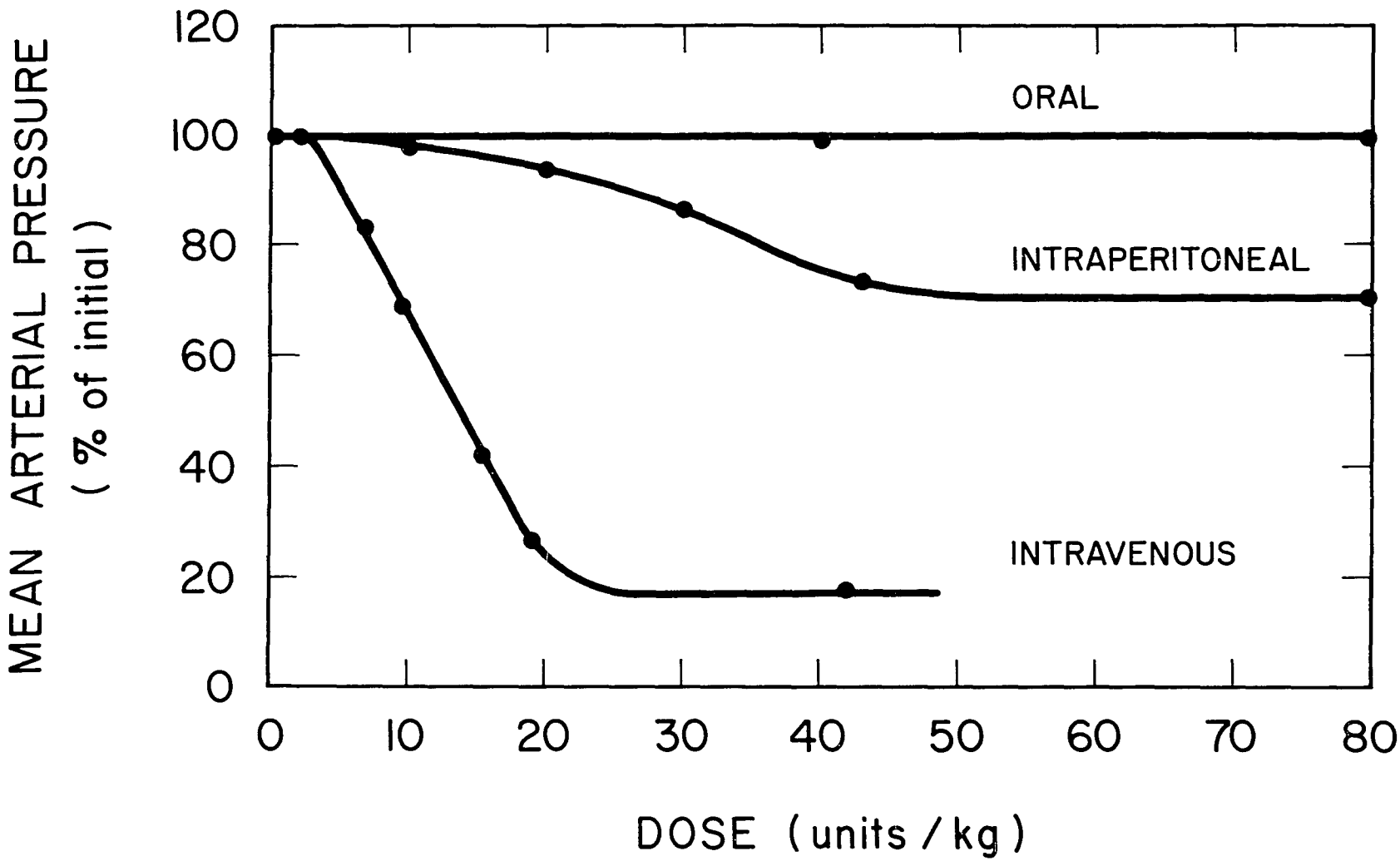

MEAN ARTERIAL PRESSURE
(% of initial)
120
100
80
60
40
20
0
ORAL
INTRAPERITONEAL
INTRAVENOUS
0
10
20
30
40
50
60
70
80
DOSE (units / kg)

TABLE IX. EFFECT OF THE EUCALYPTUS FACTOR ON BLOOD PRESSURE WHEN ADMINISTERED TO THE HEAD ONLY

Animal	Number of Trials	Dose (u/kg)	Average Weight[a] (Grams)	Mean Arterial Pressure mmHg		Treated as % of Initial
				Initial	Five Minutes After Treatment	
Donor	3	30	534	65	30	46
Recipient		0	453	134	96	71

[a]The heavier animal of each pair was used as the donor.

Effect of pharmacological agents on the hypotensive action of EF. The effects of the prior administration of pharmacological blocking agents on the action of EF is shown in Table X. Hexamethonium, reserpine, and pronethalol did not alter the action of EF. Atropine and phenoxybenzamine (respectively) seemed to inhibit and enhance the hypotensive action of EF but not significantly. Tripellenamine and diphenhydramine significantly attenuated the hypotensive action of EF, as did repeated pretreatments with 48/80 or EF.

The influence of EF on the action of several pharmacological agents is shown in Table XI. The pressor actions of norepinephrine, phenylephrine, epinephrine, and angiotensin were not antagonized by EF but instead were enhanced, possibly because of an increased potential available for a pressor response following the depressor action of EF. The actions of the depressor substances isoproterenol and methacholine were diminished by EF, probably because of the lessened potential for a depressor response following EF. The action of histamine was reversed by EF. Instead of a depressor response, as is normally seen with histamine, there was a pressor response. The same histamine reversal was obtained with 48/80 in place of EF.

Effect of pharmacological agents on the action of EF in smooth muscle. The effect of EF, alone and with several blocking agents, on intragastric pressure is shown in Table XII. It can be seen that EF produces a marked relaxation. The antihistamines diphenhydramine and tripellenamine blocked

TABLE X. EFFECT OF PHARMACOLOGICAL BLOCKING AGENTS ON THE HYPOTENSIVE ACTION OF THE *EUCALYPTUS* FACTOR IN RATS

Drug	I.V. Dose (mg/kg)	Number of Animals	Mean Blood Pressures (mmHg) Initial	After Drug	*E.* Factor After Drug[e]	Extract Hypotension as % of Initial Pressure
None (Control)	-	5	153	-	66	43 (±7)
Hexamethonium	20.0	5	120	72	59	49 (±3)
Reserpine	4.0[d]	6	138[a]	69	59	43 (±5)
Phenoxybenzamine	3.0	4	138	115	47	34 (±3)
Pronethalol	4.0	5	134	93	60	45 (±5)
Atropine	0.3	5	134	122	71	53 (±3)
Tripellenamine	8.0	5	123	92	79	64 (±6)[s]
Diphenhydramine	10.0	5	150	115	108	72 (±6)[s]
48/80	0.5[b]	5	138[a]	137	115	83 (±7)[s]
E. factor	13.6[c]	9	125[a]	99	81	65 (±5)[s]

[a]Because pretreatment preceded surgical preparation, precluding the measurement of normal pressures, the average values for rats of the same weights were used.

[b]48/80 given intraperitoneally for 5 days in doses increasing by 0.1 mg per day, beginning with 0.1 mg, such that on day 5 the rats were getting 0.5 mg.

[c]*Eucalyptus* factor given intraperitoneally for 5 days in doses increasing by 13.6 u/kg, per day, beginning with 13.6 u/kg, such that on day 5 the rats were getting 68 u/kg.

[d]Reserpine was given intraperitoneally 24 hours before the experiment.

[e]Blood pressure reading taken five minutes after *Eucalyptus* factor given intravenously at a dose of 15 u/kg.

[s]Indicates a significant difference from the control ($P<.05$).

TABLE XI. EFFECT OF THE EUCALYPTUS FACTOR ON THE BLOOD PRESSURE RESPONSE TO PHARMACOLOGICAL AGENTS IN RATS

Drug	I.V. Dose (ug/rat)	Number of Animals	Mean Arterial Pressure - (mmHg) A. Initial	B. Drug Only	C. E. Factor Only [a,c]	D. Drug After E. Factor[d]	B/A x 100	D/C x 100
Norepinephrine	1	6	127	179	62	99	142 (±7)	160 (±7)
Phenylephrine	1	4	125	158	66	99	127 (±2)	150 (±2)
Epinephrine	2	5	120	173	75	130	144 (±3)	173 (±19)
Angiotension	1	5	124	180	59	103	145 (±4)	175 (±5)
Isoproterenol	5	4	124	63	76	62	51 (±1)	81 (±4)
Methacholine	10	5	125	26	50	23	21 (±3)	46 (±6)
Histamine	50	5	115	77	71	85	67 (±2)	119 (±3)
					48/80 Only[b]	Drug After 48/80		
Histamine	20	4	126	92	56	68	73 (±5)	121 (±3)

[a]Eucalyptus factor given at a dose of 15 u/kg, iv.

[b]48/80 given at 1 mg/kg.

[c]The Eucalyptus factor was given after all effects of the drug had disappeared.

[d]The drug was given when the animal was clearly under the action of the Eucalyptus factor.

TABLE XII. EFFECT OF PHARMACOLOGICAL AGENTS ON THE ACTION OF THE *EUCALYPTUS* FACTOR IN RAT INTRAGASTRIC PRESSURE

Drug	I.V. Dose (mg/kg)	No. of Trials	Change in Pressure --- mmHg: Drug Only		Change in Pressure --- mmHg: E. Factor After Drug[a]	
None	---	7	---		-9.0	(±3.0)
48/80	0.1	8	-2.6	(±1.7)	---	
"	0.5[b]	5	---		-1.3	(±1.0)[s]
Diphenhydramine	10.0	6	+ .8	(±1.3	- .5	(±1.2)[s]
Tripellanamine	8.0	4	+2.6	(± .8)	0.0	(±1.5)[s]
Phenoxybenzamine	3.0	4	+ .6	(±1.0)	-9.4	(±1.8)
Pronethalol	4.0	5	+ .3	(± .8)	-3.0	(±1.3)
Atropine	0.3	5	+ .5	(±1.6)	-6.8	(±3.3

a The *Eucalyptus* factor was given intravenously at a dose of 15 u/kg.

b 48/80 given intraperitoneally for five days in doses increasing by 0.1 mg per day, beginning with 0.1 mg, such that on day 5 the rats were getting 0.5 mg.

s Indicates a significant difference from the control ($P < .05$).

this action of EF as did pretreatment with 48/80. Atropine, pronethalol, and phenoxybenzamine did not significantly alter the action of EF.

The effects of EF on smooth muscle in vitro are shown in Table XIII. Here it can be seen that EF caused the rabbit aorta and guinea pig taenia coli to relax, but caused the rat aorta to relax only when it was in a contracted state. In the rabbit aorta, prior administration of guanethidine, MJ1998, or reserpine had no significant effect on the relaxation produced by EF. Angiotensin and norepinephrine produced strong contractions which were reversed by EF. Histamine was without effect in the rat aortic strips and commercial tannic acid had no effect on rabbit aortic strips.

DISCUSSION

At the outset, the information available from the Polygraph assays indicated that the hypotensive action of EF was more likely due to a peripheral vasodilatation than to a cardiac effect, since the smooth muscle of the gut was relaxed by the extracts and there was no change in the EKG or heart rate. The cross-circulation experiments (as well as some spinal rat experiments not reported) indicated that the site of action was peripheral. Blockade of peripheral effector pathways with a ganglionic blocking agent, a catecholamine depleting agent, or a beta-adrenergic receptor blocking agent did not decrease the effect of EF either. A post-

TABLE XIII. EFFECT OF THE *EUCALYPTUS* FACTOR ON THE ACTION OF PHARMACOLOGICAL AGENTS ON SMOOTH MUSCLE IN VITRO

Tissue	Drug	Dose	Number of Animals	Change in Muscle Tension - g[a]	
				Drug Only	*E.* factor[c] After Drug
Rabbit aortic strips					
	none	---	3	---	-0.4
	guanethidine	15 mg/kg[b]	4	---[b]	-0.3
	MJ1998	$5x10^{-5}$M	2	0.0	-0.3
	reserpine	5 mg/kg[b]	4	---[b]	-0.5
	angiotensin	$5x10^{-7}$M	2	+1.9	-1.2
	norepinephrine	$1x10^{-6}$M	2	+2.2	-1.1
	tannic acid	$1x10^{-3}$ g/ml	5	0.0	---
Rat aortic strips					
	none	---	3	---	0.0
	histamine	10^{-3}M	2	0.0	---
	angiotensin	$5x10^{-8}$M	2	+0.5	-0.5
Guinea pig taenia coli					
	none	---	2	---	-0.9

[a]Muscle strips were under 1.5 grams tension initially.
[b]Animals pretreated before sacrifice.
[c]*Eucalyptus* factor given at a dose of 0.2 u/ml.

ganglionic cholinergic blocking agent (atropine) and an alpha-adrenergic receptor blocking agent (phenoxybenzamine) had weak effects on the action of EF. On the other hand, antihistamines significantly blocked the action, implicating a histaminic mechanism. Three possible mechanisms were considered--histaminase inhibition, histamine liberation and a histaminomimetic action. Atropine is known to have a mild antihistaminic action (Innes and Nickerson, 1966) and this could explain its slight blockade of EF. Similarly, potentiation by phenoxybenzamine is understandable because the administration of histamine is known to cause the release of catecholamine (Rocha e Silva, 1966a), an action that would lessen the hypotensive effect of histamine. An alpha-adrenergic blocking agent would then inhibit the pressor effect of catecholamine. Catecholamine release would also explain the biphasic response (Fig. 1) of blood pressure to the administration of EF. Histamine liberation by EF could explain the respiratory deaths of guinea pigs; the bronchiolar smooth muscle of this animal is known to be extremely sensitive to histamine (Parrot and Thouvenot, 1966).

Of the three possible mechanisms for the action of EF, histamine release or a histaminomimetic action are more probable than histaminase inhibition, as the latter would not be so prompt in producing a hypotensive effect, nor would it be so strong (inactivation of histaminase has only a slight effect on the action of histamine; Rocha e Silva,

1966a). It was possible to decide between the two remaining potential mechanisms by testing the extract in rats depleted of their mast cell histamine. The loss of the hypotensive action after pretreatment with 48/80 indicated that EF acts by liberating histamine, rather than as a histamine analogue. Support for this interpretation came from the tolerance observed after daily injections of EF for five days, and from the fact that the response of untreated rats to 48/80 was nearly identical to that seen with EF.

EF did not block the action of alpha- and beta-adrenergic receptor stimulants, angiotensin, or a cholinergic stimulant. The action of histamine, however, was reversed by EF (Table XI). This unexpected result, which was also obtained with 48/80, is probably attributable to the medullary catecholamine-releasing property of histamine. If histamine liberation takes place in the tissues, where it acts and is then metabolized, less histamine would be found in the blood than the level required to produce the same response by its intravenous administration. Thus in an animal maximally depressed by a histamine liberator, intravenous administration of histamine might have a catecholamine-liberating action with a consequent pressor effect that outweighed the depressor effect of the histamine. This action parallels the action of histamine in the pheochromocytoma test. In this test the histamine reveals the presence of the chromaffin cell tumor by causing the release of unusually large amounts of catecholamine, causing hypertension instead of hypotension. The

only difference between these two situations is that histamine in the latter causes hypertension because of elevated catecholamine levels, whereas in the former there is a reduced responsiveness to histamine.

The results of the smooth muscle experiments are not easily interpreted as it is well known that histamine is a smooth muscle stimulant. It has been shown that histamine has a strong stimulatory effect on gastric motility (Graham, 1949; Singh and Singh, 1950) and intestinal smooth muscle (Dale and Laidlaw, 1911; Fujita, 1954; M. M. Dale, 1958). As there is little doubt that histamine causes these tissues to contract, it is necessary to find some additional theory to explain the relaxation of smooth muscle produced by the Eucalyptus extracts.

One explanation could be that histamine liberation is not the only action of EF; other pharmacological actions are obtained from 48/80 and other histamine liberators. Rothschild (1966) reviewed the pharmacology of 48/80 and discussed its ganglionic and neuromuscular blocking actions. If EF also possesses these activities, but on smooth muscle, then its action could be explained. However, Feldberg and Smith (1954) reported an increased tone and motor activity in guinea pig colon in vitro following the administration of 48/80.

Another possible explanation is that the relaxation of smooth muscle caused by EF is due to the action of some other compound present in the extracts. This possibility is

enhanced by a report in the literature of a smooth muscle relaxing property of tannic acid. Begovic (1959) reported that tannic acid inhibits the effect of histamine and acetylcholine on the isolated intestine of guinea pigs. He goes on to state that atonia of the GI tract following ingestion of tannin is due to a reduction in the sensitivity of nerve terminals and end organs. However, this is not a general action for tannins, since the author found that commercial tannic acid did not have this effect on rabbit aortic strips. Additional experiments are required before this smooth muscle data can be explained.

Because of the undesirable side effects of histamine (headache, visual disturbances, diarrhea and dyspnea; Roth and Tabachnik, 1965), it is obvious why histamine liberators are not used in the treatment of hypertension. Therefore, it is unlikely that EF will be of any use in the therapy of hypertension. However, these compounds are useful in the study of the phenomenon of histamine liberation. Because histamine is a very important naturally occurring substance, new agents that cause its liberation can be useful in furthering our understanding of the role of histamine and the mechanism of its release.

In addition to being a new histamine liberator, EF could turn out to be more potent than 48/80, "the most powerful chemical basic histamine releaser" (Rothschild, 1966). At a dose of 1 mg/kg (Table XI), 48/80 produced a hypotensive response slightly greater than that seen with crude EF

at a dose of 5.2 mg/kg (Table VIII). If the crude Eucalyptus extracts contain less than 10 - 20% EF (a likely possibility judging by the chromatographic data), then EF would be more potent than 48/80.

It is concluded that histamine liberation is the mechanism of the hypotensive action of EF.

APPENDIX

Organic Substances Known to Occur in the genus *Eucalyptus*

This list does not include fatty acids, amino acids, or carbohydrates common to "energy" metabolism.

Compound	Reference
acetone	Ripa *et al.*, 1944
afzelechin	Hillis and Carle, 1967
amyl alcohol	Labo, 1931
arbutin	Hillis, 1967a
aromadendrene	Treibs and Barchet, 1949
aromadendrin	Hillis and Carle, 1960a; Hillis, 1951, 1967d
aromadendrin-7-methyl ether	Pinhey and Ritchie, 1958
astringin	Hillis, 1967a, 1967b, 1967c, 1967d
australol	Penfold, 1927
azulene	Lysenko, 1967
calcium oxalate	Smith, 1907
caffeic acid	Bland *et al.*, 1967; Hillis and Hingston, 1963
caffeoyl glucose	Bland *et al.*, 1967
calythrone	Birch and Elliot, 1956
cannabiscitrin	Hillis and Hingston, 1963
catechin	Hillis and Isoi, 1965; Hillis and Carle, 1960a
chlorogenic acid	Hillis and Isoi, 1965; Hillis, 1967a
cinnamic acid	Ramos and Ramos, 1964
citral	Neethling *et al.*, 1963; Labo, 1931
citriodorol	Satwalekar, 1957
citronellal	Cristini *et al.*, 1966
citronellol	Penfold and Morrison, 1948; Cristini, 1966
corilagin	Hillis and Carle, 1960b
p-coumaric acid	Bland *et al.*, 1967; Hillis, 1967d
o, m, and p-coumaroyl glucose	Bland *et al.*, 1967
p-coumarylquinic acid	Hillis and Isoi, 1965; Hillis, 1967a
cryptal	Penfold, 1927
cryptone	Robinson, 1963

cuminal	Penfold, 1927
cyanidin	Hillis and Hingston, 1963; Hillis, 1954
cycloeucalenol	Cox et al., 1959
cymene	Martelli et al., 1967
dehydroanguistione	Birch and Elliot, 1956
delphinidin	Hillis and Hingston, 1963; Hillis, 1954
dihydrokaempferol rhamnoside	Hillis and Isoi, 1965
3,4-dimethoxycinnamoyl glucose	Bland et al., 1967
ellagic acid	Hillis, 1967c; Swan and Akerblom, 1967
engelitin	Hillis, 1967d; Hillis and Hingston, 1963
epicatechin	Hillis and Carle, 1960a
eucalyptin	Jain et al., 1966; Horn et al., 1964
eucalyptol	Martelli et al., 1967; Labo, 1931
eudesmol	Arora et al., 1967
ferulic acid	Hillis and Hingston, 1963; Hillis, 1967d
feruloyl glucose	Bland et al., 1967
gallic acid	Hillis, 1967c; Swan and Akerblom, 1967
gallocatechin	Hillis and Carle, 1960a
gentisic acid	Hillis and Hingston, 1963
geraniol	Cristini, 1966
glucogallin	Hillis and Carle, 1960b
guaiacol	Ripa et al., 1944
hexanal	Robinson, 1963
hydrogen cyanide	Finnemore et al., 1936
p-hydroxycinnamic acid	Pinhey and Ritchie, 1958
isoamyl alcohol	Robinson, 1963
isoquercitrin	Hillis and Hingston, 1963
juglanin	Hillis and Carle, 1960b
kaempferol	Hillis, 1967b; Hillis and Carle, 1960a
leucocyanidin	Hillis, 1967b; 1967d; Hillis and Carle, 1960b
leucodelphinidin	Hillis, 1967b; Hillis and Carle, 1960b
leucopelargonidin	Hillis, 1967b; Ganguli and Seshadri, 1961
lignin	Bland et al., 1947; Hasegawa and Higuchi, 1960
macrantherin	Hillis, 1967d
methanol	Ripa et al., 1944
morolic acid	Hart and Lamberton, 1965
myrcene	Martelli et al., 1967

myricetin	Hillis, 1967a, 1967b, 1967c, 1967d
myricitrin	Hillis and Hingston, 1963
myrtan	Anderson and Steeden, 1950
myrtenal	Schmidt, 1942
myrtenol	Schmidt, 1942
naringenin	Pinhey and Ritchie, 1958
oleanolic acid	White and Zampatti, 1952
pelargonidin	Hillis, 1954
3,5,3',4',5'-pentahydroxystilbene	Hillis and Hingston, 1963
phellandral	Penfold, 1927
phellandrene	Martelli et al., 1967; Cristini, 1966
phloracetophenone-4,6-dimethyl ether	Penfold, 1927
phlorglucinol	Hillis and Carle, 1960b
piceid	Hillis and Hingston, 1963; Hillis, 1967a
pinene	Martelli et al., 1967
pinocarveol	Schmidt, 1944
pinocarvone	Schmidt, 1944
piperitol	Penfold and Morrison, 1935
piperitone	Penfold and Morrison, 1924
proanthocyanin	Nisi and Panizzi, 1966
protocatechualdehyde	Marquard and Koch, 1947
protocatechuic acid	Capito and Panizzi, 1959
pruasin	Finnemore et al., 1936
pyrogallol	Anderson and Steeden, 1950
quercetin	Hillis, 1967c; Hillis and Isoi, 1965
quercitol	Plouvier, 1961; McCasland et al., 1967
quercitrin	Elkiey et al., 1964; Brooker and Eble, 1966
quinic acid	Anet et al., 1957
renantherin	Hillis, 1967a, 1967d
rhaponticin	Hillis and Hingston, 1963; Hillis, 1967a
rutin	Hillis, 1967d; Humphreys, 1944
sabinene	Martelli et al., 1967
sanosine	Labo, 1931
shikimic acid	Hasegawa and Higuchi, 1960; Anet et al., 1957
sideroxylon	Hasegawa and Hillis, 1966
sinapic acid	Bland et al., 1967; Hillis and Hingston, 1963
sinapoyl glucose	Bland et al., 1967

spathulenol	Bowyer and Jeffries, 1963
syringaldehyde	Hasegawa and Higuchi, 1960
syringoylmethyl ketone	Hasegawa and Higuchi, 1960
tasmanone	Birch et al., 1966
taxifolin	Hillis, 1967a, 1967b, 1967c, 1967d
3,5,3',4'-tetrahydroxystilbene	Hasegawa and Hillis, 1966
thuzene	Martelli et al., 1967
3,3',5'-trihydroxy-4'-methoxystilbene	Hillis and Hasegawa, 1962
3,4',5-trihydroxystilbene	Hillis and Hasegawa, 1962
ursolic acid	Theodosious, 1962
valeraldehyde	Labo, 1931
vanillin	Hasegawa and Higuchi, 1960
vanilloyl-methyl ketone	Hasegawa and Higuchi, 1960

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