I N V E S T I G A T I O N S
O F S O M E
P L A N T W O U N D H O R M O N E S

Thesis by

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DEDICATION

To my wife, Marjorie, who has shared my sorrows and joys and has helped so much with this problem and manuscript and to our son, Rik, who in his own way has enriched both our lives, I gladly dedicate this thesis.

A C K N O W L E D G E M E N T S

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ABSTRACT

Research has shown that renewed cell division and growth may be induced in many mature living cells either by wounding or treating with an extract of certain tissue macerates. During the past decade there have been no publications in the field of wound hormones along the lines of Haberlandt (4) (5) or Bonner, English and Haagen-Smit (11-13). The latter authors isolated a natural wound hormone of beans, traumatic acid; the present investigation seeks to extend their work by the study of a wound hormone from another source. The modified Wehnelt test used as an assay in the earlier isolation work was adopted. Variety of beans but not environment was shown to influence the test. Of a number of natural products investigated for wound hormone activity, citrus products were found exceptionally active. From lemon peel infusion it was possible to prepare a very active concentrate. This concentrate could be subjected to catalytic hydrogenation without losing its activity. Pressed Valencia Orange Oil contains an active component which upon purification was found to be a mixture of unsaturated fatty acids. Preparations of pure linoleic and linolenic acids were found active as wound hormones. In lemon peel infusion there was a water soluble complement, which though inactive itself, was able to augment the activity not only of the wound hormone of lemon peel infusion but also those of orange oil, linoleic and linolenic acids. Several purified water soluble factors which enhanced the activity of wound hormones were found, the most effective being Coenzyme A and Cytochrome C. Several positional and geometrical isomers of linolenic acid were found only slightly active.

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INVESTIGATIONS OF SOME PLANT WOUND HORMONES

PART I INTRODUCTION

A) HISTORICAL PREFACE

Wound tissue formation at the site of an injury in higher plants has often been observed and described. Less conspicuous responses arise when plants suffer internal injuries affecting essential tissues. Such responses of a compensative and regenerative nature are seen in both animals and plants. A substantial amount of information on these processes has come from studies on tissue and organ cultures, regeneration and vegetable propagation.

There are several well-known types of wound healing: 1) Regeneration of the lost parts in injuries of the terminal growing regions of stems or roots (in the zone of undifferentiated meristematic tissue), 2) Repair effected by secondary meristems such as cork phellogens, cambia or calluses at injuries some distance from the apex and 3) Repair manifested in cell wall changes by injured cells in an effort to restore the prewound pattern.

Through wound healing it has been possible to study the physiological and structural changes preceeding and following the initiation of meristematic activity, for example the degeneration and necrosis of cells, the effect of such cells on adjacent healthy ones as well as the effects of other internal and external factors on the entire healing process. A large proportion of mature vacuolate plant cells are capable of rejuvenation; i.e. they can to a certain degree, depending upon cell types and orientation in the plant, be induced to divide and resume growth (dedifferentiate).

This behavior can be readily utilized as a tool to examine the relative potentialities of cells, cell layers, and tissues at any stage of development.

Robert Bloch (1,2) has emphasized the importance of wound healing in the experimental method,

> "Experimentally induced wound healing is a valuable tool in the analysis of the cytological, histological, and physiological sides of tissue development and the causes and mechanics of cell division and differentiation. By this method it is possible to reproduce under controlled conditions, tissue structures within limited areas, and to investigate cell division and growth conveniently in large, more or less vacuolate cells. Though only in its beginning this method has already yielded tangible results and thrown light on fundamental processes, such as changes which precede the rejuvenation of cells , the mechanism and plane of nuclear and cell division, and the problem of subsequent controlled redifferentiation."

Mohl's early description (3) of the cicatrization processes in plant wounds was the first of numerous works describing and analyzing some of the complex changes following wounding. It was found that external conditions, humidity, light, temperature and access of oxygen directly influenced wound responses. Moreover it appeared that degenerating cells influenced the metabolism of adjacent healthy cells. Wiesner (4) is believed to be the originator of the concept of "wound substances". Degenerating cells were assumed to form and release such substances to diffuse to adjacent healthy cells and therein effect the meristematic activity resulting in tissue regeneration and callus formation.

Two decades later Haberlandt (5) in a series of classical experiments was able to provide satisfactory evidence in support of the Wiesner hypothesis. He found that the cells making up the surface layers of unwashed discs cut from kohlrabi roots and potato

tubers showed considerable cell division whereas washed discs showed negligible cell division. Haberlandt also provided evidence that both, a diffusible substance from phloem cells (lepto-hormone) as well as the diffusible substance from injured cells was necessary for the stimulation of cell division. To show that this wound phenomenon was not specific but more general in nature, Haberlandt demonstrated renewed cell division in leaf tissues of various succulents, Bryophyllum, Crassula, Sedum and others and in the individual hair cells of Coleus and Pelargonium by treatment with plant tissue extracts.

Reiche (6) confirmed Haberlandt's results by demonstrating callus tissue growth after injection of tissue macerates into stems and petioles of various plants, Gratiola, Solanum and others. She believed however, that the substance activating cell division was associated with the particulate rather than the soluble fraction of the tissue macerates.

Several years later Wehnelt (7) utilizing Haberlandt's methods and techniques sought to corroborate and extend the work of his predecessors but soon realized that the discs from potato tubers and kohlrabi roots were variable in their response and not satisfactory for assay purposes. Uninjured cells of succulant leaves, exposed by careful dissection, manifested satisfactory responses but the skill and painstaking care necessary was a serious handicap. The injection technique of Reiche was no better since it was not readily adaptable to large scale assay procedures. While searching for wound hormone responses in other plants Wehnelt found that the uninjured parenchyma cells lining the seed chambers of pods of certain varieties

of beans, when treated with bean tissue extract, produced sizeable intumescences. He found also that cell division was directly proportional to the quantity of wound substance. This discovery provided a method, readily adaptable for assays with easily available material. Wehnelt used this test to investigate the wound activity of several plant and animal products. He showed that the wound substance from Phaseolus was water soluble, alcohol soluble and thermostable. A clear filtrate obtained by passing tissue juice from Phaseolus leaves or pods through an ultra filter was nearly as active as the unfiltered juice. Tissue juice or filtrate could be heated under pressure (4 hours, 128°C) with no loss of activity. Neither could, however, be heated under pressure in the presence of alkali without serious loss of activity. Alcohol (70-90%) extracts of Phaseolus tissue were found active. He also established that the Phaseolus pod response to wound substances was not species specific. Preparations of egg white, horse serum, hemoglobin, deuteralbumose, insulin and agar were found to give responses comparable to tissue extracts. Water and sugar and Knop solutions on the other hand, were found to give negligible responses.

Several years later Bonner and English (8,9,10) modified Wehnelt's qualitative test to one which was quantitative and easily amenable to routine large scale testing. These authors, in an investigation of the wound substances of Phaseolus, were able to prepare a highly active, water soluble, oily concentrate. Shortly thereafter Bonner, English and Haagen-Smit (11,12,13), using a different approach, isolated and characterized a lipoid soluble active agent from the same source. Their work revealed the existence of at least

one water soluble factor, itself inactive, that greatly enhanced the activity of the active agent, traumatic acid. Traumatic acid was identified as 1-decene-1,10-dicarboxylic acid. The water soluble factor was not characterized, but it was found that glutamic acid and sucrose possessed similar properties. The accomplishment of these workers was the last published effort of a wound hormone isolation from natural products.

B) THE PROBLEM

The approach adopted by Haberlandt, Wehnelt, Bonner,English and Haagen-Smit appears to be the most satisfactory in studying the wound substances inducing tissue generation. Bonner, English and Haagen-Smit (8,9,10) established the relative potency of a number of plant products and prepared a concentrate of water soluble material from Phaseolus pods containing an active component, traumatin. Later Bonner, English and Haagen-Smit (11,12,13), by a different procedure isolated an active lipoid soluble material, traumatic acid, from the same source. The former concentrate was active by itself whereas the activity of the latter substance was found to be enhanced by water soluble factors. Inasmuch as these investigations had shown the existence of water and lipoid soluble hormones and water soluble factors capable of enhancing the hormone response, it was of interest to investigate the nature of the wound substances from other sources.

PART II

THE ASSAY

A) INTRODUCTION

Resumption of meristematic activity by otherwise mature cells is the primary expression of the phenomenon of wound hormone activity; any tissue therefore, manifesting such a response may be used in an assay. Haberlandt (5) used several plants for his determination of wound hormone activity, kohlrabi roots, potato tubers, Bryophyllum leaves and Coleus hairs. Basically the test with each plant was the same; from kohlrabi roots and potato tubers, sections would be cut, washed free of debris, treated and incubated for a week or so, then examined in cross section through the treated surface for any increase in cell division in the uppermost 10 layers. Bryophyllum leaves could not be used when cut but had to be torn. The numerous intercellular spaces resulting from a random arrangement of cells made it impossible to wash away all of the cytoplasmic material from the cut surface. However, a careful tear, taking place along cell walls, gave negligible cell rupture and the dry surface so exposed could be treated directly, incubated and examined in cross section for increase in cell division. In the case of Coleus the basal hair cells were treated directly and the adjacent region examined for cell division after proper incubation.

Reiche used the injection technique in her investigations. Materials were injected into stems or petioles, and after incubation the injected region was examined for cell division and growth. Wehnelt also based activity upon increased cell division induced

in the parenchymatous cells lining the seed chamber of the bean pod. His test, however, was a step forward in several respects for there were: (a) a thick layer of reactive cells available, (b) an orderly arrangement of cells with a lack of intercellular spaces, and (c) ready availability as well as ease and simplicity of an assay preparation.

B) THE TEST

Estimation of increased cell division, a cumbersome aspect of the Wehnelt test in routine large scale assays, was overcome by the work of Bonner and English (9). These workers found that the height of the intumescence produced by a drop of active extract on the responsive cell layer of the bean pod could be used as a satisfactory measure of cell division. Furthermore they were able to demonstrate that the height of the intumescence was, under prescribed conditions, directly proportional to the amount of wound hormone present in the extract.

In a practical application of the test one simply slices the immature bean pod along suture and midrib and removes the unripe seeds to expose the layer of uninjured parenchyma tissue. A drop (0.01 ml.) of solution containing the substance to be tested is applied to the center of the seed cavity and, after a 48 hr. incubation at 25°C, a cross section through the reactive zone is prepared and the height of the intumescence measured under a low power binocularscope (Fig.1).

C) EFFECT OF BEAN VARIETY

The response of beans in the Wehnelt test varies markedly with the variety. Since the reaction is carried out by the parenchyma-

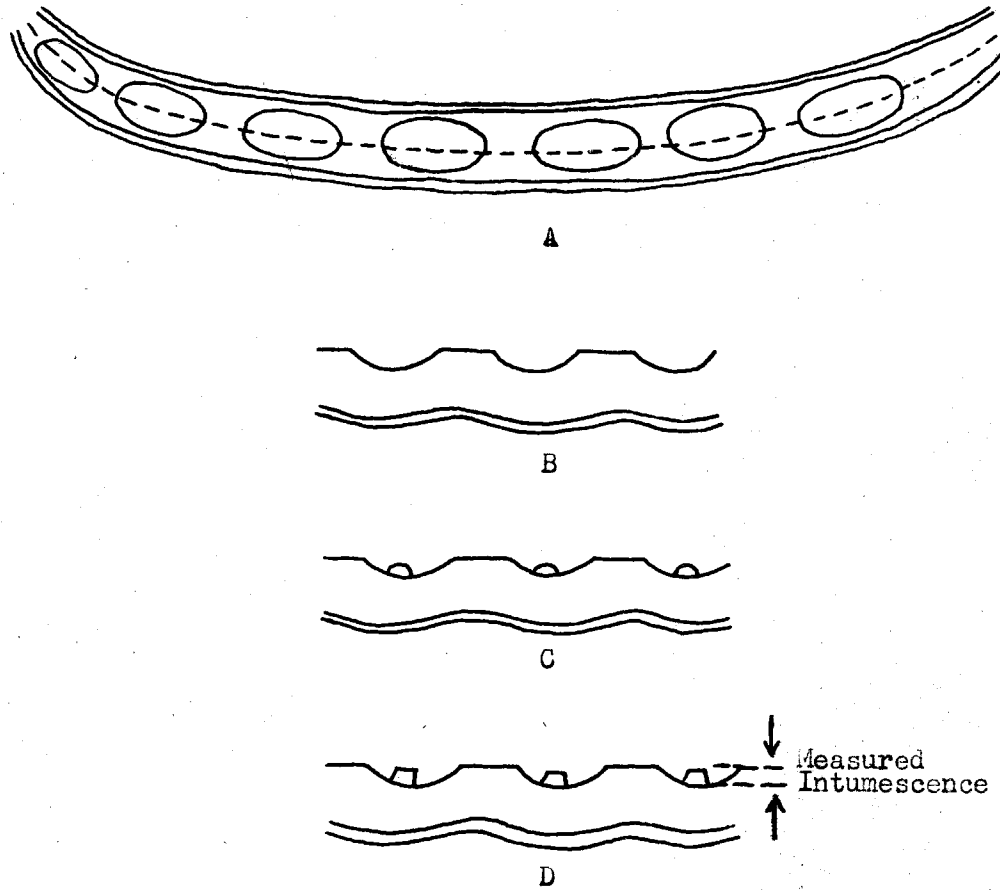

Figure 1. Schematic Representation of the Steps in the Wehnelt test.

A. Fresh immature pod slit along both sutures and seeds discarded; B. Longitudinal section of A along dotted line; C. A drop (0.01 ml.) of active solution in the center of the bean cavity; D. Intumescence measured under low power binocularscope.

tous lining of the bean pod, it is not surprising that other leguminous pods; peas, lima beans, chinese peas, etc., with only a rudimentary parenchymatous lining are not reactive.(9). There is no such simple explanation however, for the difference in response of beans whose pods have similar histological characteristics. Bonner and English (9) examined five varieties of Phaseolus with respect to their response to a standard test sample and found marked differences in reactivity even between such closely related varieties as Kentucky Wonder brown seed and Kentucky Wonder white seed (Table 1).

TABLE 1

Maximum Height of Intumescence Given by Different Varieties of Beans to a Standard Test Sample (9).

Variety	Average Height (mm.)
Kentucky Wonder, Brown Seed	1.9
Kentucky Wonder, White Seed	0.45
Florida Black Valentine	0.3
Golden Wax	0.15
Green Pod	0.00

Since the response of the pod in Wehnelt's test is obviously dependent not only upon the concentration of active component but also upon properties of the pod itself, it would be desirable in extending the work of Bonner and English to investigate the reactivity of pods of other bean varieties. Ferry-Morse* and Aggler & Musser** Seed Companies kindly provided several varieties of bean seeds for experimental purposes, and the plants were raised in the Earhart Plant Research Laboratory (14).

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* Ferry-Morse Seed Company, San Francisco 24, California
** Aggler & Musser Seed Company, Los Angeles 21, California

The seed samples were germinated and grown in an inert medium (one-half vermiculite and one-half gravel in two gallon earthen crocks) with the standard nutrient of the laboratory (Table 2), supplied when needed at watering periods. The physical conditions were those of the greenhouse, normal day photoperiod at a temperature of 20°C. from 8:00 a.m. to 4:00 p.m. and 14°C. from 4:00 p.m. to 8:00 a.m. Upon reaching a height of 5 to 8 inches (2 to 3 weeks) the plants were selected for uniformity and permitted to mature and fruit. After some 12 weeks immature pods could be harvested and their reactivity to a stock test solution* determined in the usual manner.

TABLE 2

Standard Nutrient Solution Employed at the Earhart Plant Research Laboratory

Substance	Concentration (grams / 1000 liter)
$Ca(NO_3)_2 \cdot 4H\ O$	820
$MgSO_4 \cdot 7H_2O$	490
KH_2PO_4	140
KNO_3	500
H_3BO_3	2.86
$MnSO_4 \cdot H_2O$	1.38
$ZnSO_4 \cdot 7H_2O$	0.22
$Fe(C_6H_5O_7) \cdot 3H_2O$	5.00
$CuSO_4 \cdot 5H_2O$	0.08
$H_2MoO_4 \cdot 4H_2O$	0.09

* See Lemon Peel Infusion page 25(for stability)

The results of such tests are shown in Table 3. Of the beans tested the distinction between the activity of bush beans and pole beans is noteworthy; the most active bush bean is only about one-half as reactive as the poorest pole bean. It appears that only pole beans are sufficiently sensitive to be used in Wehnelt's test. Even among the pole beans tested the sensitivity varies as much as three fold (see Ferry-Morse's Early White Seeded Kentucky Wonder and Aggeler & Musser's Kentucky Wonder Green Podded Brown or White Seeded). Since all varieties were grown under the same conditions one could conclude that genetic factors are very important in determining the reactivity of a bean. Early White Seeded Kentucky Wonder and Blue Lake White Creaseback varieties from Ferry-Morse Seed Company are the most sensitive and would be excellent for assay purposes.

D) ENVIRONMENTAL CONDITIONS OF THE PRODUCING PLANT AS A FACTOR IN BEAN RESPONSE.

The experience of this author confirmed the findings of Bonner and English (9) that the reactivity of commercial beans varied during the growing season. The reasons for such variations may have been manifold, but such considerations as genetics, environment and nutrition of the producing plant, maturity of the pods, time lapse between picking and purchase of beans and environmental conditions of testing appeared to be the primary ones.

The environmental conditions of testing from the procedure of Bonner and English (9) were adopted. The samples were incubated at 25°C. in the dark at 80-90% humidity. The drying of the pods with resultant poor tests was prevented by the high humidity. The time lapse between the picking and purchasing of commercial beans was not known but was kept as low as possible by selection of the firmest and freshest

TABLE 3.

Reactivity of Different Varieties of Beans to a Stock Test Solution*

Variety	Average Intumescence (mm.)	Plant Type
Ferry-Morse Seed Company		
1. Early White Seeded Kentucky Wonder	1.35	Pole
2. Blue Lake White Creaseback	1.20	Pole
3. Black Seeded Blue Lake	1.01	Pole
4. Pinto	1.01	Pole
5. Coaster	0.77	Pole
6. Oregon Giant	0.77	Pole
7. Kentucky Wonder Rust Resistant (brown seed)	0.72	Pole
8. Pink	0.72	Pole
9. Morse's #191	0.72	Pole
10. London Horticultural	0.53	Pole
11. Bachicha	0.24	Bush
12. Red Kidney	0.24	Bush
13. Tendergreen	0.19	Bush
14. Stringless Refugee	0.10	Bush
15. Pencil Pod Black Wax	0.10	Bush
16. Bountiful	0.10	Bush
17. Stringless Black Valentine	0.05	Bush
18. Stringless Kidney Wax	0.05	Bush
19. Top Notch Golden Wax	0.05	Bush
20. Dwarf Horticultural	0.05	Bush
21. Round Pod Kidney Wax	0.05	Bush
22. Full Measure	0.00	Bush
23. Landreth's Stringless Green Pod	0.00	Bush
24. Plentiful	0.00	Bush
Aggeler & Musser Seed Sompany		
1. Kentucky Wonder Rust Resistant White Green Podded	0.91	Pole
2. Kentucky Wonder White Seeded Special Rust Resistant	0.72	Pole
3. Kentucky Wonder Wax Pole Yellow	0.58	Pole
4. Kentucky Wonder Rust Resistant Brown Seeded	0.48	Pole
5. Kentucky Wonder Brown Seeded Green Podded	0.43	Pole
6. Kentucky Wonder White Seeded Green Podded	0.43	Pole

* Lemon Peel Infusion page 25

pods. Qualitative observations on green house beans, however, indicated that pods aged one day after picking were more satisfactory than freshly picked ones. Such pods could then be stored satisfactorily in plastic bags in a refrigerator for several days when fresh beans were not available. With greenhouse beans, immature pods about 2 weeks old from the time of fruit set were the most satisfactory; older or younger bean pods were less responsive. Selection of commercial beans was largely a matter of experience; usually, Kentucky Wonder pods of dark green color, large size with large seed chambers but small seeds were satisfactory.

In Southern California market beans come mainly from the desert, Coachella Valley, in the spring, the coast during the summer, etc., according to the seasonal conditions for best growth. The seasonal variation in reactivity which occured could be explained by genetic factors (as exemplified by the response of different varieties--see previous section), but there remained a definite possibility that some of these variations could be effected by different conditions of environment and nutrition of the producing plant. The findings of Osborne and Went (15) have shown that with the same nutrient, properties of the tomato fruit, flavor, color, texture, etc., are dependent upon the environmental conditions in which the fruit is produced. A similar observation has been made in the case of strawberries (16). In view of these observations an investigation to assess the effect of temperature and photoperiod upon bean response was conducted under the supervision of F. W. Went.* The work was carried out in the Earhart Plant Research Laboratory (14) where it was possible to arrange suitable conditions of temperature and photoperiod.

* Frits W. Went, Professor of Plant Physiology, California Institute of Technology

The Kentucky Wonder Rust Resistant Beans (Ferry-Morse Seed Company) were selected for these tests because of their intermediate level of response. The effect of environment might then be reflected in either an increase or decrease in reactivity. Plants were germinated and grown at all combinations of day temperatures (20°C., 23°C., 26°C.) and night temperatures (14°C., 17°C., 20°C.) for short days (8 hours) and long days (14 hours). The planting medium consisted of equal amounts of vermiculite and gravel; the nutrient was the standard one for the laboratory (Table 2.).

Upon harvest the immature pods were tested with a stock wound hormone solution.* The wound response was largely the same for all beans grown in this test. Photoperiod and temperature variations therefore do not affect the reactivity of the bean pod though they do markedly affect growth and fruit production. Assuming that the response of other varieties of beans is as little affected by the environmental conditions, one may conclude that environmental factors are not the major ones involved in the variations found in the wound tests. The effect of nutritional factors on the response of beans was not examined.

E) LIMITATIONS AND RESTRICTIONS IN THE ASSAY

1/ Use of Commercial Beans

Beans obtained from local sources responded less uniformly in the test than those grown in the Earhart Research Laboratory. Nevertheless in view of the large amounts of beans necessary for testing, market beans had to be used in this investigation. The commercial beans used were of the local varieties of Kentucky Wonder

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* See Lemon Peel Infusion page 25

Pole; another variety occasionally available, Kentucky Wonder Bush beans, was unsatisfactory. In the early spring and late fall the market beans came from Mexico, and though these were of a Kentucky Wonder variety, they also were unsatisfactory.

2/ Variability in Response

In an investigation of this type it is desirable to establish a reproducible unit of activity as early as possible. It has been mentioned that the response of beans shows a seasonal variability. Such a variation could be compensated, but what makes the definition of an activity unit difficult is the fact that the response of market beans frequently shows a batchwise variation, that is , the response of such pods to a stock solution * often varies from batch to batch. A stock solution was therefore always tested with each batch of bean pods to permit comparison of the activities of various fractions tested at different times.

Tests have shown that the height of intumescence induced by equal volumes of solutions of active material is a function of the concentration. If the height of intumescence is plotted against the logarithm of the concentration of test material, an s-shaped curve is generally obtained which has a linear portion. If this is done for a number of materials, a set of nearly parallel lines can be selected (within certain concentration ranges). If a stock solution is included in the test, the activity of the test material relative to the stock solution can be estimated by taking an intumescence common to the linear portions of the two respective plots and calculating the

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* See Lemon Peel Infusion, page 25 (for stability)

inverse ratio of the corresponding concentrations at that intumescence. This inverse ratio is then the relative potency. For example in Fig. 2, curve A is the plot of the response to a stock solution; curve B, to a methanol soluble material; curve C, to a methanol insoluble material and curve D, to a proportional (1:1) recombination of methanol fractions. It can be seen that B has about 60% of A's potency, that C has 30% and curve D has 90%. The error of the test is such that curves A and D cannot be said to be significantly different. In practice the linear portions of the curves may be difficult to select and the choice of the common intumescence becomes necessarily somewhat arbitrary.

3/ Toxicity and Non-Specific Reaction

This aspect of the assay has been adequately described by Bonner and English (9); all fractions examined for activity during the investigation were tested in a concentration series calculated to span the difference between a non-specific response (slight intumescence such as is caused by water and other non-specific agents) and a toxic response (crater shaped intumescence such as is caused by release of wound hormone from cells killed by toxic material).

During the final phases of the investigation it became important to have some idea of the buffer capacities of the parenchyma cells lining the interior of the bean pod, since some of the compounds necessitated a slightly alkaline medium to remain in solution. Alkaline solutions of pH 7.0, 7.5, 8.0, and 8.5 were prepared with M/15 phosphate buffer and one solution pH 9.0 was prepared with M/50 borate buffer. The wound response of these buffer solutions was not greater than that of water.

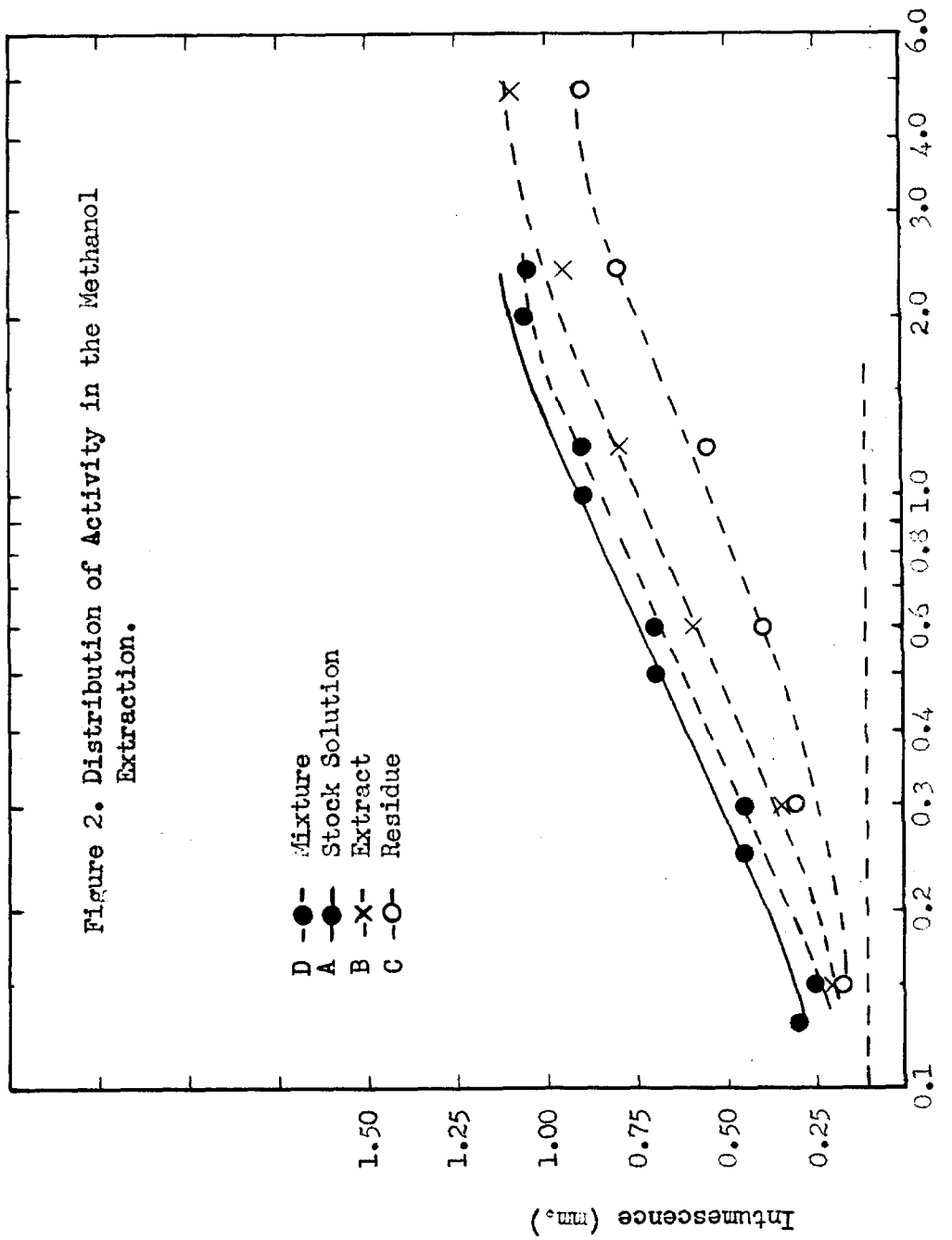
Figure 2. Distribution of Activity in the Methanol Extraction.
D Mixture
A Stock Solution
B Extract
C Residue
Intumescence (mm.)
1.50
1.25
1.00
0.75
0.50
0.25
0.1
0.2
0.3
0.4
0.5
0.8
1.0
2.0
3.0
4.0
6.0
Percent Concentration of Test Sample

PART III

SOURCE MATERIAL

A) GENERAL SURVEY

The work of Wehnelt (7) indicated that wound active materials were widespread in nature. Bonner and English (9) carried out a more extensive investigation and found activity in many diverse products (Table 4.). In a search for a suitable starting material for the isolation of wound active substances, a number of products were examined and their activity compared on a wet as well as a dry weight basis. The results of these investigations are summarized in Table 5.

TABLE 4

Occurrence of the Wound Hormone

Source	Activity	Source	Activity
Bean Pod	++	Corn Meal	-
Brussel Sprouts	++	Soybean Meal	-
Sweet Potato	+	Wheat Germ	+
Potato	+	Molasses	+
Orange	++	Yeast, bakers	-
Lemon	++	Yeast, brewers	+
Tomato	++	Vitamin B Concentrate	-
Lettuce	++	Urine, Human	-
Spinach	+	Urine, Cow	-
Pea Plant, etiolated	++	Peptone, Difco	-
		Beef Extract	-
Pea Plant, green	++	Liver Extract	-
		Milk	-
Pea Seed	-	Egg Albumin	-
Hay, Alfalfa	+	Serum	-
Malt	+	Emulsin	-
Rice Polishings	-		
Cabbage	+		

To test these products the material was crushed and filtered through cheese cloth. The watery suspension was neutralized with 5% aqueous KOH then centrifuged; the supernatant was used for the test and for determination of solids. In the case of citrus peels it was necessary

to add an equal weight of water to the tissue to get an aqueous suspension. The figures for relative activity are approximations but they are considered adequate for comparative purposes.

TABLE 5.

Relative Activity and Percent Solids of Various Plant Extracts

Plant Source	Percent Solids	Relative Activity* Juice	Relative Activity* Solids
Orange fruit peel (colored portion)	7.9	120	80
Orange fruit (fresh juice)	11.0	110	54
Kentucky Wonder Beans (pods)	5.4	100 (control)	100 (control)
Lemon (fruit peel)	5.5	90	90
Florida Green Beans		80	
Tangerine fruit (fresh juice)		70	
Russet Potato (tuber)	3.9	70	100
Carrot (root)	6.3	70	60
Onion (bulb)	6.3	70	60
Tomato fruit (fresh juice)	5.4	70	70
Orange juice (canned)	13.0	60	25
Orange fruit peel (albedo portion)		60	
Lemon fruit (fresh juice)	8.5	50	30
Pineapple fruit (fresh juice)		50	
Pineapple juice (canned)	14.0	50	20
White Rose Potato (tuber)	3.6	40	60
Beet (root)	8.5	40	30
Pippin Apple (fresh)	11.0	30	15
Coconut milk		30	
Grape (berry)	24.0	20	5
Pomegranate (fresh)		0	
Lemon Peel Infusion**	1.0	80	430

* Relative Activity of source materials to beans (100) on the basis of natural juice and dry solids.

** See page 25 of this thesis.

B) SELECTION

From the data (Table 5.) it is apparent that citrus products are a potent source of activity. In fact the natural juice is comparable to that of the pod extract of Kentucky Wonder beans. It is interesting to note that in the case of the lemon as well as the orange, the peel extract is more active than the natural juice. It should be pointed out however, that none of the citrus extracts are as as active on a dry weight basis as the extract of Kentucky Wonder Beans or the Russet potato, but that the extract of lemon peels is nearly so. These observations indicate that the orange, the lemon and the Russet potato are satisfactory source materials.

PART IV

MATERIALS AND REAGENTS

This section is devoted to the description of materials used in the present work.

A) PURIFIED SOLVENTS

Acetone, Reagent, Merck and Company.
This solvent was purified before use by boiling with $KMnO_4$ and then distilling over dry K_2CO_3 (17).

Diethyl Ether, Reagent, Merck and Company.
The peroxides were removed by shaking ether with a slurry of ferrous sulfate and calcium hydroxide followed by direct distillation of the ether from the slurry (17).

Petroleum Ether (30-60°C) and (60-70°C)
Both fractions were purified by permitting them to sit over 5-10% fuming sulfuric acid for a week. The solvent was then washed with alkali and dilute permanganate and dried with anhydrous K_2CO_3 before distillation over a fresh portion of anhydrous K_2CO_3 (17).

B) ORGANIC REAGENTS USED DIRECTLY IN TESTS

Ascorbic Acid, Eastman Kodak White Label
Biotin, Nutritional Biochemical Corporation
Calcium Pantothenate, Wm. T. Thompson Company
Folic Acid, Nutritional Biochemical Corporation
Nicotinic Acid, Merck and Company
Para-Aminobenzoic Acid, Merck and Company
Pyridoxine · HCl, Merck and Company
Riboflavin, Merck and Company
Thiamine · HCl, Merck and Company

D-Fructose, Pfanstiehl Chemical Corporation
D-Galactose, Nutritional Biochemical Corporation
D-Glucose, Reagent, Merck and Company
D-Mannose, Nutritional Biochemical Corporation
Sucrose, Reagent, Merck and Company

DL-Alanine, Eastman Kodak White Label
L-Arginine, Van Camp Laboratories
L-Asparagine, Merck and Company
L-Aspartic Acid, H. M. Chemical Company
L-Cysteine, Van Camp Laboratories
L-Cystine, Van Camp Laboratories
L- Glutamic Acid, H. M. Chemical Company
Glycine, Merck and Company

L-Histidine · HCl, Merck and Company
DL- Isoleucine, Merck and Company
L-Leucine, Nutritional Biochemical Corporation
L-Lysine, Van Camp Laboratories
L-Methionine, H. M. Chemical Company
DL-Norleucine, Pfanstiehl Chemical Corporation
L-Ornithine, Van Camp Laboratories
L-Phenylalanine, Nutritional Biochemical Corporation
L-Proline, H. M. Chemical Company
L-Serine, California Federation for Biochemical Research
L-Threonine, General Biochemical Corporation
L-Tryptophane, Van Camp Laboratories
L-Tyrosine, Eastman Kodak White Label
L-Valine, Nutritional Biochemical Corporation

Decane-1,10-dicarboxylic Acid, Dr. James English, Jr. Dept. of Chemistry, Yale University
Fumaric Acid, Eastman Kodak Practical
α-Keto-Glutaric Acid, Nutritional Biochemical Corporation
Maleic Acid, Eastman Kodak White Label
Malonic Acid, Eastman Kodak White Label
Sebacic Acid, Eastman Organic Chemicals
Succinic Acid, Merck and Company
Tartaric Acid, Analytical Reagents, Mallinckrodt Chemical Works
Traumatic Acid, trans, Abbott Laboratories
Traumatic Acid, cis, Dr. James English, Jr. Dept. of Chemistry, Yale University

Adenosine Triphosphate, Pabst Chemical Company
Adenylic Acid, Nutritional Biochemical Corporation
Benzoic Acid, Primary Standard, Merck and Company
Casein Hydrolysate, Nutritional Biochemical Corporation
Cinnamic Acid, Eastman Kodak Practical
Citric Acid, Reagent, Merck and Company
Coenzyme I, Pabst Chemical Company
Coenzyme II, Sigma Chemical Company
Coenzyme A, Pabst Chemical Company
Cytidylic Acid, Nutritional Biochemical Corporation
Cytochrome C, Nutritional Biochemical Corporation
3,5-Di-iodotyrosine, Eastman Kodak White Label
Furoic Acid, Quaker Oats Company
Glutathione, Eastman Kodak White Label
Guanylic Acid, Nutritional Biochemical Corporation
Indole-3-acetic Acid, Eastman Organic Chemicals
Ion-Exchange, Resin, Amberlite IR400, Resinous Products and Chemical Company

Phenylhydrazine, Matheson Chemical Company
Pyruvic Acid, Fisher Scientific Company
DL- Tropic Acid, Eastman Kodak White Label
Yeast Hydrolysate, Nutritional Biochemical Corporation

Propionic Acid, Eastman Kodak White Label
Butyric Acid, Eastman Kodak White Label
Caproic Acid, Eastman Kodak White Label
Caprylic Acid, Nutritional Biochemical Corporation
Capric Acid, Eastman Kodak White Label
Lauric Acid, Eastman Kodak White Label
Myristic Acid, Eastman Kodak White Label
Palmitic Acid, Eastman Kodak White Label
Stearic Acid, Eastman Kodak White Label
Oleic Acid, U.S.P. Merck and Company
Linoleic Acid, C. P. Eimer and Amend
Linolenic Acid, Bios Laboratories
Chaulmoogric Acid, Eastman Kodak Practical

C) RAW MATERIALS

Pressed Valencia Orange Oil, Dr. J. Kirchner U.S. Dept. of Agriculture Fruit and Vegetable Laboratory, Pasadena, Calif.
Linseed Oil, Fuller Paint Company
Corn Oil, Mazola brand, Corn Products Refining Company
Tung Oil, Argentine, Pacific Vegetable Oil Corporation
Lemon Peel Infusion, California Fruit Growers Exchange

PART V

PRELIMINARY INVESTIGATIONS

A) ORANGE JUICE

Orange juice was found to possess a high level of activity and was selected for a preliminary study of the properties of the active factors. The extract was prepared simply by squeezing fresh oranges in a household squeezer and centrifuging the juice.

1/ Solvent Partition

The active component of orange juice was not extractable from acidic, neutral or basic solutions by ether or ethyl acetate. The active material was quantitatively recoverable from the aqueous fraction.

2/ Adsorption

The active component of orange juice was completely adsorbed by successive treatments with Norite A. It was however, difficult to elute from the Norite and methyl alcohol, ethyl alcohol or a mixed solvent, water-acetone-ammonia could only elute it partially.

3/ Stability

a/ Heat

Orange juice could be heated in a boiling water bath for several hours or boiled for an hour with no loss of activity.

b/ Oxidants

Extracts could be treated with dilute hydrogen peroxide or with a stream of oxygen at room temperature or at 100°C. without loss of activity.

4/ Metal Precipitation

Treatment of the juice extract with solutions of

barium or lead acetate led to the formation of precipitates. The soluble and insoluble fractions, separated from metallic ions by treatment with dilute sulfuric acid in the case of barium and hydrogen sulfide in the case of lead, showed no activity.

B) LEMON PEEL INFUSION*

Since the stability characteristics of the active principle of oranges were favorable, a search was made for some industrial citrus preparation that would preclude the necessity of large scale processing of fresh fruits. A Fruit Grower's Exchange preparation, Lemon Peel Infusion, appeared to be a suitable substitute source material. The data of Table 5. show that an aqueous lemon peel extract is quite active and in fact on a dry weight basis it is the most favorable of the citrus products tested. Precluding the possible destruction of the active principle during manufacture, the infusion should be active. Activity tests (Table 5.) proved this supposition correct. In fact on an activity per dry weight basis, the infusion is far superior to all the other natural products, and therefore, was chosen as source material for further experiments.**

According to the Fruit Grower's Exchange Pharmaceutical Division (18), the infusion is prepared in the following manner:

> "Freshly ground lemon peel is mixed with water and sufficient calcium hydroxide to prevent the solution of pectin, which, if extracted, would interfere with subsequent vacuum concentration due to the high viscosity

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* This material is now marketed under the name of Lemon Bioflavonoid Complex by the Fruit Grower's Exchange Products Division , Ontario, California.

** Tests on this product have shown no detectable loss in activity over a three year period.

> it would impart. The infusion is effected with warm water, the solution is strained through the mass of peel serving as a filter bed but not completely clarified, and is then vacuum concentrated by single effect evaporation to a thick sirup. The sirup is passed over specially constructed steam rolls where, in a few seconds, it is reduced to a solid. Dryness is maintained during further handling, dry granulation, chemical testing, blending and packaging, by the use of silica gel beads."

1/ Properties

a/ Appearance

The infusion as manufactured is tan to brown in color and passes a 10 mesh screen. It possesses a caramelized odor and, being very hygroscopic, must be stored in the presence of a desiccant (18).

b/ Moisture and Ash

The loss of weight after 4 hours at 60°C. in a vacuum oven (27-28" of mercury) is considered as "moisture". This value varies between 0.5% and 3.0%. Ash is determined by heating for three hours at 500°C.; values vary between 7% and 15%, about one-third of which is added calcium and the balance natural ash constituents of lemon peel (18). Samples of the infusion used in this laboratory were ignited and burned in air to a gray flaky residue which amounted to 10% of the weight of the original material. Only 22% of the residual ash dissolved in water to form a 1% solution; which after neutralization was inactive in Wehnelt's test.

c/ Solubility

In accordance with the method of preparation one should expect water insoluble matter to be present. Solubility deter-

minations show that 10% of the infusion is water insoluble. A solution of soluble infusion material is only slightly acidic; the pH varies from 5 to 7.

d/ Stability

1/ Heat

The activity of lemon peel infusion is relatively stable to heat, for an aqueous solution of the infusion can be heated or boiled for several hours with no apparent ill effects.

2/ Oxidants

The active agent in an aqueous solution of the infusion is not harmed by short time contact with mild oxidizing agents. A water solution of the infusion treated with dilute hydrogen peroxide or a stream of gaseous oxygen for several hours shows no change of activity over untreated material.

3/ Acids and Bases

Lemon peel infusion, heated for several hours in the presence of dilute acid or alkali, shows no loss of activity.

2/ Experimental Purification Procedures

a/ Dialysis

Dialysis of the infusion (cellulose dialysis tubing, Visking Corporation) against twice distilled water replaced at regular intervals is nearly complete in one day. The dialysate obtained at the end of the first day contained most of the activity of the starting material. Approximately 10% of the starting material remains as undialyzable residue (Table 6.).

TABLE 6

Distribution of Material and Activity in Dialysis.

	Weight (grams)	Activity (mm. Intumescence of 0.25% sol'n)	Percent of Initial Activity Present in Fraction*
Lemon Peel Infusion	2.00	0.82	
Dialysate 0-24 hours	1.77	0.53	60
Dialysate 24-48 hours	0.02	1.06	1
Dialysate 48-72 hours	0.01	0.91	0.5
Residue	0.20	0.82	10

b/ Solvent Partition

1/ Methyl Alcohol

The dry infusion when extracted continuously is 90% soluble in Methyl alcohol; methanol therefore, is no better for extraction than water. If, however, a methanolic suspension of 10% infusion is shaken overnight, only 50% of the starting material is soluble. The methyl alcohol soluble as well as the methyl alcohol insoluble fraction gives a response which is lower than that of the original material. A proportional recombination of the two fractions, however, shows activity comparable to that of the starting material (Fig. 3).

* The overall loss in activity is probably only an apparent one. Subsequent findings established that several factors are involved in the wound response. Differential rates of dialysis would alter the ratio of factors present in each fraction and hence the wound response. Combination of all fractions would undoubtedly have shown the original activity.

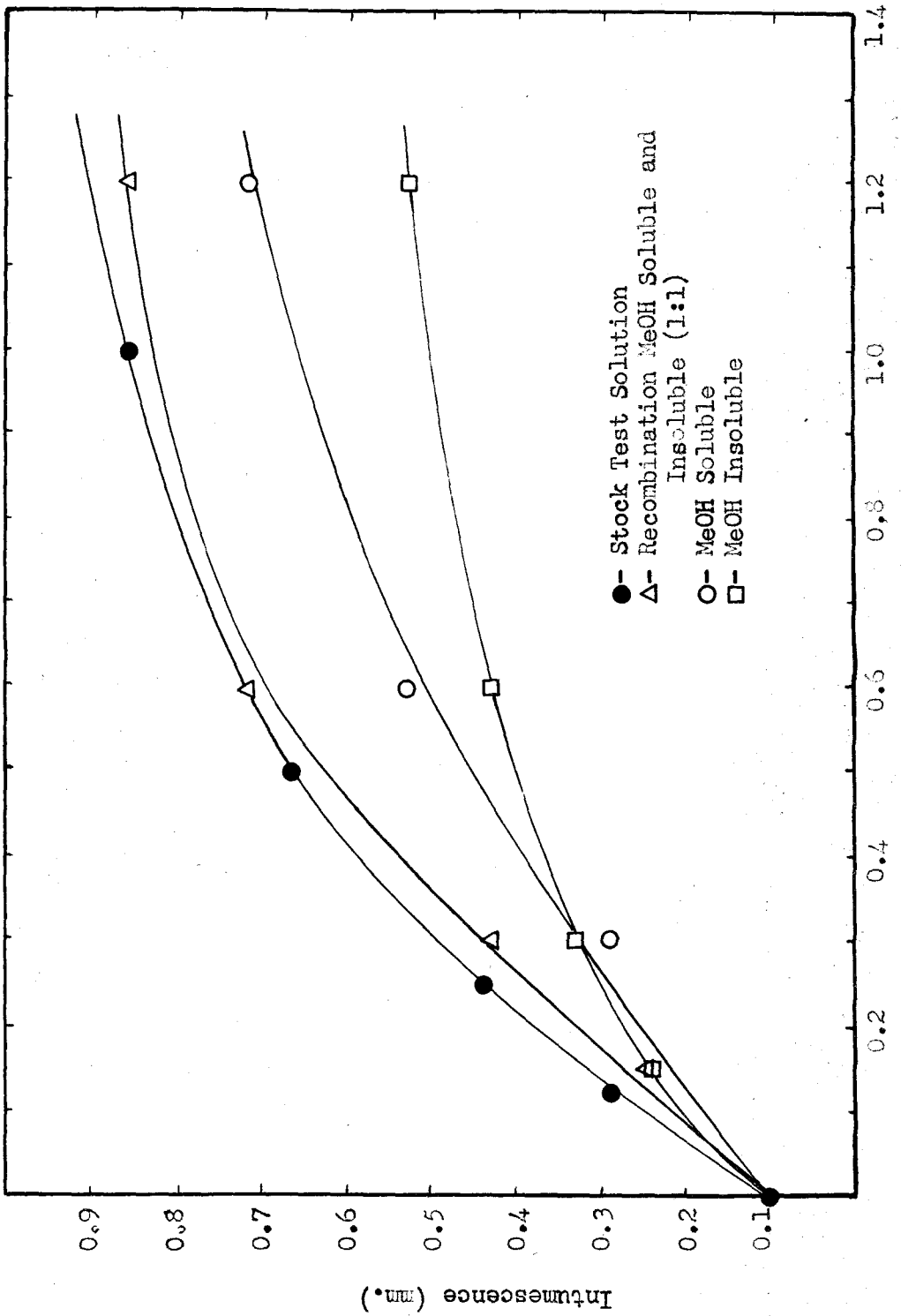

Figure 3. Activity of Methanol Fractions

The batchwise extraction procedure with methyl alcohol indicates the existence of at least two factors involved in the wound response.

The methanol insoluble fraction containing predominantly water soluble factors was tested with a series of purified acids (Table 7.), and also used in subsequent tests-- see diethyl ether.

TABLE 7.

Acids Tested for Wound Hormone Activity in Combination with the Methanol Insoluble Fraction of Lemon Peel Infusion.

Tartaric Acid	Sebacic Acid
Citric Acid	Indole-3-acetic Acid
Oxalic Acid	Nicotinic Acid
Pyruvic Acid	Para-aminobenzoic Acid
Succinic Acid	Traumatic Acid
Pantothenic Acid	

These pure acids could not replace the methanol soluble fraction, so one may conclude that the active component is different from any of the acids tried.

The converse system utilizing the methanol soluble material as the wound hormone factor was also considered. Here the more lipoid soluble factor was combined with several purified preparations (Table 8). None of the preparations, tested over a range of concentrations, was capable of replacing the methanol insoluble fraction. This fact indicated that these materials were not the active components found in this fraction.

2/ Diethyl Ether

Lemon peel infusion was dissolved in water to form a 3% solution, the pH adjusted to about 1 and the solution submitted to continuous ether extraction. Upon drying with anhydrous

TABLE 8

Preparations Tested for Cofactor Properties

Glucose	Proteose Hydrolysate of Milk**
Sucrose	Casein Hydrolysate
Glutamic Acid	Yeast Hydrolysate
Vitamin Solution*	

sodium sulfate, and removal of solvent under reduced pressure the ether fraction contained about 5% of the original infusion. Ether extracts of acidic infusion solutions occasionally manifested slight activity, but this was believed to be due to emulsion carry over since emulsion formation was a serious problem. In ether extractions of alkaline infusion solutions (pH 13) the extract contained about 4% of the starting material. The ether extractable material from an alkaline infusion was not active by itself or in combination with the factors from the methanol insoluble fraction. The aqueous fractions, neutralized and dried under reduced pressure, were always as active as the original infusion, and therefore the ether extraction was considered ineffective.

3/ Ethyl Acetate

Ethyl acetate was substituted for ether in the continuous extraction procedure described above. About 5% of the infusion was extractable. After drying each fraction the tests showed that on a dry weight basis there was no difference in activity of the individual fractions, the recombined fractions or the original infusion. There was no fractionation.

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* This preparation was 5 times the strength normally used for a microbiological assay.

** This material was obtained from Dr. Edwin Goldsmith, Research Fellow in Biology, 1951, California Institute of Technology

4/ Glacial Acetic Acid

Even though the active component of lemon peel infusion could withstand boiling in water or dilute acid for a short time, it was considered inadvisable to subject the infusion to a continuous extraction with boiling glacial acetic acid. Consequently the infusion was extracted by making up a 4% suspension in glacial acetic acid. After shaking overnight at room temperature, 80% of the infusion was found soluble in acetic acid. Upon removal of the solvent, activity tests on the individual fractions and the recombined fractions (ratio of yields) showed not only poor partition but also a loss of activity (Fig. 4.). Acetic acid partition was less favorable than that achieved with methyl alcohol.

5/ Amyl Alcohol

Lemon peel infusion at room temperature mixed overnight on a shaker as a 10% suspension in n-amyl alcohol is less than 2% soluble. Activity tests show no separation of components or enrichment. This procedure is useless for partition purposes.

c/ Precipitation from Aqueous Solution

1/ With Barium and Lead

The activity of fresh orange juice disappeared upon treatment with barium and lead acetate solution. This disappearance of activity may have been due to a separation of active factors and it was of interest to investigate this possibility with lemon peel infusion. Accordingly a 15% aqueous suspension of infusion material was treated successively with saturated solutions of barium acetate, neutral lead acetate, and basic lead acetate (Chart I). The precipitate of each fraction was separated by centrifugation, and then

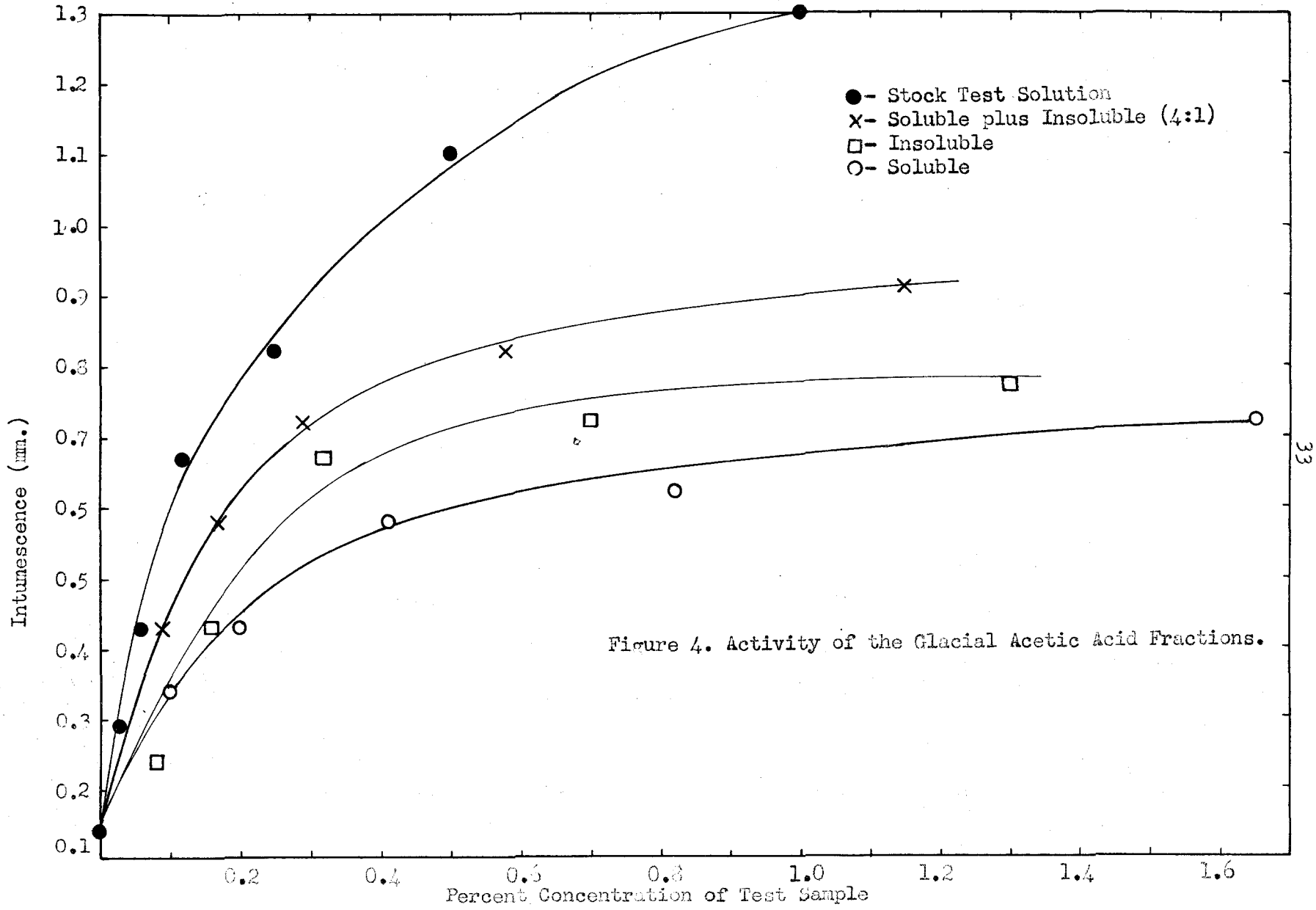
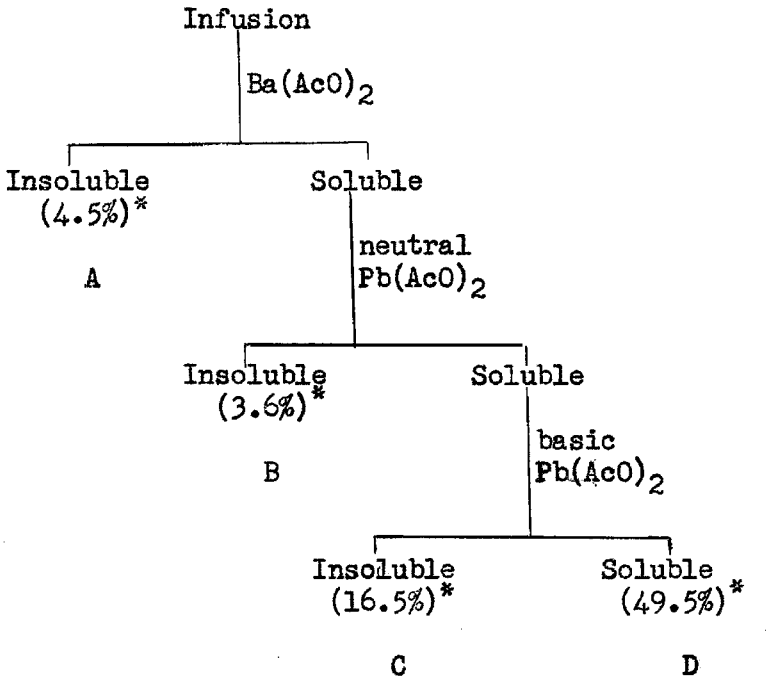


Figure 4. Activity of the Glacial Acetic Acid Fractions.

regenerated by removing barium and lead in the usual manner. Each regenerated fraction was vacuum dried, weighed then tested for activity (Fig. 5. and 6.).

CHART I



Fraction A is relatively inactive at the lower concentrations but its activity is raised considerably by combination with filtrate D. The neutral and basic lead precipitates, B and C, show by themselves an activity comparable to that of the original infusion and are not raised significantly by the addition of the filtrate D. The barium precipitation effects a separation of factors but the large loss of material does not warrant acceptance of this procedure at present.

2/ With Ethyl Alcohol

In view of the partition of active fractions achieved with methanol extraction, and the lower solubility of infusion

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* Percent of initial material found in this fraction. 25.8% is lost.

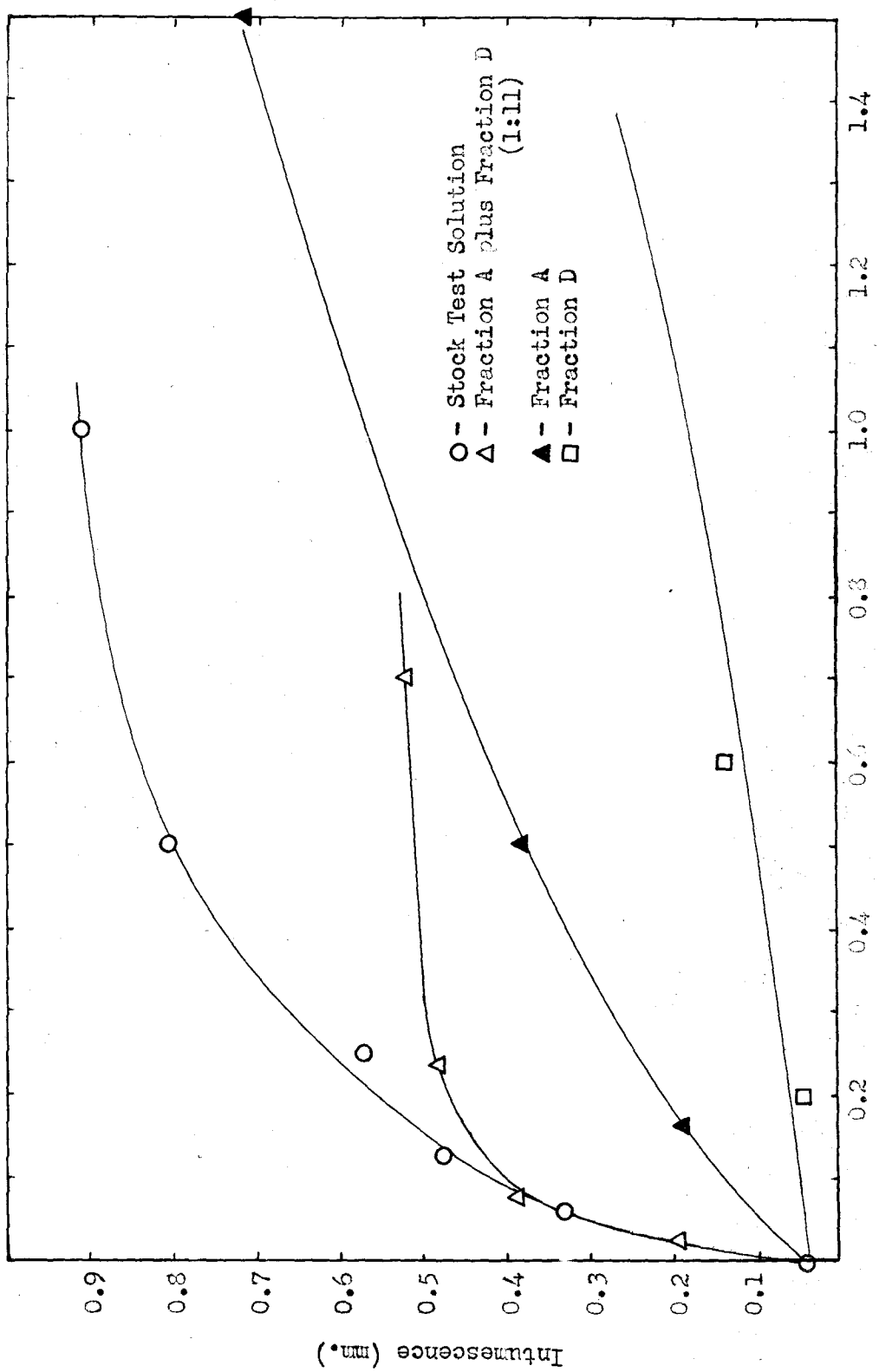


Figure 5. Activity of the Barium Precipitate.

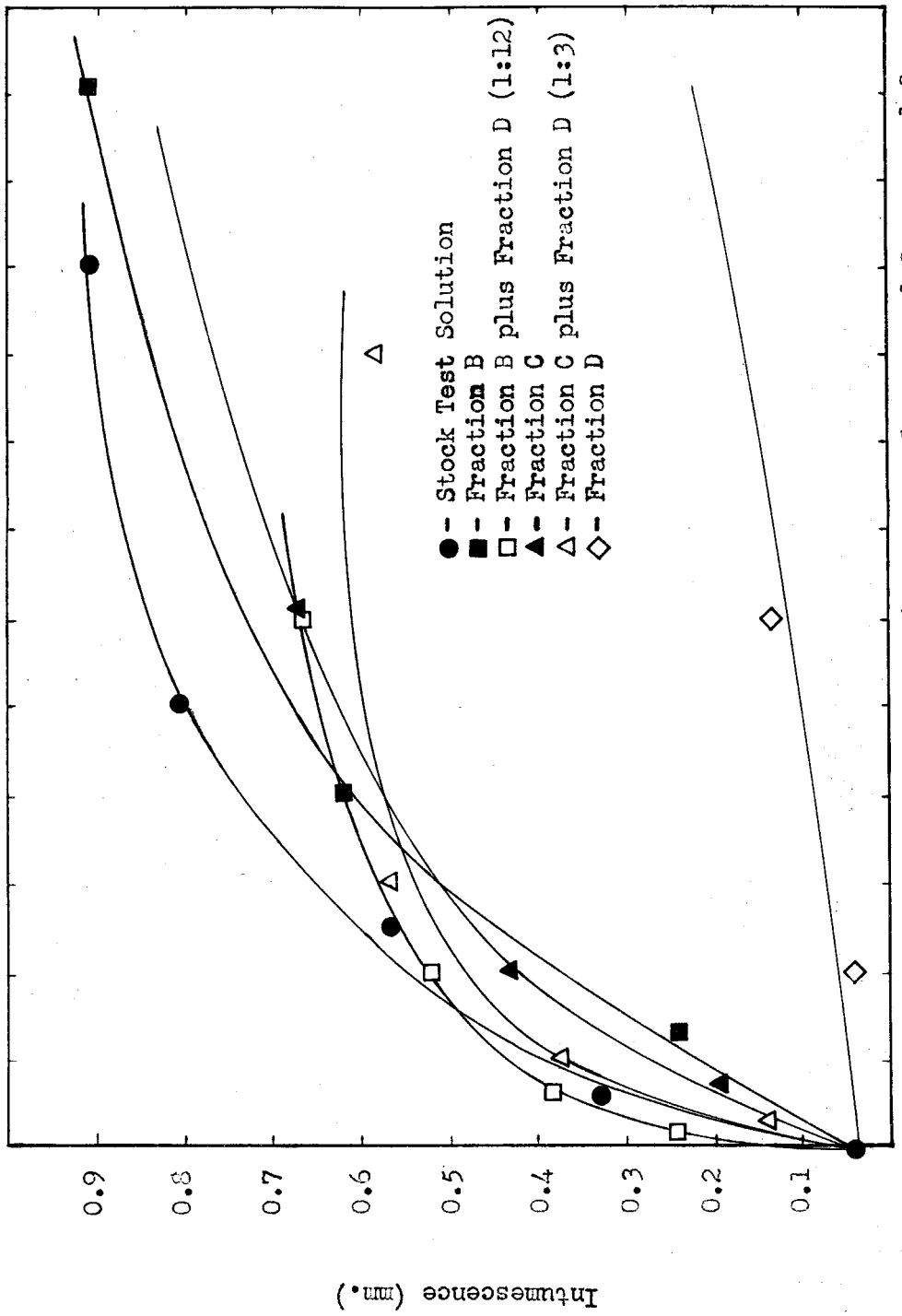


Figure 6. Activity of the Lead Fractions.

in ethanol, the latter solvent was used in an attempt to enrich the active system of the infusion by precipitation from an aqueous solution. A concentrated aqueous stock solution was prepared, the water insoluble matter removed and a portion diluted to the desired concentration of mixed solvent (Table 9.). The supernatants were separated by centrifugation then dried, weighed and tested for activity (Fig. 7.). There is a slight enrichment in a mixture of equal volumes of water and alcohol but it hardly compensates for the loss of soluble material. For example, in going from 40% to 50% ethyl alcohol, 20% of the soluble solids precipitate whereas the activity remains unchanged. The behavior of the supernatant during vacuum drying indicates that at least some of the surface active materials appear to have been precipitated by a 20% ethanol solution.

3/ With Acetone

The experiments with the water-ethyl alcohol mixtures were repeated with water-acetone mixtures (Table 10.). Tests (Fig. 8.) indicate that acetone precipitation offers no promise of enrichment. The behavior of the supernatant on vacuum drying shows that precipitation with 20% acetone appears to remove some of the surface active material.

d/ Removal of Sugars

So far the investigations have not excluded sugars as active compounds and in view of the large amounts present in lemon peels (18,19) it would be desirable to evaluate their effect. This can be done by removing the sugars as osazones after the method of Maquenne (20). The limitations of the biological assay make it imper-

TABLE 9

Ethanol Precipitation of Aqueous Lemon Peel Infusion

Volume % EtOH	0	10	20	30	40	50	60	70	80	90	95	100
EtOH Added (ml.)	0	4	8	12	16	20	24	28	32	36	38	40
Stock Solution (ml.) 27.5%	4	4	4	4	4	4	4	4	4	4	2	1.10*
H_2O Added (ml.)	36	32	28	24	20	16	12	8	4	0	0	0
Total Volume (ml.)	40	40	40	40	40	40	40	40	40	40	40	40
Character of Insoluble Material	....	cloudy	floc.	floc.	floc.	floc.	floc.	firm	firm	firm	firm	firm
Color of Supernatant	dk.red brown	..... gradual change to									pale yellow	pale yellow
Behavior of Supernatant on vacuum desication	froth	froth	no froth	.....								
Percent Soluble Solids	100	96.5	94.8	82.3	94.8	74.3	74.3	62.8	56.7	51.3	51.1	16.3

* This amount of lemon peel infusion, in grams, was mixed with ethanol

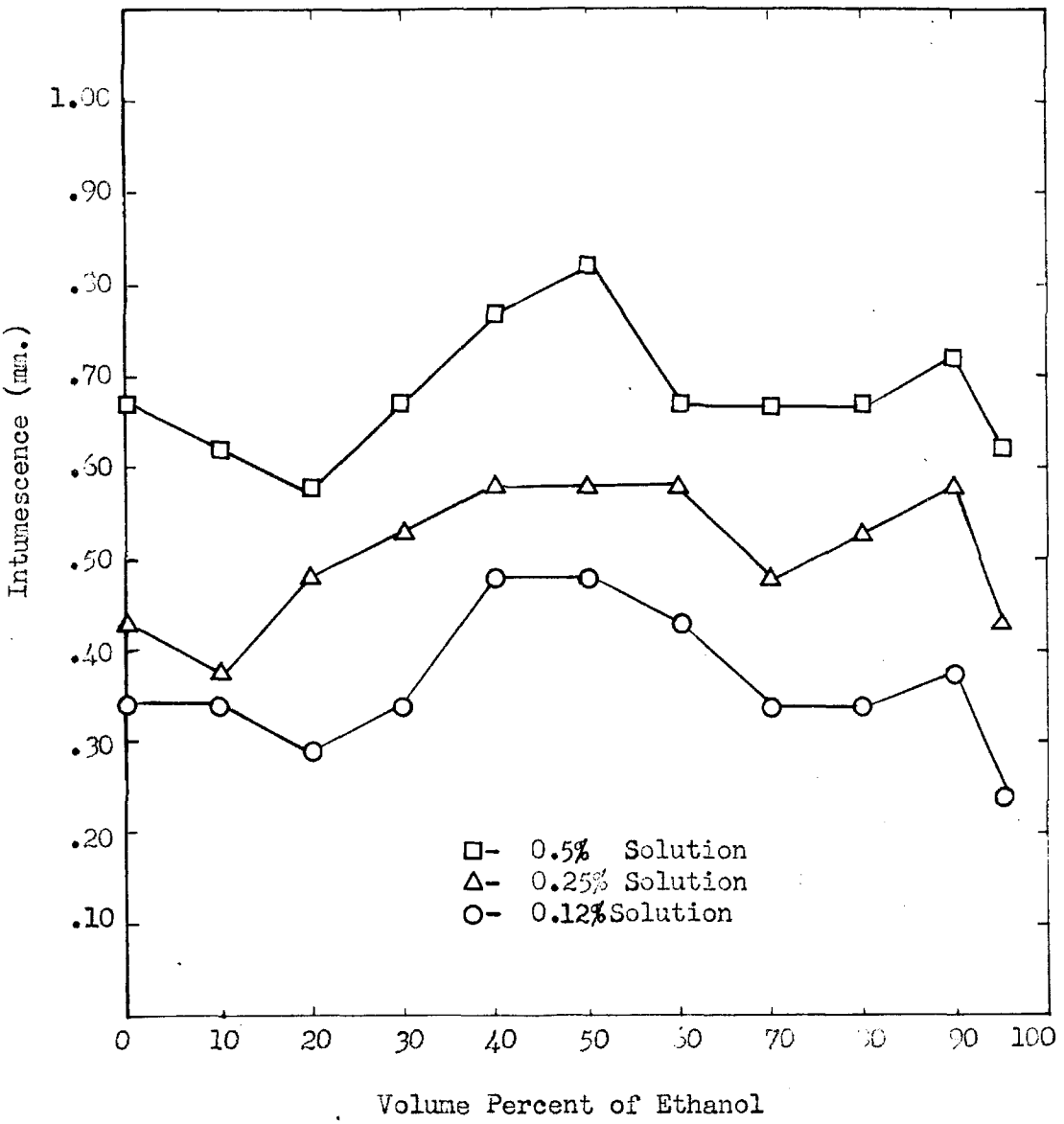


Figure 7. Relative Activity of the Water-Ethanol Supernatants at Three Concentrations.

TABLE 10

Acetone Precipitation of Aqueous Lemon Peel Infusion

Volume % Acetone	0	10	20	30	40	50	60	70	80	90	100
Acetone Added (ml.)	0	4	8	12	16	20	24	28	32	36	40
Stock Solution (ml.) 24%	4	4	4	4	4	4	4	4	4	4	0.96*
H_2O Added (ml.)	36	32	28	24	20	16	12	8	4	0	0
Total Volume (ml.)	40	40	40	40	40	40	40	40	40	40	40
Character of Insoluble Material	....	floc.	floc.	cloudy.....		firm	.....				
Color of Supernatant	dk.red brown	.....		gradual change to			.....				pale yellow
Behavior of Supernatant upon vacuum desication	froths	froths	no froth	.....							
Percent Soluble Solids	100	88.7	96.0	89.8	92.8	83.5	65.0	65.0	60.8	33.0	1.7

* This amount of dry infusion, in grams, was mixed with acetone.

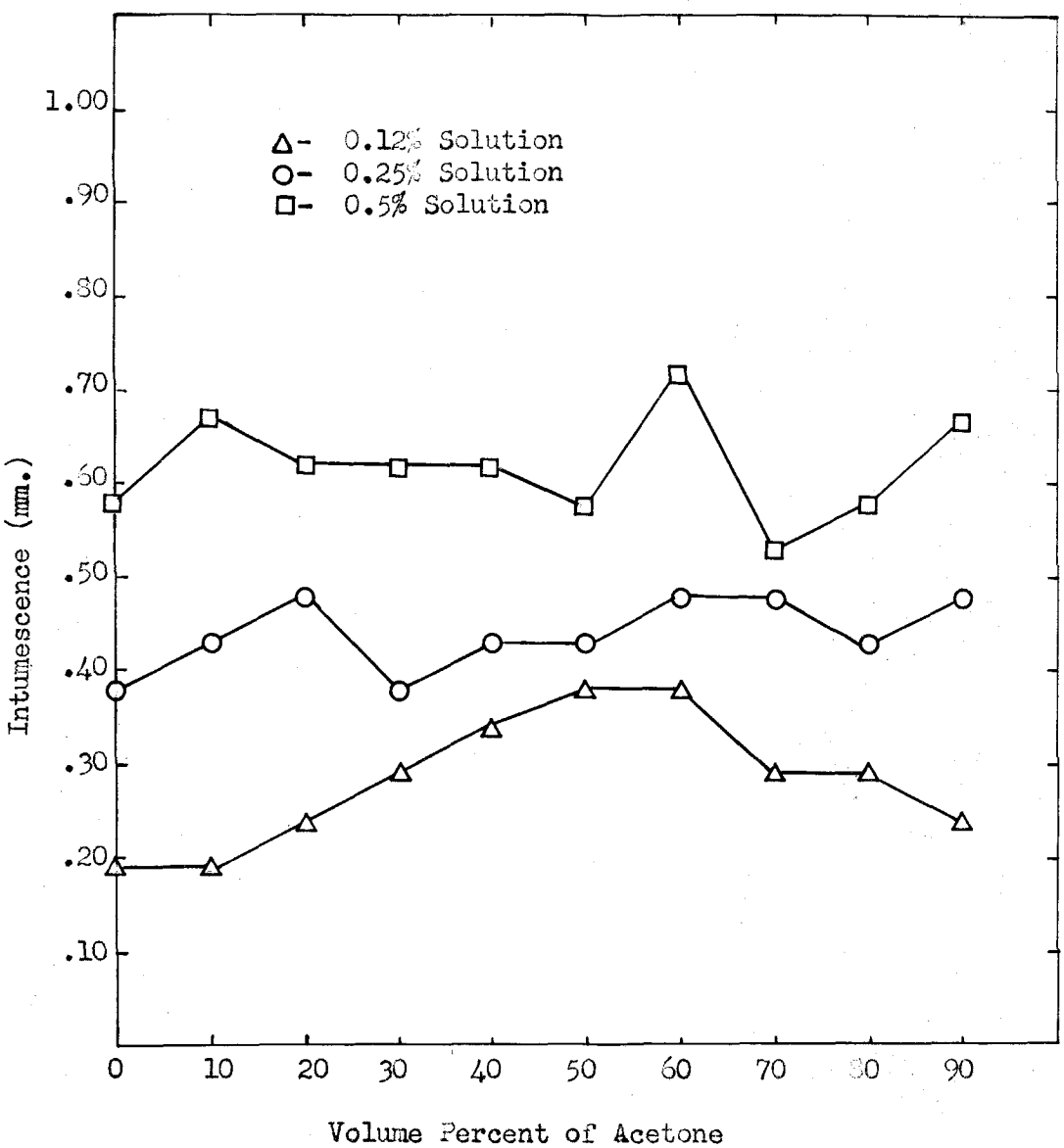


Figure 8. Relative Activity of the Water-Acetone Supernatant at Three Concentrations.

ative to keep the mineral salt concentration to a minimum and Maquenne's procedure utilizing phenylhydrazine and glacial acetic acid permits a subsequent extraction of phenylhydrazine and distillation of acetic acid. According to this method a mixture of 2 parts of phenylhydrazine, 2 parts of glacial acetic acid and 1 part of water, was added to a solution containing approximately 1 part of sugar in 20 parts of water and heated over a boiling water bath for one hour. The yellow to orange-brown precipitate was removed by filtration and the solution made alkaline with barium hydroxide. The small amount of added precipitate was again removed by filtration and the filtrate then continuously extracted with benzene overnight to remove phenylhydrazine. Upon completion of the extraction (disappearance of phenylhydrazine odor) the aqueous solution was made acidic to pH 2 with dilute sulfuric acid, the $BaSO_4$ removed by centrifugation, the supernatant repeatedly evaporated to a small volume after addition of water, and the fraction vacuum-dried after neutralization. The sugar free material, tested in the usual fashion, was found nearly as active as the original infusion (Fig.9). Sugars, therefore, are not involved in the wound response inducing system of lemon peels.

e/ Esterification

Lemon peel infusion was made up to a 9% suspension in absolute ethyl alcohol and refluxed for three hours with 5.3% sulfuric acid (97%). The rest of the procedure was carried out in the manner shown on Chart II. The activity distribution was determined in the usual manner (Fig. 10,11). From the curves in Fig 10 it can be seen that all activity disappeared from fraction A and from the water soluble portion of fraction B as a result of the reaction. On the

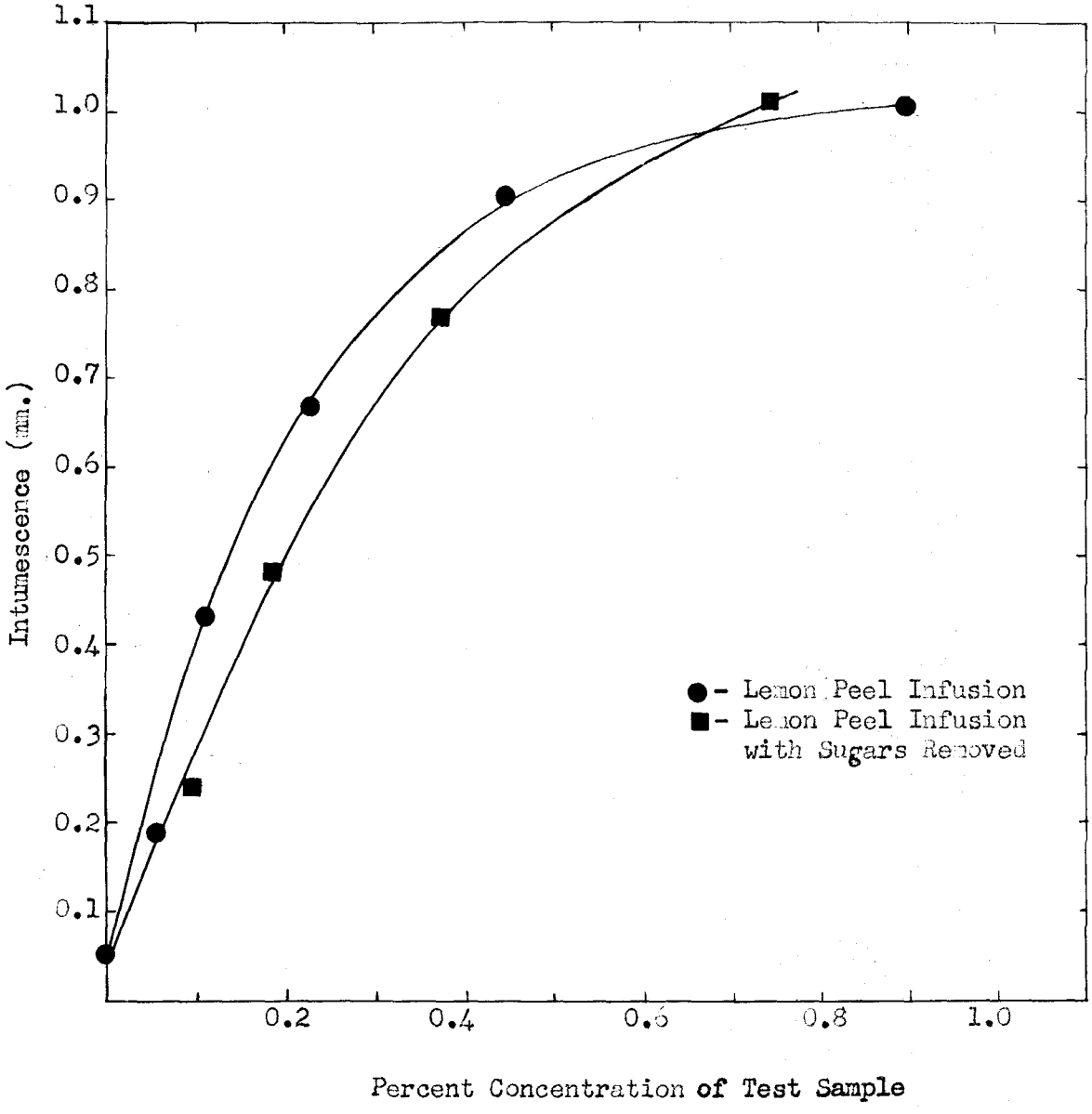
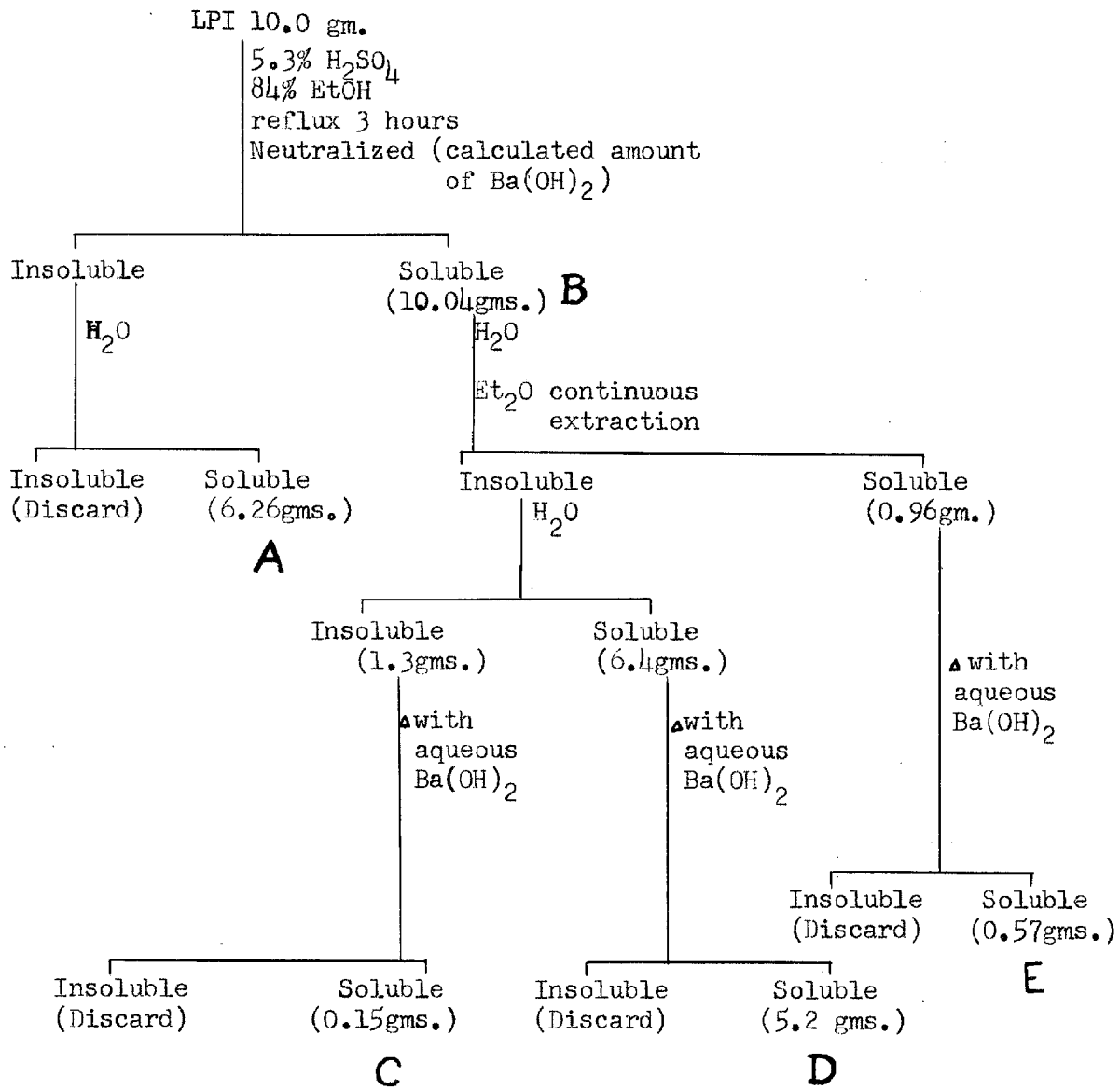


Figure 9. Activity of Lemon Peel Infusion and Infusion with Sugars Removed.

CHART II



Lemon Peel Infusion Reaction with Ethyl Alcohol; sequence of steps to final testing fractions. Barium was removed in the usual manner.

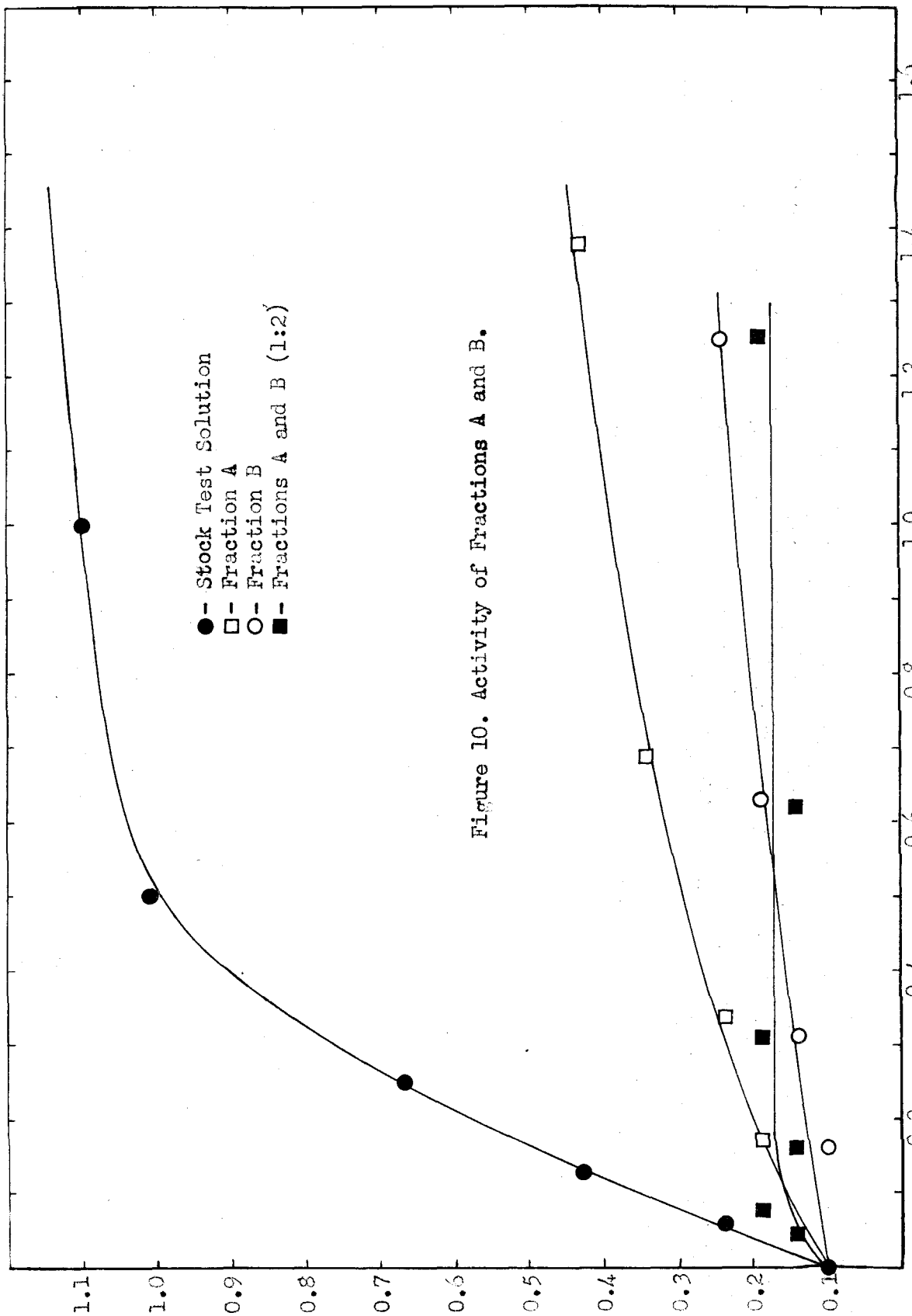


Figure 10. Activity of Fractions A and B.

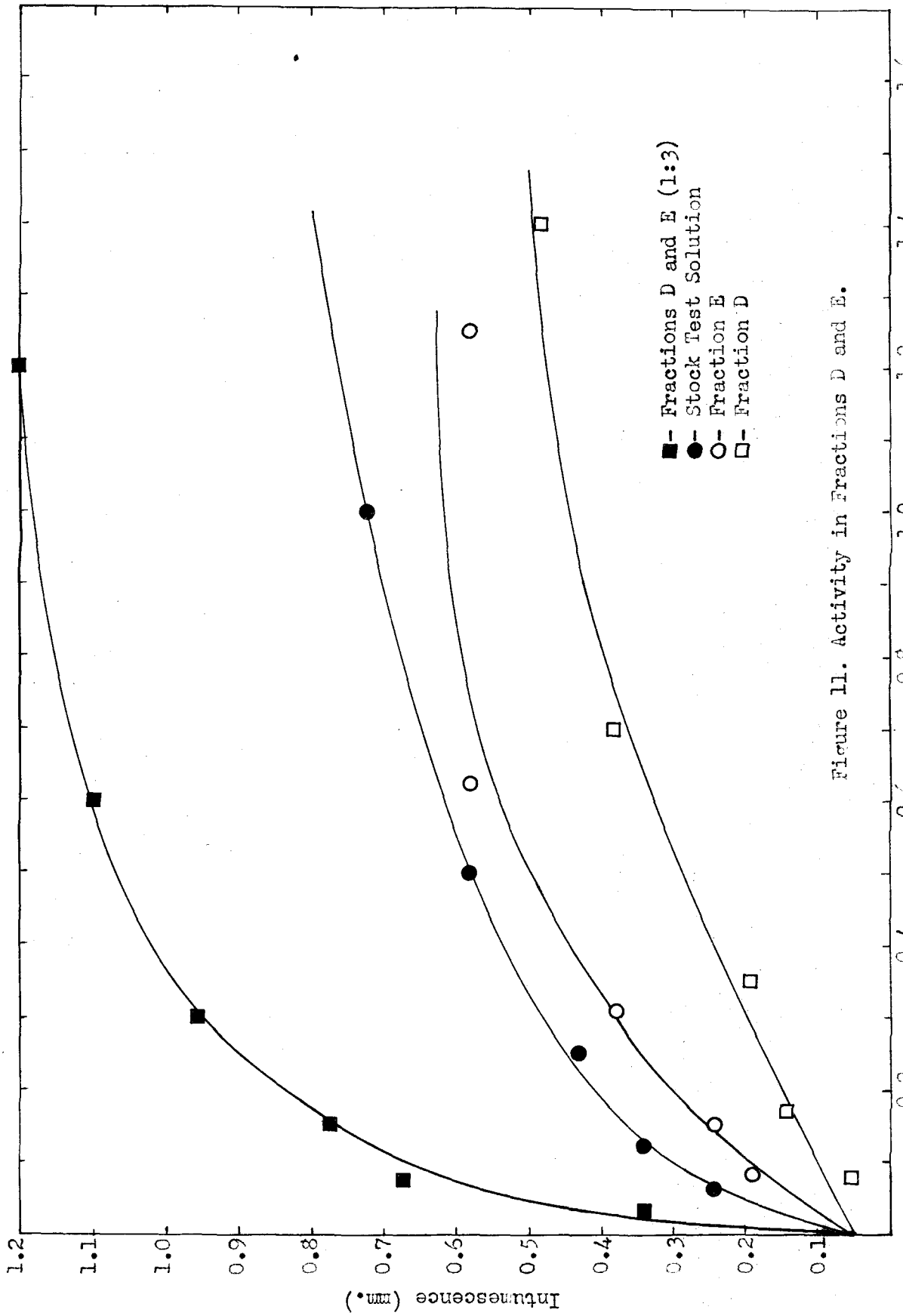


Figure 11. Activity in Fractions D and E.

other hand Fig. 11 shows that on further processing of B, the active components reappear and that they lie in fractions D and E. Recombination of D and E shows a tremendous enhancement of activity over the individual fractions. This evidence provides strong support for the indication from the methanol partition that there are at least two factors involved in the inducement of the wound response. Upon comparison of the curves of recombined D and E (ratio of yields) and the standard infusion there appears to be about a seven fold enrichment (Table 11). But a comparison of the total initial activity of the infusion and that recovered in fractions D and E indicates approximately a 300% increase in the amount of active material in the latter fractions (Table 12). *It appears as if active material were released* by the partition procedure.

TABLE 11

Enrichment of Fraction D and E

Intumescence (mm.)	Concentration (%) LPI*	D plus E	Enrichment
0.45	0.24	0.04	6.0
0.50	0.33	0.045	7.3
0.65	0.70	0.10	7.0
0.80	1.45	0.17	8.5
0.90	1.95	0.25	7.8

f/ Adsorption with Norit -A

Fifteen grams of infusion powder were mixed with 50 ml. of water, treated with 5 grams of Norit -A, filtered with the aid of Hyflo-Supercel and the carbon cake washed until the washings were colorless (filtrate—fraction I'). The filter cake was sub-

* LPI--Lemon Peel Infusion

sequently eluted with 100 ml. of N/2 hydrochloric acid (eluate--fraction II), 100 ml. of absolute methanol (eluate--fraction III) and 100 ml. of mixed solvent, (2.5 ml. concentrated ammonia-density 0.90, 60 ml. acetone and made up to 100 ml. with water) (eluate--fraction IV).

TABLE 12

Comparison of Total Activity of Lemon Peel Infusion and Fraction D plus E at Specific Intumescences.

Intumescence (mm.)	Lemon Peel Infusion Equivalents (in grams) LPI (10.0 gm.)	D plus E (5.70 gm.)
0.45	10	34
0.50	10	40
0.65	10	40
0.80	10	48
0.90	10	45

Fraction I' was resuspended in water (50 ml.) and treated with a second portion of Norite A (5 gm.) and as before filtered with the aid of Hyflo-Supercel and washed (filtrate--fraction I). This time the filter cake was eluted directly with 100 ml. of the mixed solvent (eluate--fraction V). The solvent was removed from each fraction at reduced pressure; neutral test solutions were prepared and activity determinations made (Table 13).

From the data (Table 13) there appears to be a considerable loss of activity (71%) while there is only a small loss of solids (1.8%). On the other hand if one assumes:

a) the presence of at least two different factors,

b) the presence of the water soluble component W in fraction I in a non-limiting concentration, and

c) that the non-limiting concentration of W is achieved by recombination in the ratio of yields,

then it is possible to recalculate the equivalents of activity (Table 14).

TABLE 13

Activity Distribution in the Norite Fractionation.

Fraction	Concentration mg./ml.*	Relative Enrichment	Weight of Fraction (mg.)	LPI Equivalents (in mg.)**
I	23.0	0.074	2490	185
II	2.9	0.59	260	153
III	0.9	1.9	110	210
IV	2.0	0.85	580	490
V	4.5	0.38	1060	400
		Total	4910	1438
LPI***	1.7	1.0	5000	5000

TABLE 14

Activity Distribution in the Norite Fractionation.

Fraction	Concentration mg./ml.*	Relative Enrichment	Weight of Fraction (mg.)	LPI Equivalents (in mg.)**
II	0.97	1.8	260	470
III	0.30	5.7	110	630
IV	0.82	2.1	580	1200
V	1.3	1.3	1060	1380
			Total	3680
LPI	1.7	1.0	5000	5000

The recoverable activity on this basis is about 75%. Here there is further support for the existence of at least two factors involved in the inducement of wound response.

It has been well established that there are at least two factors involved in the inducement of wound response in beans by lemon peel infusion. The water soluble component (factor W) can be separated

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* The concentration of each fraction necessary to give an intumescence of 0.48 mm. in the standard assay.

** The milligrams of Lemon Peel Infusion which would contain the total activity present in each fraction.

*** Lemon Peel Infusion

from the more lipoid soluble component (factor L) by treatment with Norite. Factor L is adsorbed on the Norite while factor W appears in the filtrate. In the subsequent purification of factor L the filtrate factor W will have been added to the testing solutions unless other fractions are specified.

g/ Chromatography on Anion Exchange Resin

The results obtained from the esterification indicated that the active material was acidic. Accordingly, chromatography on freshly regenerated anion exchange resin (3/4" by 6 1/4" column, Amberlite IR 400) was investigated. One gram of infusion powder (10% suspension) was put on the column and washed with distilled water; the solvent was forced through the column by maintaining a reduced pressure at the outlet. By the time 130 ml. were collected (fraction I, 300 mg.) the eluate was neutral to indicator paper; at this time the solvent was changed to 0.06 N hydrochloric acid. The eluate was collected in one fraction (II, 80 ml., 400mg.) till 20 ml. of chloride-containing orange colored solvent came through. The solvent was changed again to water and the eluate collected till it became chloride free (fraction III, 80 ml., 300 mg.). Then an additional 60 ml. was collected that was chloride-free (fraction IV, 30 mg.). The four fractions were dried under reduced pressure and the neutral test solutions assayed (Fig. 12). Fraction I is inactive by itself but does possess the properties of factor W when combined with the individually more active fraction II. Fractions III and IV are comparable in activity to the stock test solution. This separation provides additional evidence for the presence of several factors.

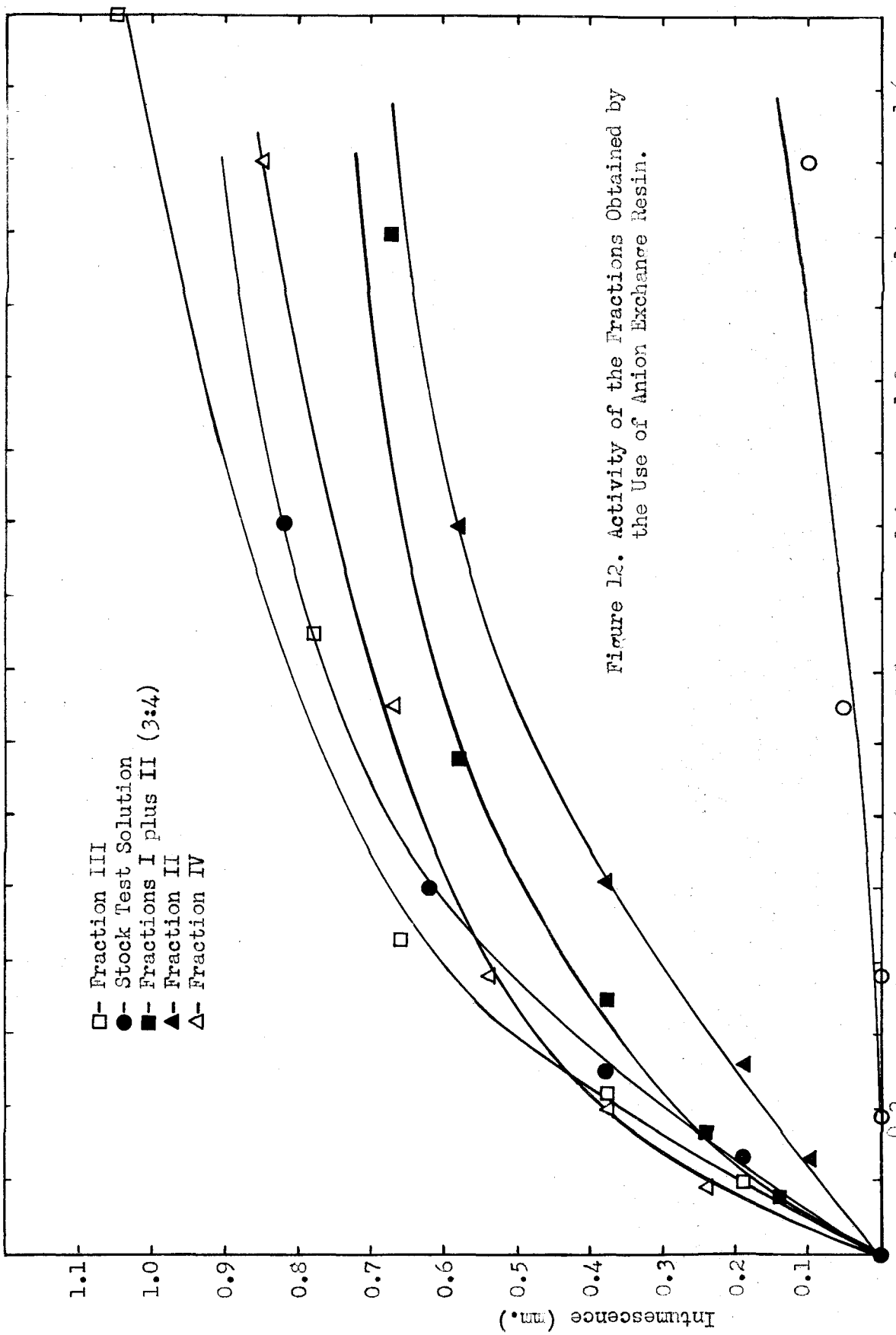

Figure 12. Activity of the Fractions Obtained by the Use of Anion Exchange Resin.

3/ Purification of the Methanol Soluble Fraction of Lemon Peel Infusion

a/ Solvent Extraction

1/ Diethyl Ether

A 6% solution of the methanol soluble material in dilute sulfuric acid (2.8%) extracted continuously with ether yielded 6% of soluble material. The results of tests comparing the relative activities of the methanol soluble and ether soluble fractions with methanol insoluble matter (ratio of yields) indicated no satisfactory separation (Fig. 13).

2/ Amyl Alcohol

A 10% suspension in n-amyl alcohol was shaken at room temperature on a shaker apparatus overnight. Only a small amount, 0.5%, was soluble under these conditions and tests showed that essentially no separation had taken place.

b/ Norite Adsorption

An aqueous solution of the methanol soluble material was treated with Norite A and then filtered with the aid of Hyflo-Supercel. The filter cake, after washing with water, was eluted with 1 N aqueous ammonia. The two fractions were then dried under reduced pressure and weighed; 70% of the starting material was recovered. Only 10% of the starting material was found in the eluate. Tests (Fig. 14) showed that in combination with the methanol insoluble material (ratio of yields) the adsorbate (1:8) was as active as the original methanol soluble material (1:1). The eluate contained 10% of the methanol soluble material with 80% of its activity.

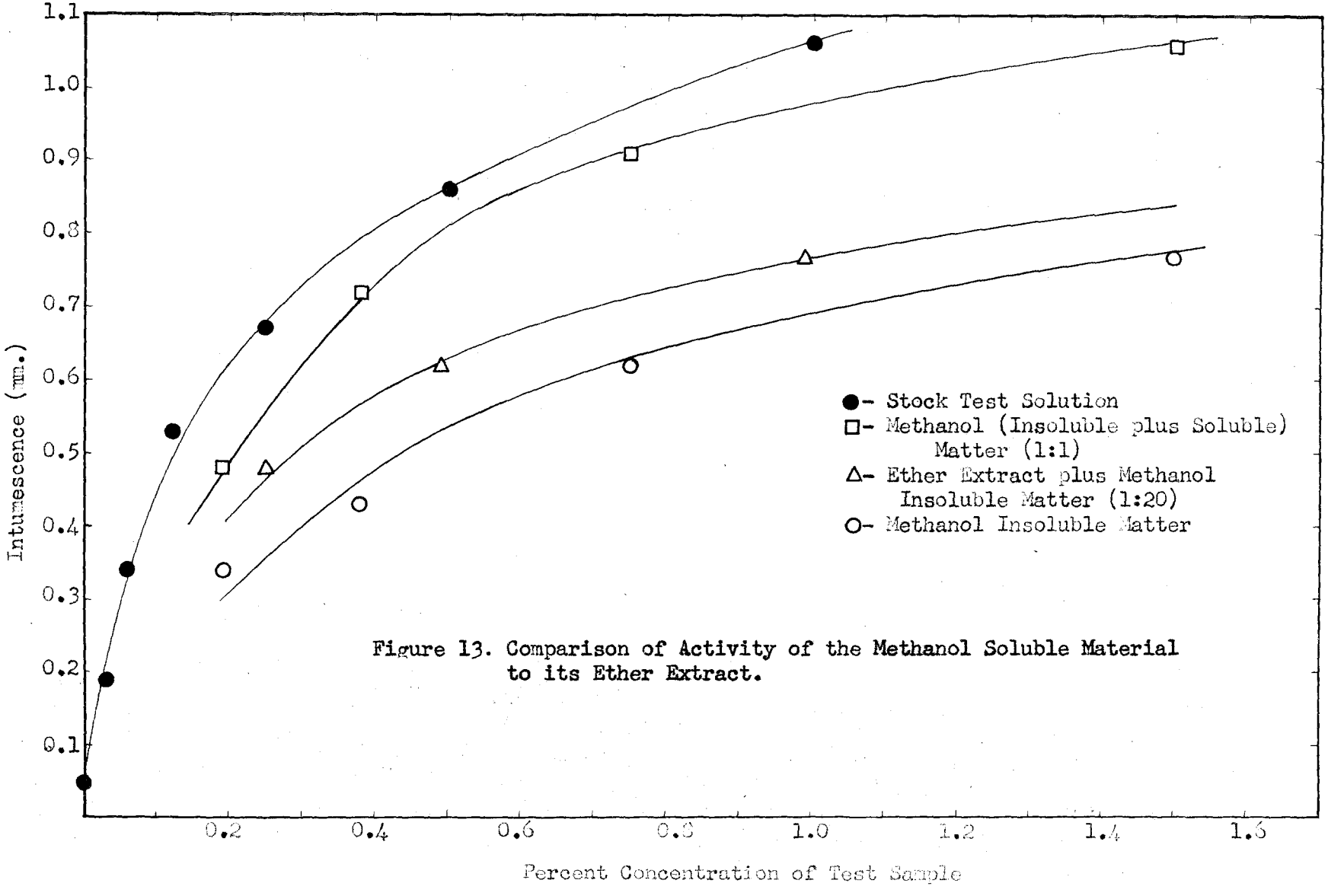

Figure 13. Comparison of Activity of the Methanol Soluble Material to its Ether Extract.

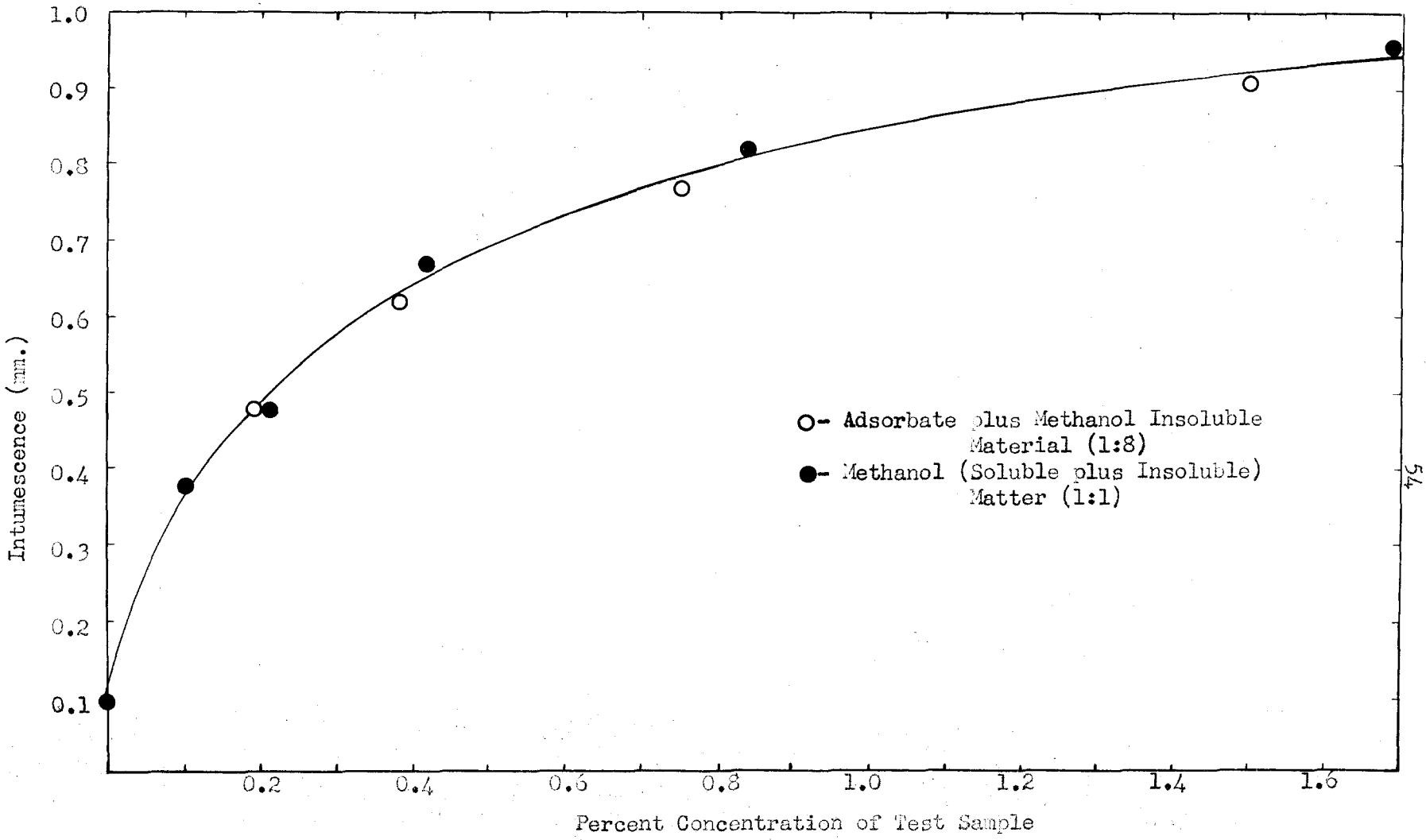

Figure 14. Activity of the Fractions Obtained in Norite Adsorption of the Methanol Extract.

4/ Purification of the Norite Adsorbate of Lemon Peel Infusion

a/ Lead Precipitation

A 9% aqueous solution of the Norite adsorbate was treated with excess basic lead acetate (saturated solution) and the resulting precipitate removed by centrifugation. Regeneration of each fraction with concomitant precipitation of lead was accomplished in the usual manner. The weighing of the dried (at reduced pressure) fractions showed that 55% of the original material was lead precipitable. Activity curves (Fig. 15) indicated that the active component is largely in the precipitate but no satisfactory separation of factors was accomplished.

b/ Solvent Extraction

1/ Ethyl Acetate

The Norite adsorbate was made up to a 9% solution just acid to Congo Red (pH 3) and extracted with ethyl acetate for 24 hours in a continuous liquid-liquid extractor. On removing the solvent from the two fractions 23% of the adsorbate was found in the ethyl acetate extract. Tests showed that the extract was about twice as active on a dry weight basis as the original adsorbate and nearly four times as active when both fractions were tested in combination with the filtrate factor W (Fig. 16).

a/ Chloroform

The ethyl acetate soluble material was dissolved in an acidic aqueous solution (pH 3) to form a 5% solution then extracted continuously for 24 hours with chloroform. The chloroform extract (20% of the ester soluble material), in combination with

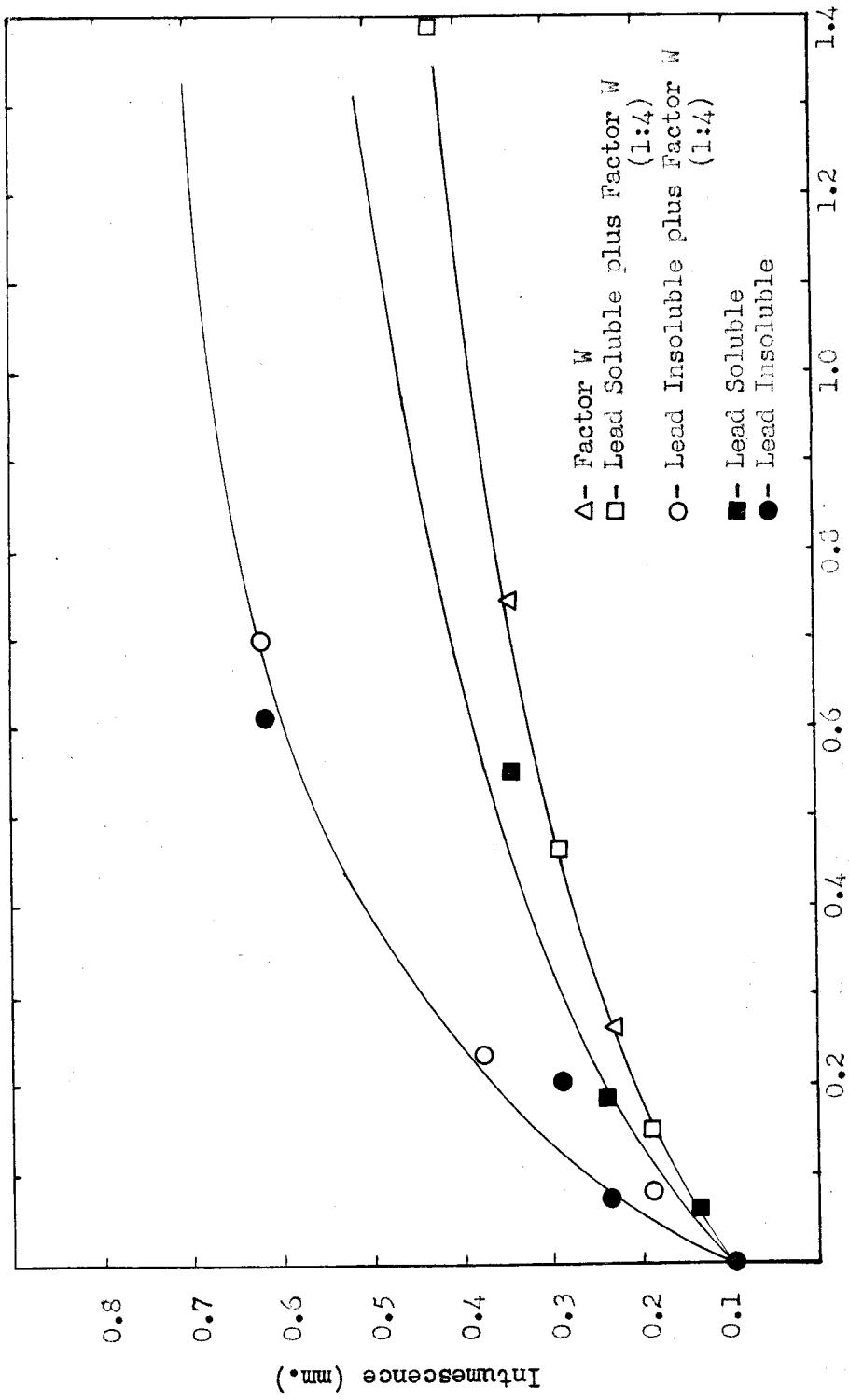

Figure 15. Activity of the Fractions Obtained by Basic Lead Precipitation.

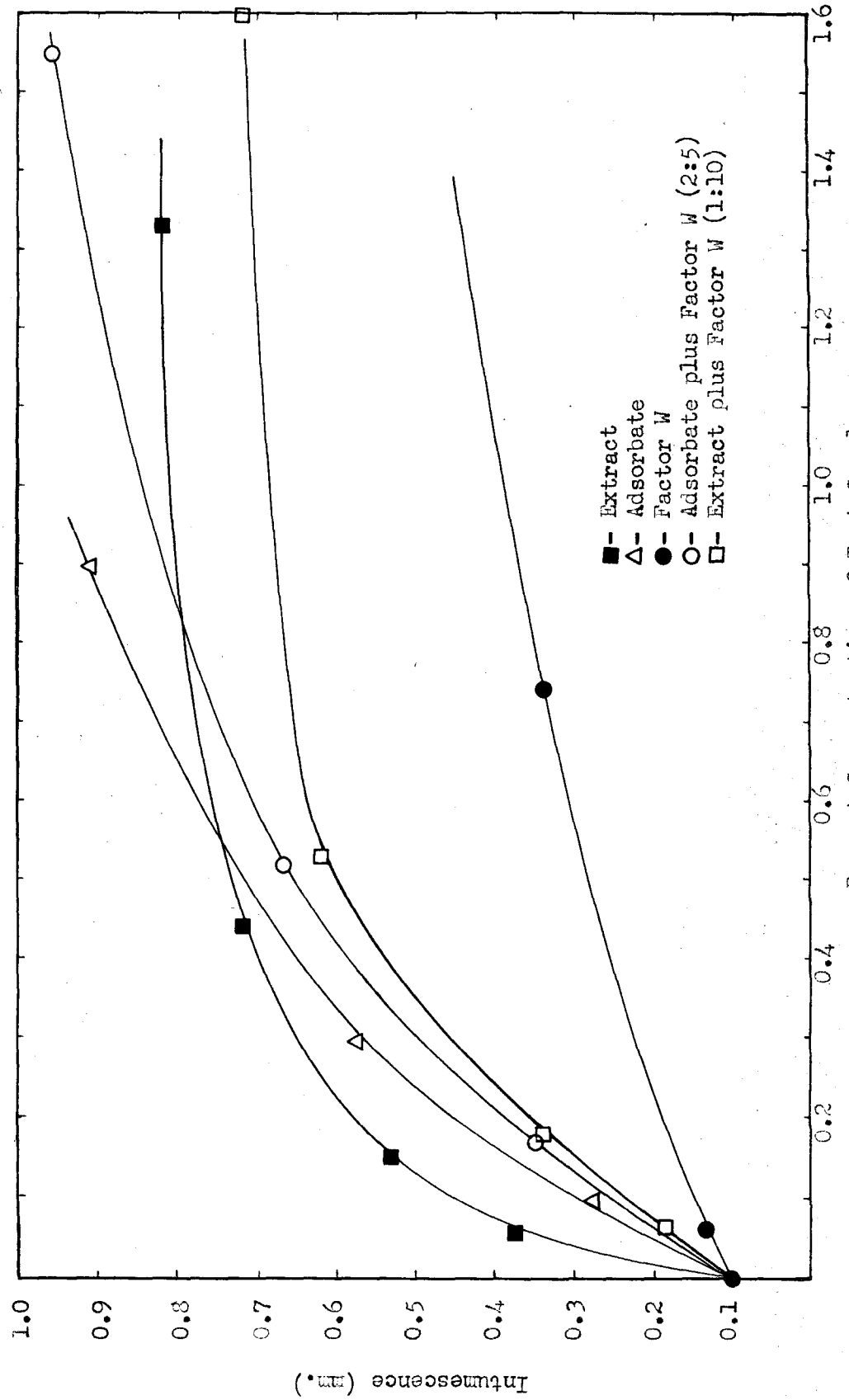

Figure 16. Activity of the Fractions Obtained by Ethyl Acetate Extraction of the Norite Adsorbate.

Norite filtrate (W) in a ratio of 1:20. has the same growth effect as the Norite adsorbate and filtrate (W) in the ratio of 1:25 (Fig. 17). This indicates that there is an 8 fold enrichment of the lipoid soluble factor L from the adsorbate and a 2 fold enrichment over the ethyl acetate extract.

The partition of the ester soluble matter achieved with chloroform suggested a direct extraction of lemon peel infusion. When a continuous chloroform extraction was carried out on a 15% aqueous solution of lemon peel infusion (pH 3), 3% of the material was extracted within 24 hours. The active material was mainly in the chloroform soluble fraction (Fig. 18). Recombination according to yields gave an activity comparable to the original infusion. This partition procedure has been by far the most selective; a 30 fold enrichment of an easily recoverable lipoid soluble factor was obtained in one simple operation.

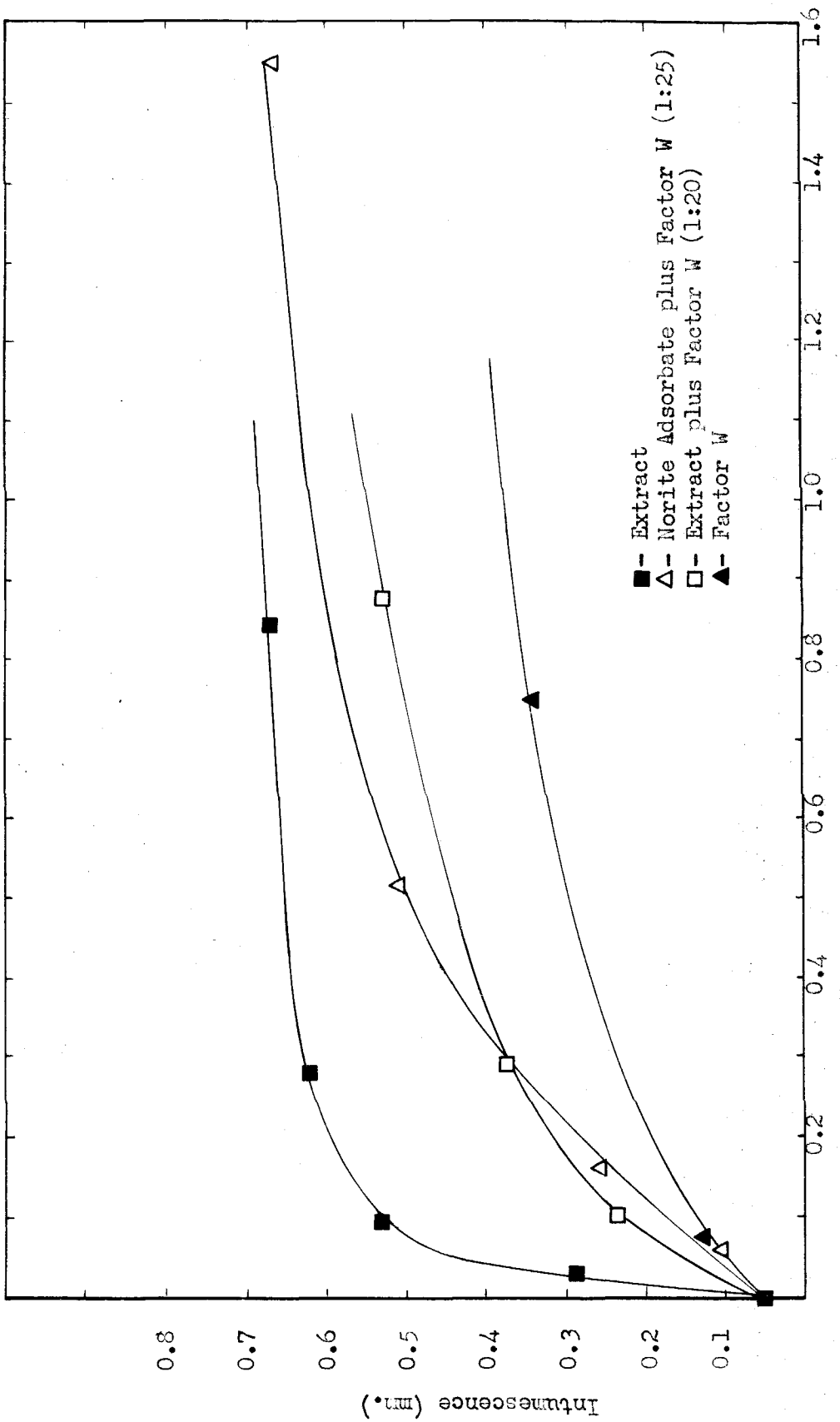

Figure 17. Activity of the Fractions Obtained by Chloroform Extraction of the Ethyl Acetate soluble Portion of the Norite Adsorbate.

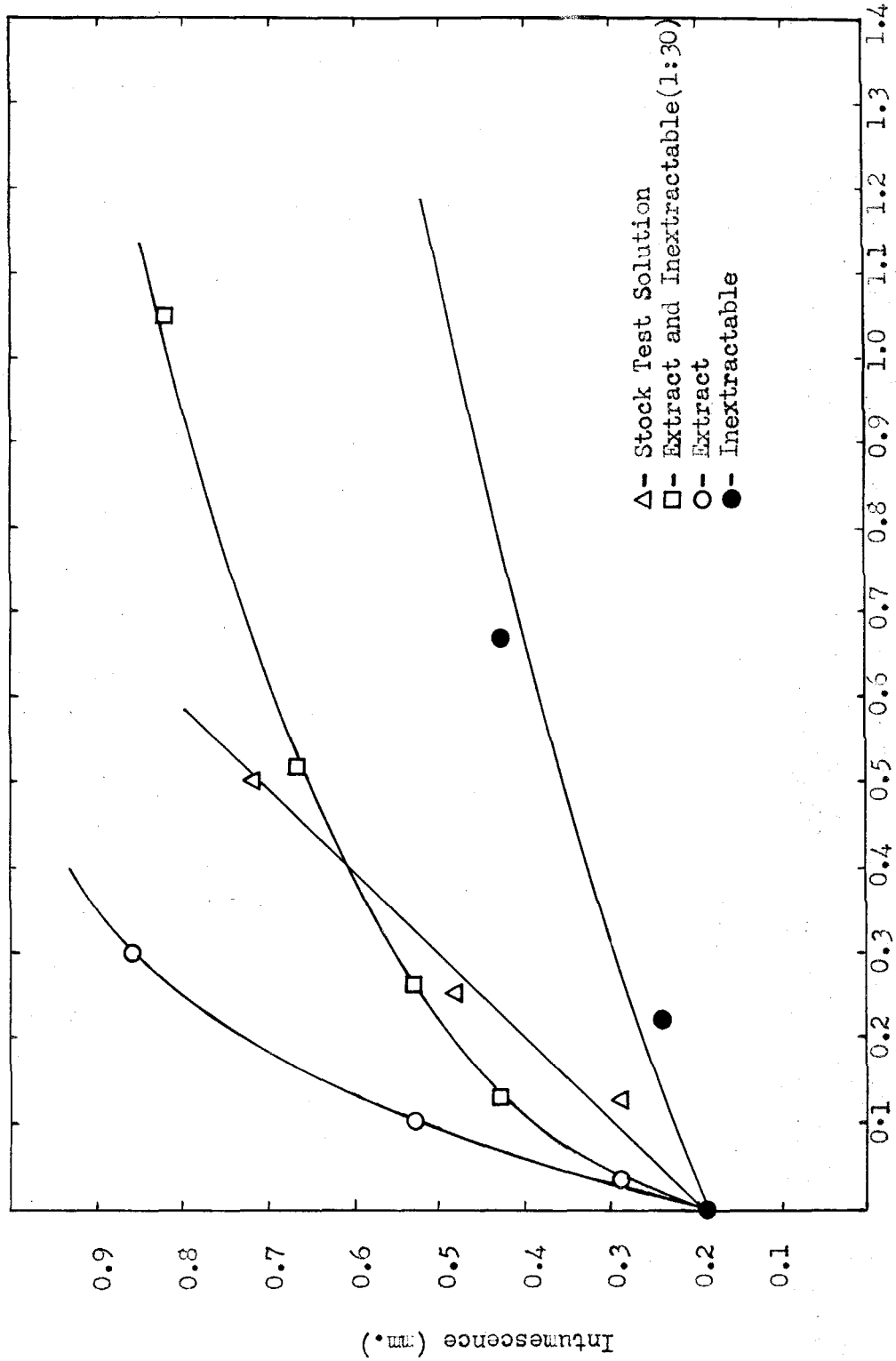

Figure 18. Activity of the Fractions Obtained by Chloroform Extraction of Lemon Peel Infusion.

PART VI

PURIFICATION OF THE LIPOID SOLUBLE HORMONE OF LEMON PEEL INFUSION

Preliminary investigations had shown that in a continuous chloroform extraction of an aqueous acidic solution of lemon peel infusion, the lipoid soluble factor could be separated from the water soluble factor with great selectivity. It was therefore decided to use this procedure on larger quantities of starting material. One hundred grams of lemon peel infusion was suspended in 500 ml. of water, the insoluble matter centrifuged and the pH of the solution adjusted to 3 with N sulfuric acid. The acidified solution extracted continuously for 3 days yielded 2% extractable material. The separation and enrichment of the two fractions were similar to those obtained in the small scale operation, and the chloroform extraction was therefore adopted as a first step in the larger scale purification process.

A) ENHANCING EFFECT OF FACTOR W

Now that the active system or systems inducing wound response in beans have been separated into 2 components, a lipoid soluble factor and[8] water soluble factor, each of which has been enriched, it becomes necessary to evaluate a criterion for recombination. The original criterion, recombination according to yields, was adopted primarily because it tended to re-establish the proportions present in the original lemon infusion. With continued purification the criterion would become more unwieldy and unreliable. To establish a recombination system on a firmer basis, a series of activity tests were

carried out with varying ratios of components (Chloroform extract, L, and Norite filtrate, W). From the plots (Fig. 19) it was evident that the concentration at which the enhancing effect of factor W is maximum is dependent upon the concentration of factor L. For practical considerations an intermediate concentration (somewhat arbitrary, 0.4%) of factor W was selected to be kept constant in tests on concentration series of factor L.

B) TREATMENT WITH BARIUM AND LEAD

Aqueous solutions of the chloroform extract (0.8%) capable of inducing strong wound responses in beans were treated with saturated solutions of barium and lead acetate; no precipitation was observed.

C) SOLVENT EXTRACTION

Crude chloroform extractable material was extracted successively with petroleum ether (60-70°C.), chloroform, acetone and water in the following manner: 1.44 grams of dark material was dissolved completely in a mixture of 50 ml. of chloroform and 5 ml. of acetone to give a dark reddish brown solution. To this solution was added a 100 ml. portion of purified petroleum ether (60-70°C.) and as insoluble matter separated from solution the mixture was evaporated to a smaller volume. More petroleum ether was added and the mixture evaporated again to a smaller volume. The process was repeated several times until the chloroform and acetone were removed. At this point the insoluble material was separated from 50 ml. of pale yellow petroleum ether solution by centrifugation and redissolved in a chloroform-acetone mixture and the previous procedure repeated to obtain two additional petroleum ether fractions. The insoluble material from the petroleum ether partition was again dissolved in a chloroform-acetone

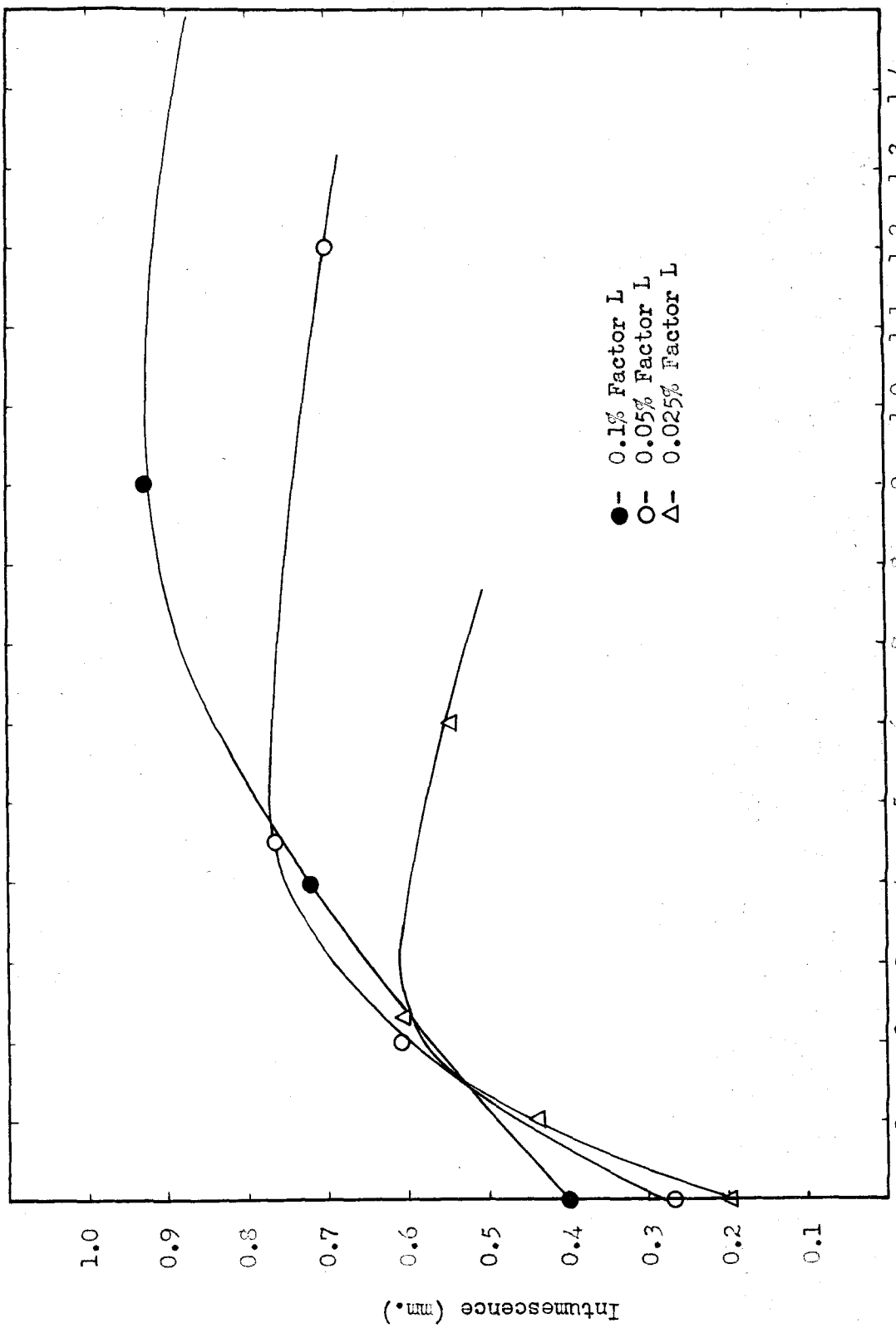

Figure 19. Enhancing Effect of Increasing Concentration of Factor W on Different Concentrations of Factor L.

solvent then, as before with petroleum ether, repeatedly evaporated in the presence of chloroform to obtain 3 chloroform fractions. The insoluble material from the chloroform fractionation was dissolved in an acetone-water mixture and the previous process repeated to obtain 3 acetone soluble fractions, then 3 water soluble fractions and insoluble residue (Table 15).

TABLE 15
Solvent Fractionation of 1.44 grams of
Crude Chloroform Extract

Solvent	Petroleum Ether	Chloroform	Acetone	Water	Residue	Total
Soluble Material (grams)	0.46	0.80	0.03	0.10	0.10	1.49
Nature of fraction	Yellow solid	Yellow sirup	Dark solid	Dark solid	Dark solid	

Tests in the presence of factor W showed that the residue, acetone soluble and water soluble fractions were inactive, that the petroleum ether fraction was somewhat active and that the chloroform fraction was very active.

D) HIGH VACUUM DISTILLATION

A preliminary experiment had shown that the active chloroform fraction obtained from the previous solvent partition procedure could be distilled in high vacuum. Both the petroleum ether and the chloroform fractions were therefore distilled in high vacuum. Petroleum ether soluble material (1.41 gm.) was distilled at a pressure of less than 10 μ. At wax bath temperatures, 90-110°C., a yellowish material sublimed and was collected separately (P_a = 0.27 gm.). From 150-170°C. (bath temperature) a tannish material distilled that

solidified upon cooling (P_b= 0.49 gm.). Since no more material distilled above this temperature, the distillation was discontinued. The residue was a gray sirupy material insoluble in water, soluble in ether (P_r= 0.56 gm.), insoluble in ether (0.07 gm.).

Chloroform soluble material (5.27 gm.) was distilled in the same manner. At bath temperatures of 100-125°C., an orange colored viscous oil distilled (C_a= 1.13 gm.). From 190-220°C. (bath temperature) a reddish brown oil distilled (C_b= 0.98 gm.). The dark colored residue which was soluble in acetone was collected (C_r= 2.38 gm.) while the remaining material (0.78 gm.), insoluble in any of the usual solvents, was discarded.

There is some active material in the low boiling distillate of the petroleum ether fraction but the major portion of the activity is in the high boiling distillate of the chloroform fraction (Fig. 20). The relative activities of the distillate fractions are shown in Table 16.

TABLE 16

Comparison of Activities of the Distillates of Petroleum Ether and Chloroform Extracts.

		Petroleum Ether		Chloroform
	LPI	Low Boiling	High Boiling	High Boiling (C_b)
Weight of fraction (gm.)	400	0.27	0.49	0.98
Concentration* (mg./ml.)	1.3	0.007	0.055	0.004
Relative enrichment	1.0	180	24	325
LPI equivalents** (in mg.)	400,000	49,000	12,000	320,000

* The concentration of each fraction necessary to give intumescence enhancement of 0.24 mm. in the standard assay with factor W.

** The milligrams of LPI which would contain the activity present in the fraction.

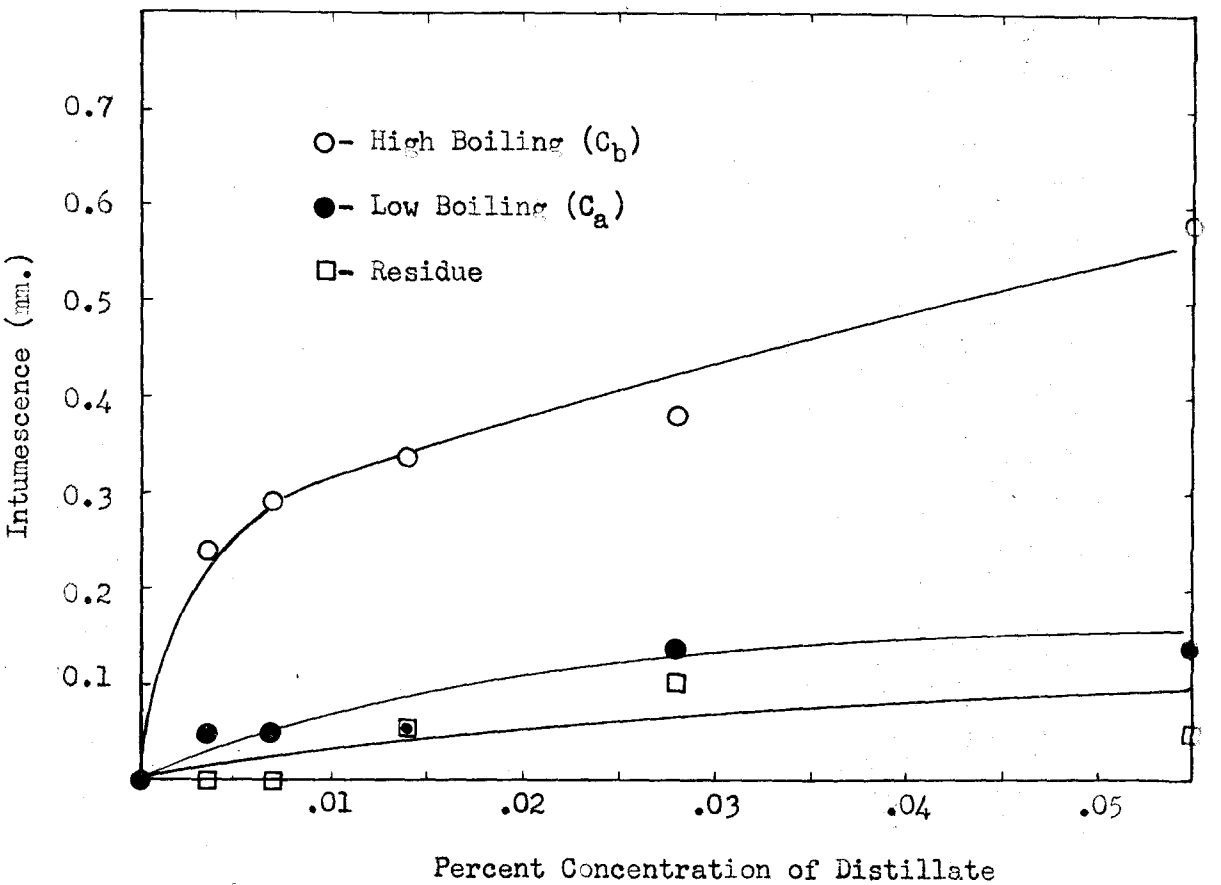

Figure 20. Activity of the Distillates Obtained from the Chloroform Fraction.
(Corrected for Factor W Activity)

E) LOW BOILING PETROLEUM ETHER FRACTION

It was observed that in the distillation of the petroleum ether fraction, the low boiling component which tended to sublime below a bath temperature of 100°C., distilled at bath temperatures of 100°-120°C. and that the distillate solidified upon cooling. The crude orange colored material was dissolved in warm petroleum ether and the substance which separated on cooling was recrystallized several times to give white crystals, (m.p. 120°-122°C.), identified with benzoic acid. Neither the purified crystals nor benzoic acid were active upon testing with factor W. Subsequent tests showed that the small amount of activity present in the crude fraction remained in the mother liquor.

F) SEPARATION OF THE ACIDIC CONSTITUENTS OF THE HIGH BOILING CHLOROFORM FRACTION (C_b)

The conditions of the initial chloroform extraction of lemon peel infusion required the active principle to be either acidic or neutral. Consequently extraction of fraction C_b with solvents of varying alkalinity could likely effect a desirable partition. Accordingly this high boiling fraction (0.94 gm.) was dissolved in 3.0 ml. of chloroform and extracted with 10 ml. of 7% sodium bicarbonate in 1-2 ml. portions, with 10 ml. of 10% sodium carbonate in 1-2 ml. portions and finally with 10 ml. of 5% sodium hydroxide in 1-2 ml. portions. Each aqueous fraction was then acidified with 1 N H_2SO_4 and repeatedly extracted with chloroform; the organic solvent was removed at reduced pressure and the fractions tested. The major portion of the activity appeared in the bicarbonate fraction (Table 17, Fig. 21).

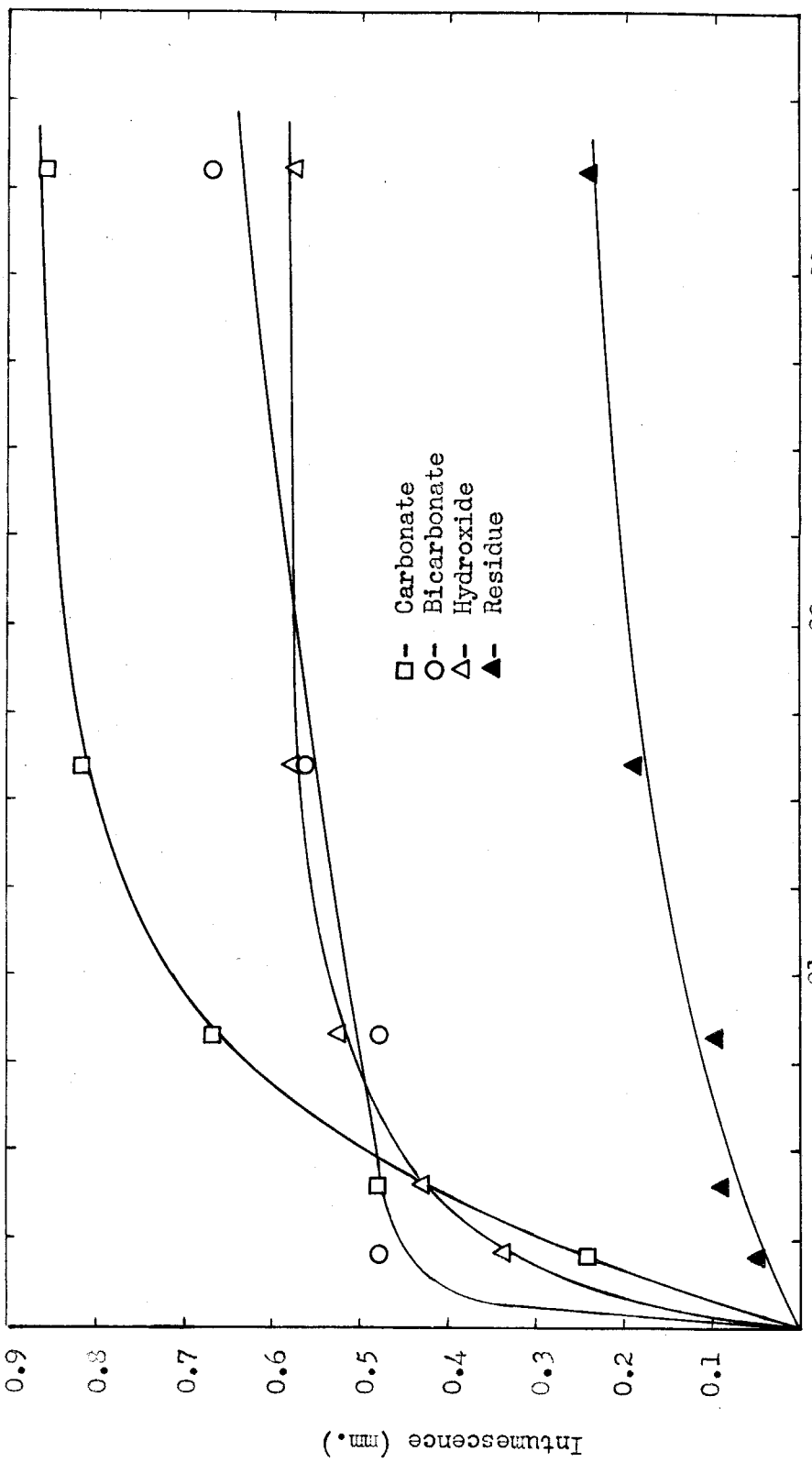

Figure 21. Activity of the Alkaline Extracts of C_b.
(Corrected for Factor W Activity)

TABLE 17

Distribution of Components Effected by Aqueous Alkaline Extraction of 0.94 grams of C_b

Extracting Agent	$NaHCO_3$	Na_2CO_3	NaOH	Alkali Inextractable	Loss
Material Extracted (gm.)	0.41	0.045	0.088	0.138	0.27
Character of Extract	Reddish brown oil	Reddish brown oil	Dark red brown oil	Orange oil	

1/ Aqueous Extraction of the Bicarbonate Fraction

The bicarbonate fraction separated in the previous procedure contains, in addition to bicarbonate soluble acids, water soluble acids and neutral substances. In an effort to separate the latter two components, a chloroform solution of the bicarbonate soluble fraction (0.37 gm. in 2 ml. of chloroform) was extracted with 50 ml. of water in 1-2 ml. portions. The chloroform residue (A 1 = 0.17 gm.) was set aside while the aqueous fraction was made just alkaline and evaporated at reduced pressure to a small volume (about 8 ml.). At this point some material (A 2) separated from solution. The solution upon acidification became cloudy and on centrifugation a trace of reddish brown sirup (A 3) separated. The clear acidic solution was then extracted with 100 ml. of chloroform in 5-10 ml. portions. After the solvent was removed, the chloroform extract (A 4) weighed 0.18 gm. Testing the fractions in presence of factor W showed the recoverable activity to be nearly equally dispersed between the original inextractable residue (A 1) and the water extractable fraction (A 4), (Fig. 22).

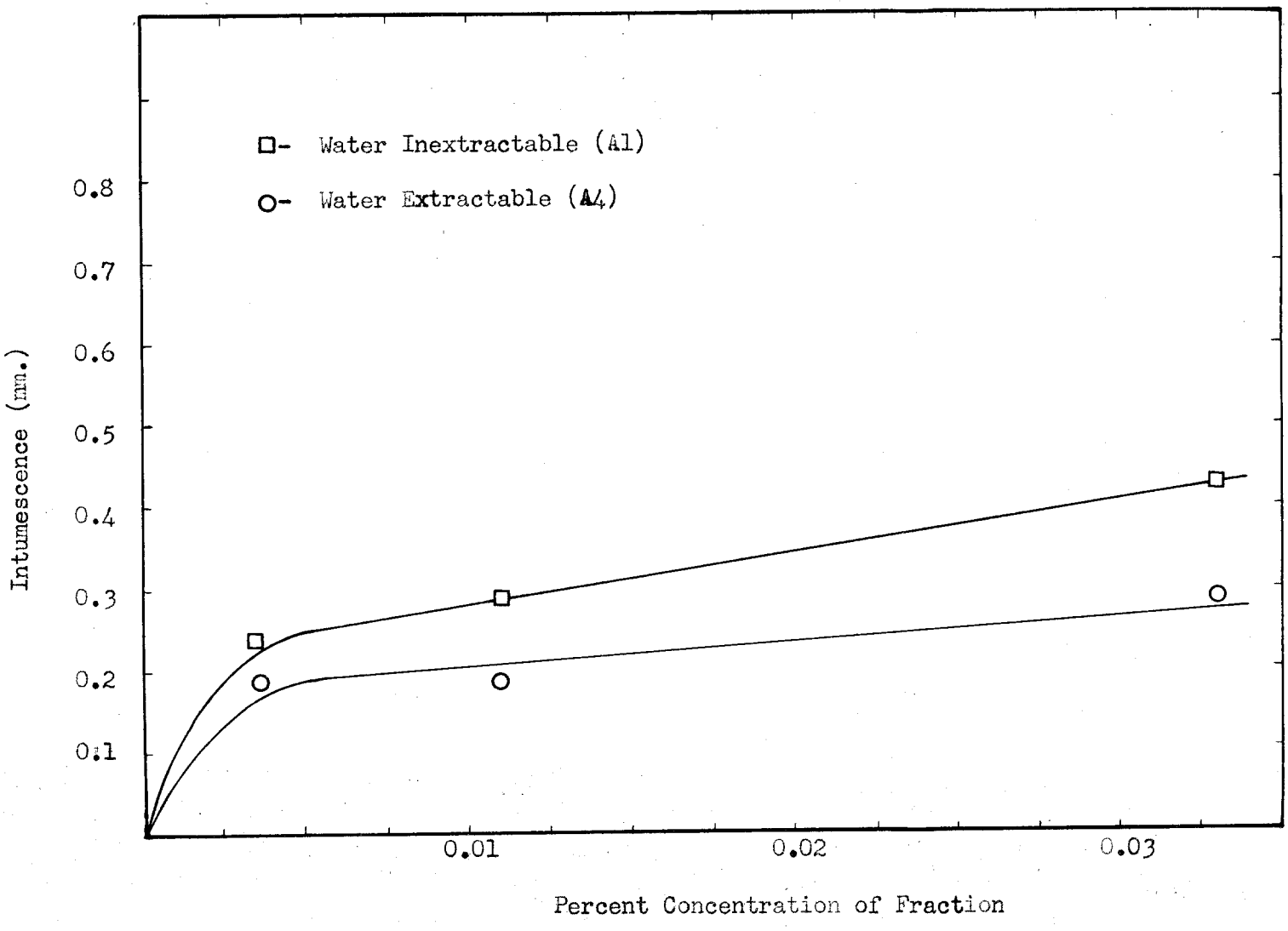

Figure 22. Activity of the Fractions Obtained by Water Extraction of the Bicarbonate Soluble Fraction of C_b.
(Corrected for Factor W Activity)

2/ Titration of Bicarbonate Fraction

Upon titration (Fig. 23 Titration Curve) a clear point of inflection appeared at pH 7.0 where 186 microequivalents of alkali had been used. With these data an average equivalent weight of 156 for the material was calculated.

3/ Hydrogenation

A sample of this orange colored material was dissolved in a water-methanol mixture and hydrogenated catalytically (H_2 plus Pt) to a colorless solution in 4 hours at room temperature. The hydrogenated product was found as active as the unhydrogenated material when tested with factor W.

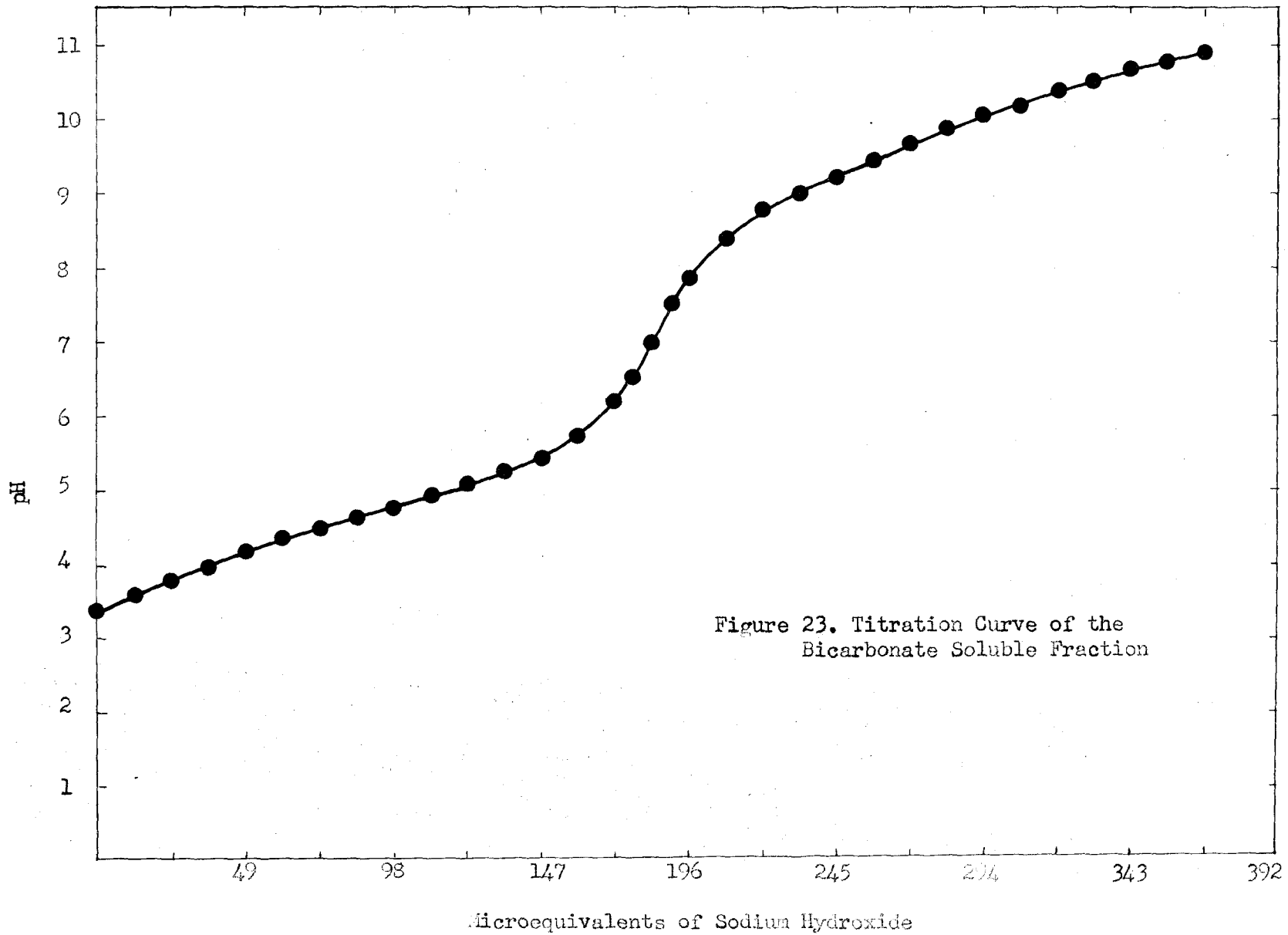

Figure 23. Titration Curve of the Bicarbonate Soluble Fraction

PART VII

LIPOID SOLUBLE FACTORS OF PRESSED OIL OF THE VALENCIA ORANGE

The high activity of the extracts from the orange peel* suggested that the lipoid soluble factors found in the peel extract should also be in the pressed oil. This was confirmed by extracting the orange oil with aqueous alkali, wherein the active material was accumulated in the acid fraction.

A) FRACTIONAL DISTILLATION

The orange oil was distilled at aspirator pressure over a boiling water bath till the major constituent, limonene (90%), was removed (21). The remaining 10 % of the oil was then distilled in high vacuum. Each fraction was extracted with 10 ml. of 10% sodium hydroxide in 2 ml. portions; the alkaline extract in turn was washed with 5 ml. of ether in 1-2 ml. portions before acidification and extraction with 100 ml. of ether in 5-10 ml. portions. The solvent from each ether extract was removed under reduced pressure; then the residual matter was weighed and tested in combination with factor W. The major portion of the activity was found in the high boiling fraction (Table 18).

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* See source material, this thesis.

TABLE 18

Distribution of Material and Activity Upon Distillation and Alkaline Extraction of 250 gm. of Orange Oil

Fraction	A-I	A-II	A-III	A-IV
Distilling Temp. C°	80-82	48-75	to 180	----
Vapor Pressure	20 mm.	100 μ	10 μ	----
Yield (grams)	221	13.0	5.7	10.0
Alkali Extract (gm.)	0.056	0.21	2.69	0.084
Concentration (mg./ml.)*	0.33	0.22	0.11	0.33
LPI Equivalents (in mg.)**	220	1200	31,800	330

B) SEPARATION OF THE ACIDIC CONSTITUENTS OF THE HIGH BOILING FRACTION OF ORANGE OIL (A-III).

Fraction A-III (2.65 gm.) containing most of the activity was dissolved in ether (100 ml.) and extracted successively with aqueous solutions of 7.5% sodium bicarbonate (30 ml.), 10% sodium carbonate (25 ml.) and 10% sodium hydroxide (10 ml.). Each fraction was then acidified and re-extracted with ether, the ether removed from the extracts and the fractions tested with factor W (Table 19). The major portion of the activity is bicarbonate soluble.

TABLE 19

Distribution of Material and Activity of High Boiling Fraction A-III of Orange Oil Upon Alkaline Extraction.

Extracting Medium	$NaHCO_3$	Na_2CO_3	NaOH	Residue	A-III
Weight of Extract (gm.)	1.13	0.37	0.10	0.81	2.65
Concentration (mg./ml.)*	0.091	0.17	0.091	0.33	0.11
LPI Equivalents (in mg.)**	16,000	2,800	1,440	3,250	31,000

* Concentration of Fraction to enhance intumescence 0.34 mm. with water soluble factor W. LPI requires 1.3 mg./ml.

** Milligrams of LPI containing the activity present in this fraction.

C) CRYSTALLIZATION OF THE BICARBONATE FRACTION

The orange colored bicarbonate extractable oil upon mixing with a 100 fold volume of petroleum ether (60-70°C.) had a cloudy appearance. On standing overnight in a refrigerator (4°C.) an orange gummy material settled out. The supernatant after evaporation at reduced pressure, gave a clear yellow oil containing all of the activity. The oil was then dissolved in a 2 fold volume of methyl alcohol and cooled to 4°C.; crystallization set in overnight. The crystals formed were separated by filtration. The oil obtained by evaporation of solvent from the filtrate was dissolved in a 2 fold volume of petroleum ether (60-70°C.) then cooled to -7°C. with an ice-salt bath to give an additional crop of crystals. These crystals were filtered off as before and the solvent removed from the filtrate under reduced pressure. Upon testing with factor W in the usual fashion it was found that the crystals from methanol were inactive, those from petroleum ether moderately active and the mother liquor very active (Fig. 24).

1/ Characterization of the Crystalline Material

In view of the distribution of activity resulting from the methyl alcohol and petroleum ether crystallizations, the entire bicarbonate fraction, obtained in the manner previously described, from one gallon of orange oil, was subjected to repeated crystallization from methanol. The purified crystals were then submitted for analysis (Table 20). Elemental analysis and neutralization equivalent determination indicated a mixture of fatty acids with palmitic predominating. Assay showed that neither the crystals nor palmitic acid were active.

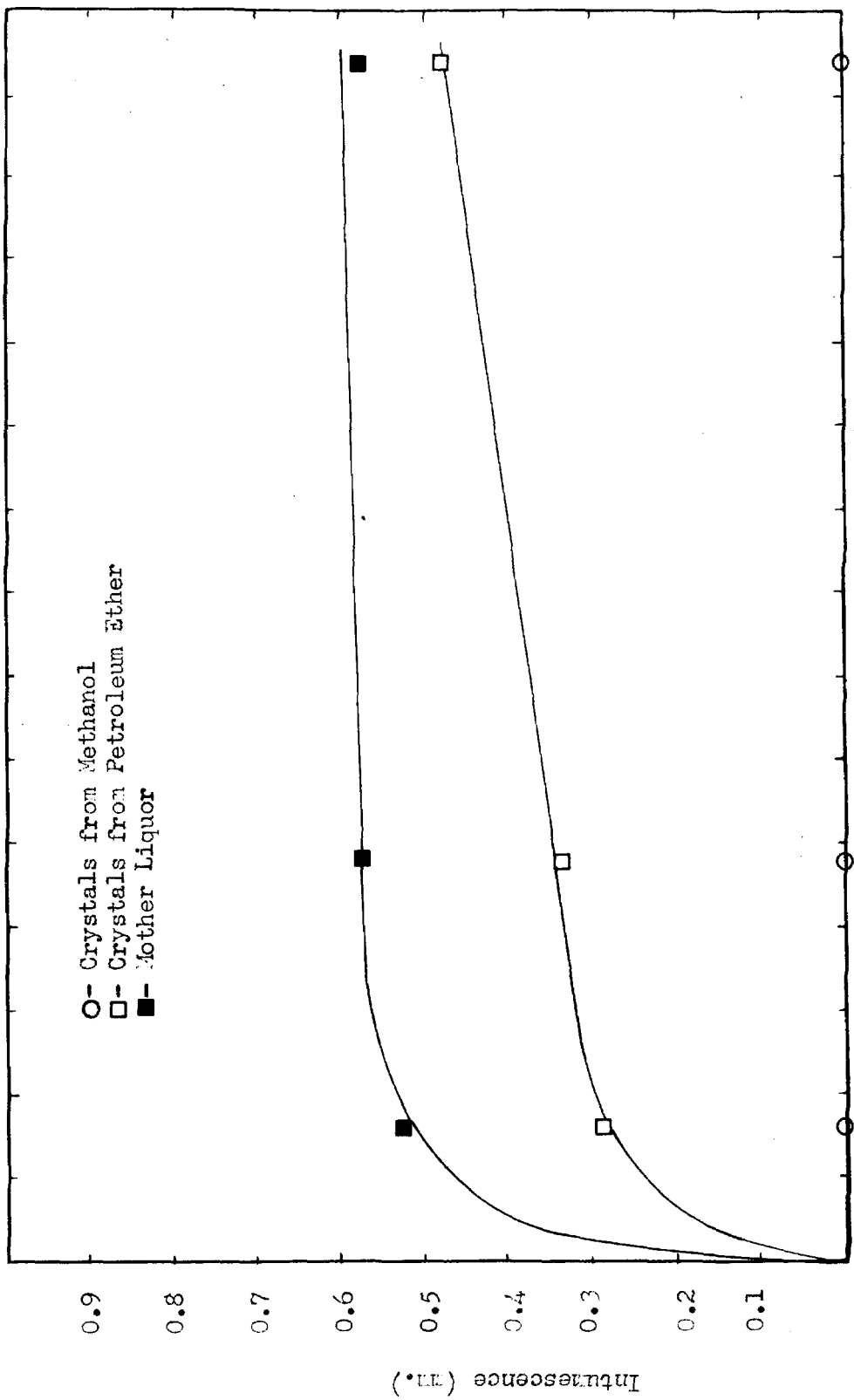

Figure 24. Activity of Crystal Fractions from Methanol and Petroleum Ether. (Corrected for Factor W Activity)

TABLE 20

Analysis*

	% Carbon	% Hydrogen	% Oxygen**	Melting Point
Crystals	75.68 75.56	11.88 12.27	12.44 12.17	52-53°C. -------
Palmitic Acid	74.94	12.58	12.48	63-64°C.

	Neutralization Equivalent	Molecular Weight***	Emperical Formula***	Iodine Number****
Crystals	250	254.6	$C_{16}H_{30}O_2$	3.0
		256.4	$C_{16}H_{32}O_2$	
Palmitic Acid	256	256.4	$C_{16}H_{32}O_2$	0.0

2/ Distillation of the Oily Residue from the Mother Liquor (C_2).

In order to further enrich the active oily material, a high vacuum fractional distillation was carried out. The results of such a distillation, are shown in Table 21. Fraction C_2-III, the highest boiling fraction, showed the highest activity (Fig. 25).

---

* Elemental analysis by G. Swinehart, Microanalyst, California Institute of Technology.

** Oxygen determined by difference.

***Calculated on basis of C and H analysis.

**** Iodine number was determined by the method described by Jamieson (22) with Wijs Solution.

Wijs Solution: This solution was prepared after the method of Jamieson (23). Upon solution of thirteen grams of powdered resublimed iodine in 1000 ml. of pure glacial acetic acid sufficient chlorine gas passed into solution to be just short of double the titer of original iodine solution (slight excess of iodine).

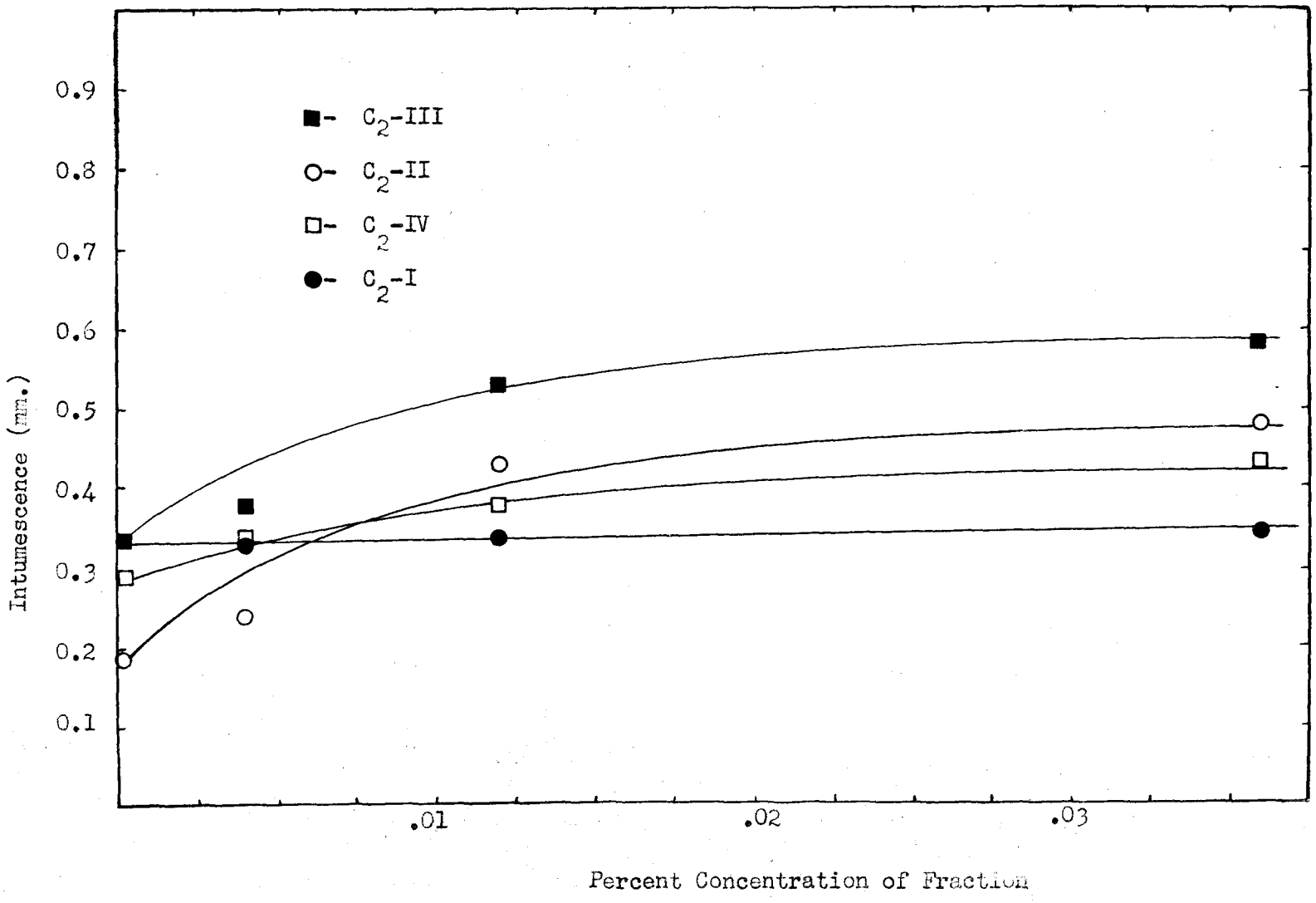

Figure 25. Activity of Fractions Obtained in Distillation of C_2. (Corrected for Factor W Activity)

TABLE 21
Fractional Distillation of the Enriched Oil Fraction (3.734 gm.) at 100μ Pressure

Fraction	C_2-I	C_2-II	C_2-III	C_2-IV
Boiling range	under 140°C.	140-155°C.	over 155°C.	over 200°C.
Weight (gm.)	0.903	1.667	0.741	0.199
Color of fraction	pale yellow oil	orange viscous oil	thin pale oil	very dark gum

D) IDENTIFICATION OF THE ACTIVE COMPONENT

Fraction C_2-I, -II, -III were liquid at room temperature (about 22°C.) but only fraction C_2-III remained liquid when stored at -10°C. Being the most active, it was examined more closely. A neutralization equivalent determination gave an equivalent weight of 320. An iodine number determination (Wijs) (22) gave a value of 153.

The general agreement of physical characteristics of the higher saturated fatty acids with those of the crystalline material from the bicarbonate fraction, as well as the general agreement of the physical and chemical characteristics of several of the more common unsaturated fatty acids with those of fraction C_2-III indicated that the latter fraction was a mixture of predominately unsaturated fatty acids (Table 22).

To establish whether the activity of fraction C_2-III could be due to unsaturated fatty acids, commercial preparations of oleic, linoleic and linolenic acids were tested in combination with factor W. It was shown that the activity of these acids increased with increasing

unsaturation (Fig. 26). The activity of the higher boiling fraction of orange oil therefore was probably due to the presence of unsaturated fatty acids, such as linolenic and linoleic acids.

TABLE 22

Physical and Chemical Characteristics of some of the Higher Fatty Acids

Acid	Boiling Point mm.	Melting Point	Molecular Weight	Iodine Number
Oleic	150-160°C. (0.01)	13°C.	282.4	89.9
Linoleic	150-160°C. (0.01)	-8°C.	280.4	181.0
Linolenic	158°C. (0.002)	-14.5°C.	278.4	273.5
Myristic	199°C. (16)	54.4°C.	228.4	
Palmitic	139°C. (1)	63.0°C.	256.4	
Stearic	160°C. (0.25)	69.6°C.	284.5	

E) PURE COMPOUNDS EXAMINED FOR WOUND HORMONE ACTIVITY

In an attempt to determine the structural specificity for wound hormone activity, a number of compounds, many of which are of biological importance, were tested (Tables 23,24).

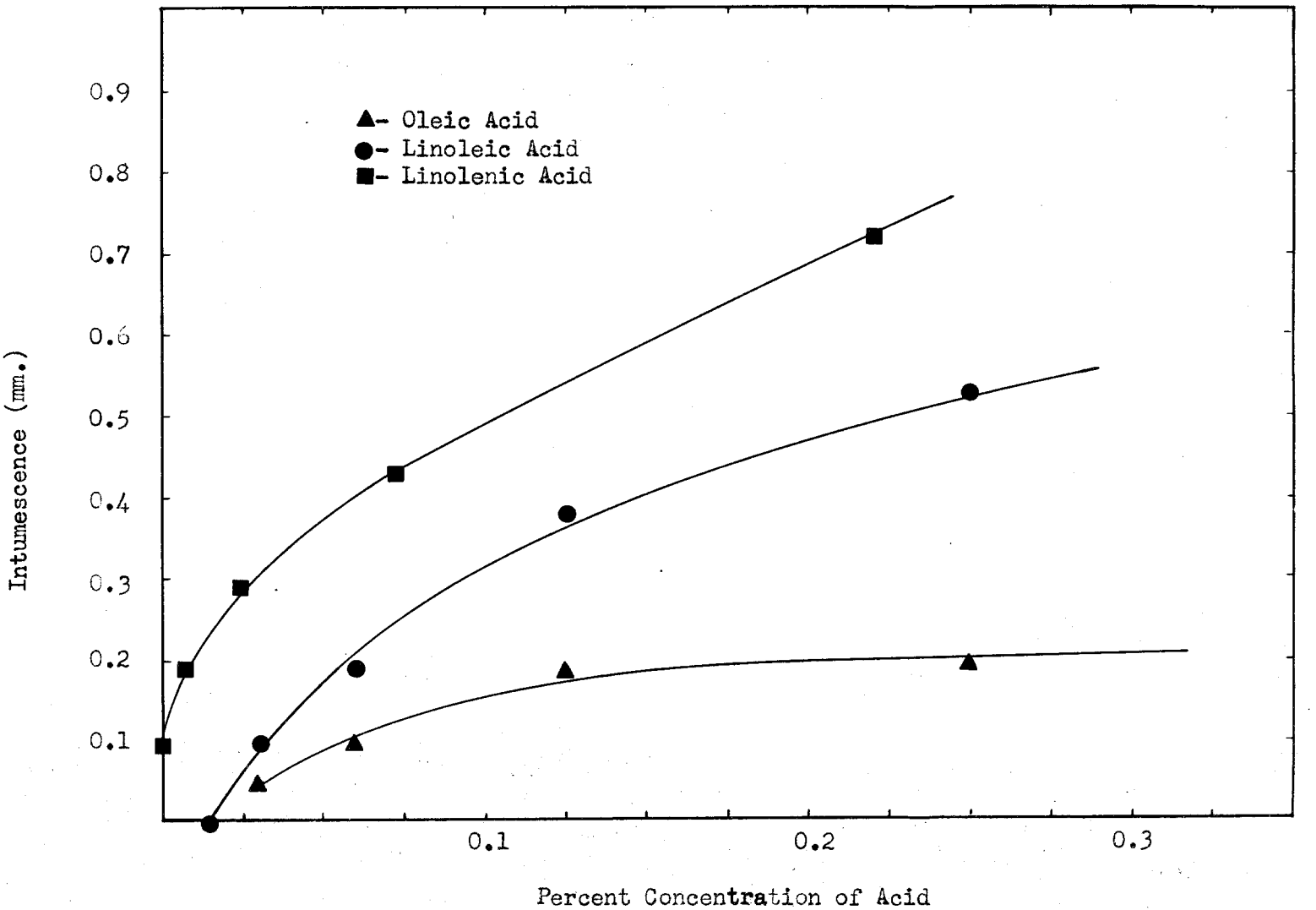

Figure 26. Activity of Commercial Oleic, Linoleic and Linolenic Acids. (Corrected for Factor W Activity)

TABLE 23

Compounds Found Active in the Wound Response Inducing System. The Compounds were Tested in a range from 80 γ to 2200 γ, in combination with Factor W.

Substances	LPI Equivalent (in mg.)	Substances	LPI Equivalent (in mg.)
Linolenic Acid	140	Malonic Acid	8
Linoleic Acid	140	Sebacic Acid	8-25
Lauric Acid	25	Decane-1,10-dicarboxylic Acid	12
Oleic Acid	50	Cinnamic Acid	25
Myristic Acid	3-8	p-Hydroxy phenyl Acetic Acid	8-25
Chaulmoogric Acid	13	Caprylic Acid	10
Succinic Acid	8-25	LPI	1
Traumatic Acid	12		
Fraction C_2-III*	100-150		

TABLE 24

Compounds Found Inactive
in the Wound Response Inducing System

Acetic Acid
Adipic Acid
Aleuritic Acid
Butyric Acid
Caproic Acid
Furoic Acid
Gallic Acid
Glycollic Acid
α-Keto-glutaric Acid
Malic Acid
Nicotinic Acid
p-Aminobenzoic Acid
Pantothenic Acid
Propionic Acid
Tropic Acid

Factor W invariably enhanced the activity of all the active compounds. The higher unsaturated acids were the most active and approximated the activity found in purified Fraction C_2-III.

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* See page 77

PART VIII

THE WATER SOLUBLE FACTORS (W)

The preparation of the standard factor W fraction has been described previously (page 49). The hydrophilic factor is not significantly extracted by organic solvents either from infusion powder or its aqueous solution, nor is it precipitable by barium or lead ions. Chromatography of factor W on an anion exchange column (Amberlite IR 400), with 0.5 N aqueous HCl gives two fractions equal in weight and activity.

A) INVESTIGATION OF COMPOUNDS FOR FACTOR W PROPERTIES

After the wound hormone activity of linolenic and related acids had been established, it was of interest to examine the nature of the water soluble factors. As a first step in this direction a number of water soluble, naturally occurring substances were tested for their ability to enhance the response of 0.01% linolenic acid. Table 25 lists compounds that were ineffective over the range tested while Table 26 lists substances which were effective.

In this investigation a number of compounds were found to enhance the growth effect of linolenic acid; outstanding among these were Coenzyme A and Cytochrome C. The concentration of Coenzyme A and Cytochrome C used in these experiments was of the order that one could expect to be present in plant extracts.* These substances could therefore, be responsible for the enhancing activity of the water soluble factors of lemon peel infusion. Definite conclusions, however, must be deferred until both Coenzyme A and Cytochrome C have been determined in this material.

*The calculations are based on the data of Mann (25) Farrell et al.(26), Heinze et al. (27) and Morgan et al. (28).

TABLE 25

Compounds Found Inactive as Water Soluble Factors with Linolenic Acid (0.01 %)

Compounds	Range Tested (% concentrations)
Adenosine Triphosphate	0.0037--0.10
DL-Alanine	0.07----2.0
p-Aminobenzoic Acid	0.0056--0.15
L-Arginine	0.037---1.0
Ascorbic Acid	0.037---1.0
L-Asparagine	0.037---1.0
Biotin	0.0056--0.15
Calcium Pantothenate	0.0056--0.15
Cinnamic Acid	0.037---1.0
Citric Acid	0.037---1.0
L-Cysteine	0.037---1.0
L-Cystine	0.037---1.0
Cytidylic Acid	0.037---1.0
3,5-Di-iodotyrosine	0.037---1.0
Folic Acid	0.0056--0.15
D-Fructose	0.056---1.5
Fumaric Acid	0.037---1.0
D-Galactose	0.056---1.5
D-Glucose	0.056---1.5
L-Glutamic Acid	0.056---1.5
Glycine	0.037---1.0
L-Histidine	0.037---1.0
Indoleacetic Acid	0.0066--0.05
DL-Isoleucine	0.07----1.0
α-Ketoglutaric Acid	0.037---1.0
L-Leucine	0.037---1.0
L-Lysine	0.037---1.0
Maleic Acid	0.037---1.0
Malonic Acid	0.037---1.0
D-Mannose	0.056---1.0
Nicotinic Acid	0.0056--0.15
L-Phenylalanine	0.037---1.0
L-Proline	0.037---1.0
Pyridoxine • HCl	0.0056--0.15
L-Serine	0.037---1.0
Sucrose	0.056---1.5
Succinic Acid	0.037---1.0
Thiamine • HCl	0.0056--0.15
L-Threonine	0.037---1.0
L-Tryptophane	0.037---1.0
L-Tyrosine	0.037---1.0
L-Valine	0.037---1.0

TABLE 26

Compounds Capable of Enhancing the Growth Response to Linolenic Acid (0.01 %)

Active Compound	Maximum Relative Activity compared to W	Concentration of Maximum Relative Activity (% concentration)	Range tested (% concentration)
Factor W	1	0.4	---------
Adenylic Acid	1	0.33	0.037 to 1.0
ATP/Ascorbic Acid	24	0.0167/0.167	0.0019/0.019 to 0.05/0.5
L-Aspartic Acid	1	0.5	0.056 to 1.5
Coenzyme A	100-200	0.0031	0.0031 to 0.05
Coenzyme I	12	0.033	0.011 to 0.1
Coenzyme II	12	0.033	0.011 to 0.1
Cytochrome C/Ascorbic Acid	700	0.00057/0.057	0.00019/0.019 to 0.005/0.5
Cytochrome C	400	0.001	0.00037 to 0.01
Glutathione	12	0.033	0.0037 to 0.1
Guanylic Acid	4	0.11	0.037 to 1.0
L-Methionine	1	0.33	0.037 to 1.0
DL-Norleucine	2	0.22	0.07 to 2.0
L-Ornithine	0.5	1.0	0.037 to 1.0
Riboflavin	24	0.0167	0.0056 to 0.15

PART IX

EFFECTS OF SOME COMPOUNDS CLOSELY RELATED TO LINOLENIC ACID

A) INTRODUCTION

The experiments with pure acids described earlier have established that wound hormone activity is shown in different degrees by higher fatty acids such as lauric, linolenic, linoleic acids as well as higher dicarboxylic acids such as traumatic, sebacic and decane-1,10-dicarboxylic acids. It was of interest to establish the relative activities of these compounds as an introduction to the study of the effect of chemical structure on wound hormone activity. Coenzyme A, one of the most active water soluble factors tested, (Table 26) was selected as the enhancing agent.

B) CONCENTRATION SERIES OF LINOLEIC AND LINOLENIC ACIDS

Linolenic and linoleic acids were tested at different concentrations to examine the response with various concentrations of Coenzyme A (Fig. 27,28). Both acids induce the wound response but tissue sensitivity to each varies. Linoleic acid is somewhat less effective than linolenic acid in inducing a growth response when combined with Coenzyme A.

C) SATURATED FATTY ACIDS

In examining the even numbered saturated fatty acid series (Table 27) only capric (C_{10}), lauric (C_{12}) and myristic (C_{14}) were active (Fig. 29,30,31). Capric and Myristic acids, at high concentration, possessed about the same moderate activity which was slightly raised by the addition of Coenzyme A. Lauric acid was also moderately

active but its activity was raised markedly by the addition of Coenzyme A.

TABLE 27

Saturated Fatty Acids Examined for Wound Hormone Activity in Combination with Coenzyme A.

1. Acetic Acid
2. Propionic Acid
3. Butyric Acid
4. Caproic Acid
5. Caprylic Acid
6. Capric Acid
7. Lauric Acid
8. Myristic Acid
9. Palmitic Acid
10. Stearic Acid

D) DICARBOXYLIC ACIDS

According to the findings of Bonner, English and Haagen-Smit (13) and English (29), the higher unsaturated dicarboxylic acids (Table 28) are capable of inducing the wound response in beans in the presence of a special cofactor preparation.

TABLE 28

Dicarboxylic Acids Found Capable of Promoting a Wound Hormone Response in Beans (13)(29)

Δ^1-Octene-1,8-dicarboxylic acid		5-Nonanone-1,9-dicarboxylic acid	
Δ^1-Nonene-1,9-	" "	5-Nonanol-1,9-	" "
Δ^2-Nonene-1,9-	" "	6-Undecanol-1,11-	" "
Δ^1-Decene-1,10-	" "	6-Undecanone-1,11-	" "
Δ^2-Decene-1,10-	" "	Δ^2-Tridecene-1,13-	" "
Δ^1-Tridecene-1,13-	" "	$\Delta^{1,7}$-Octadiene-1,8-	" "

The saturated dicarboxylic acids of less than 7 carbon atoms are inactive. Suberic (C_8) and azelaic (C_9) acids were reported to possess slight activity whereas sebacic acid and decane-1,10-dicarboxylic acid were about one-half as active as traumatic acid (trans-Δ^1-decene-1,10-dicarboxylic acid). In view of these findings several dicarboxylic acids (Table 29) were tested. Traumatic acid was found somewhat active (Fig. 32,33) sebacic and decane-1,10-dicarboxylic

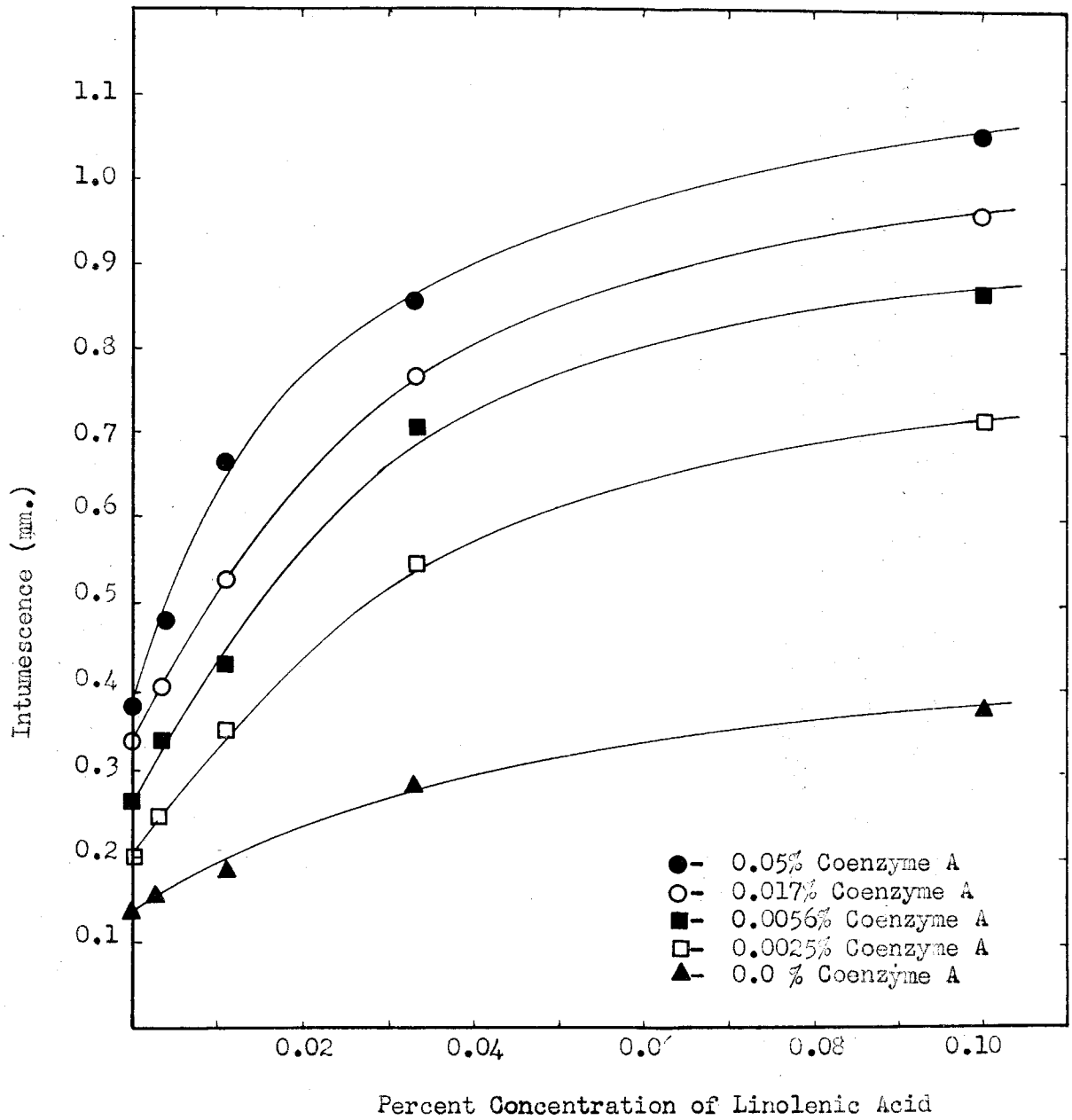


Figure 27. Coenzyme A Effect on the Growth Response to Linolenic Acid. *

* For a statistical evaluation of the points see the Appendix.

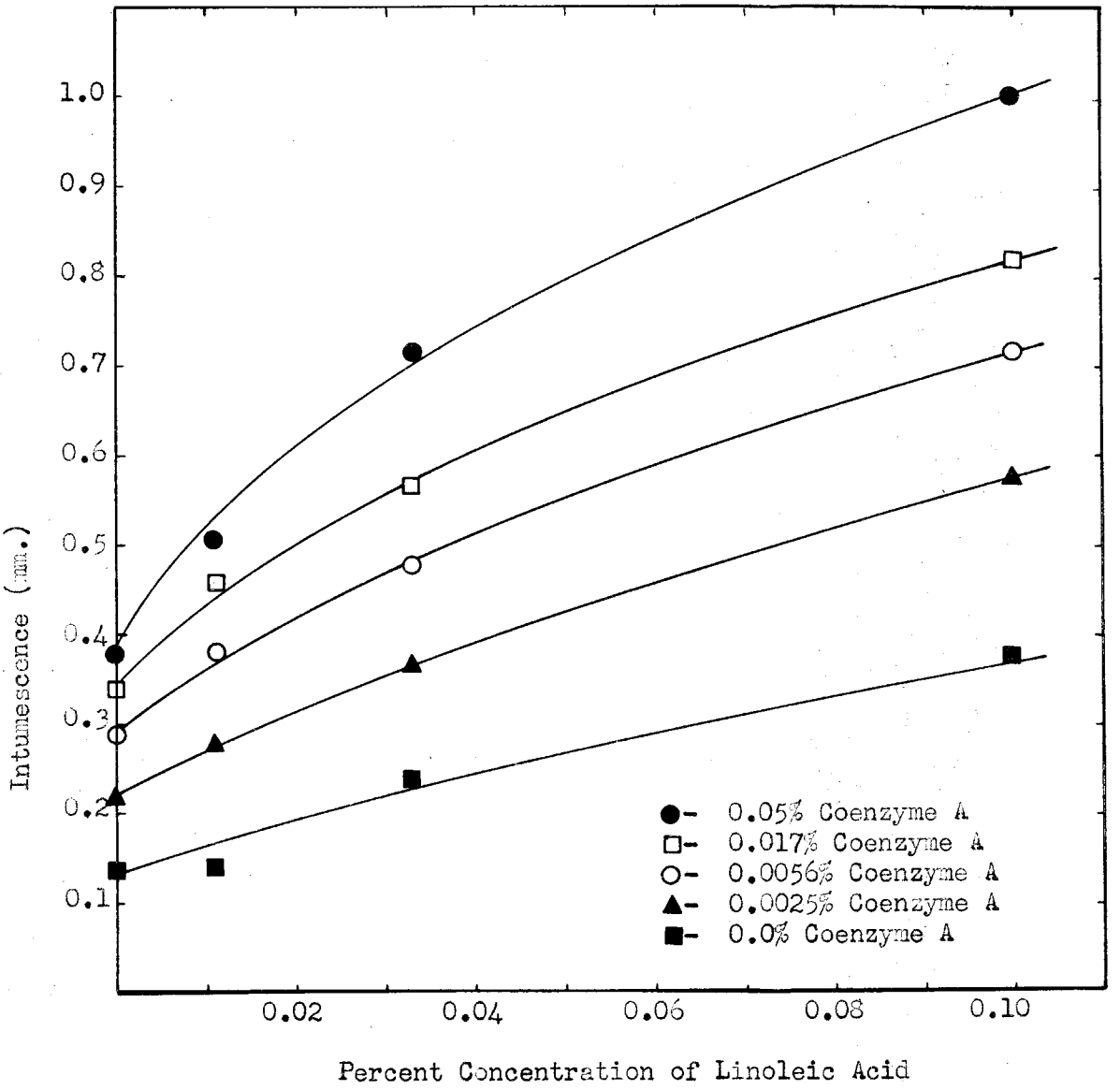


Figure 28. Coenzyme A Effect on the Growth Response to Linoleic Acid. *

* For a statistical evaluation of the points see the Appendix.

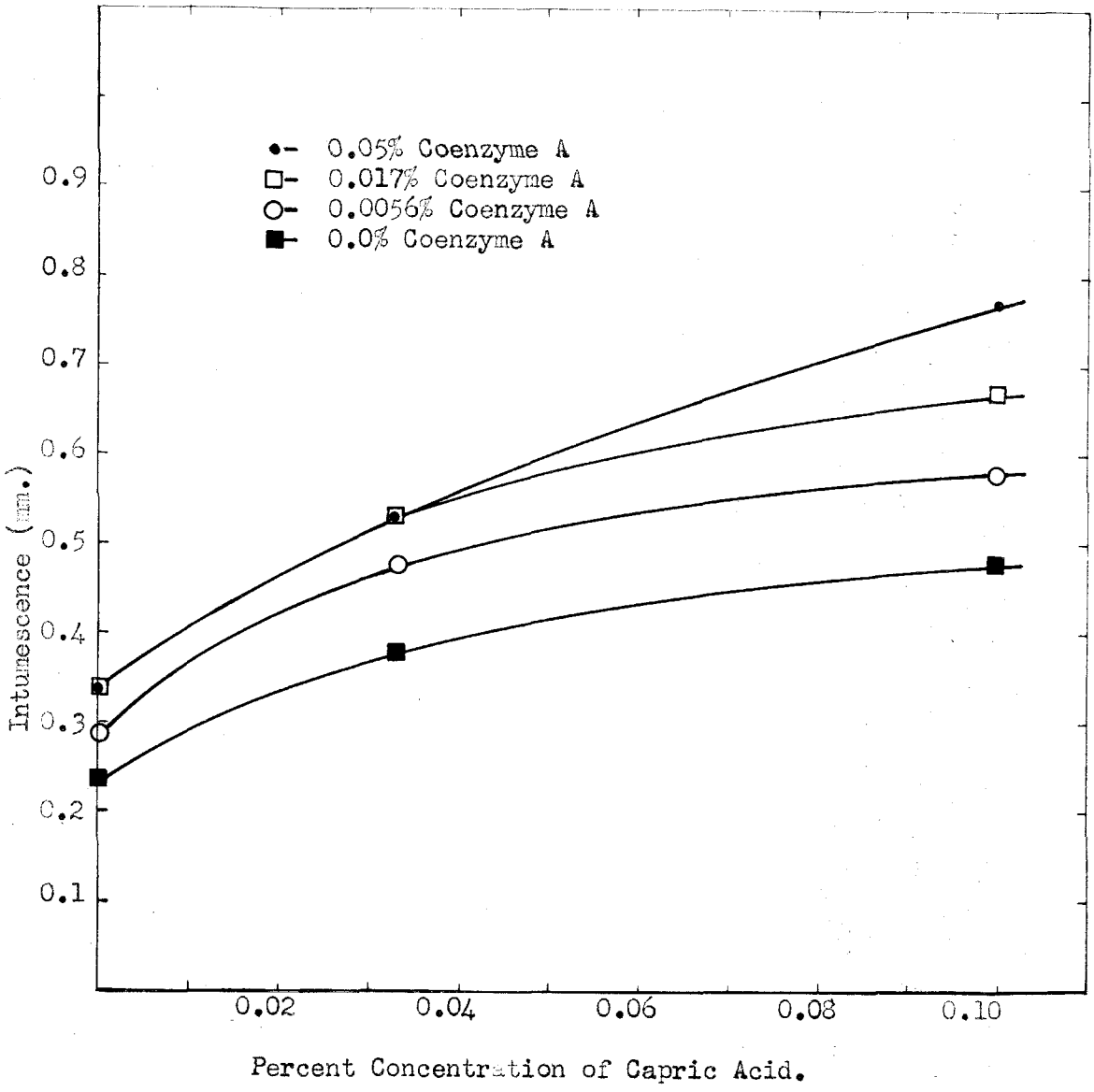


Figure 29. Coenzyme A Effect on the Growth Response to Capric Acid.

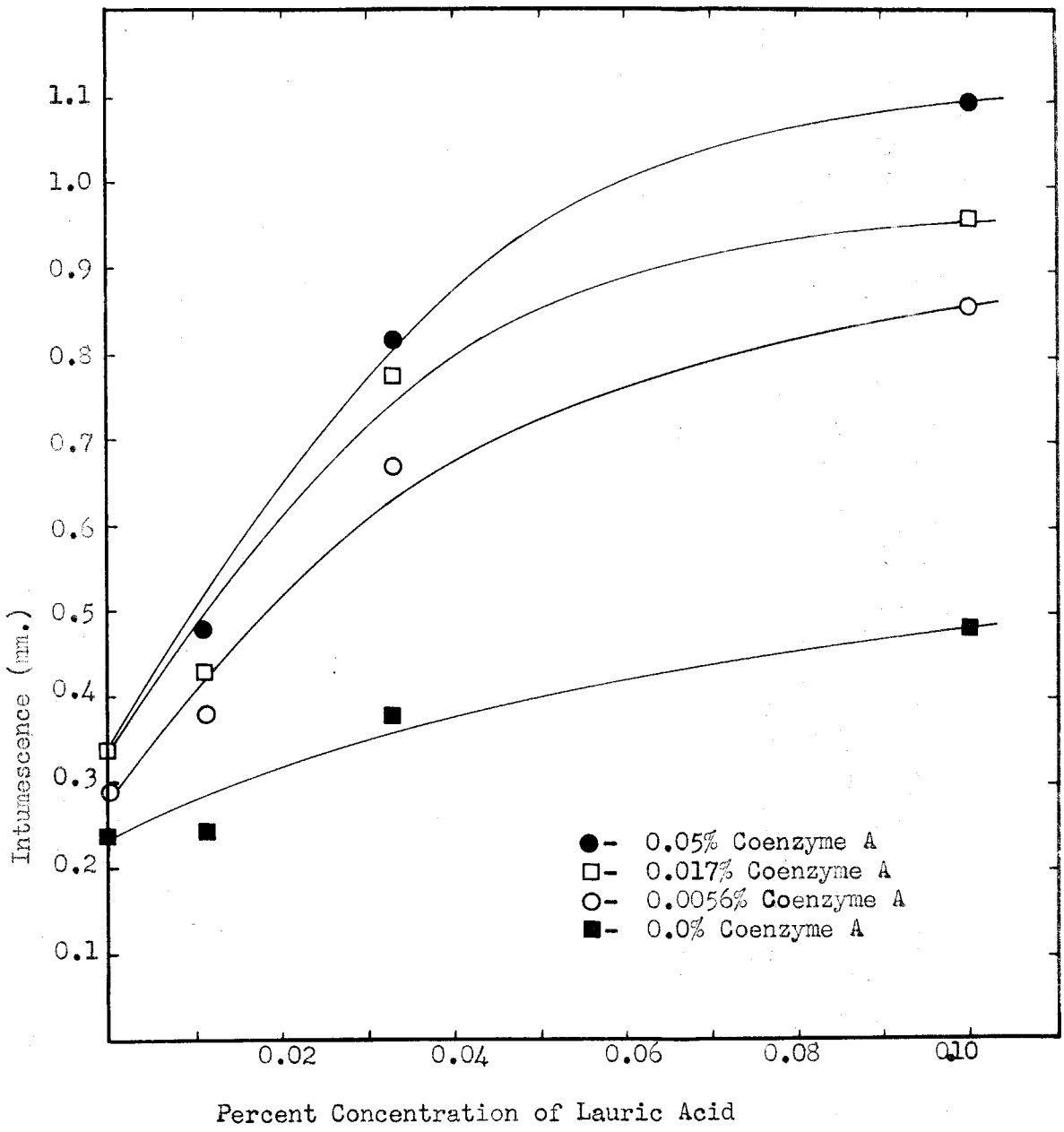


Figure 30. Coenzyme A Effect on the Growth Response to Lauric Acid.

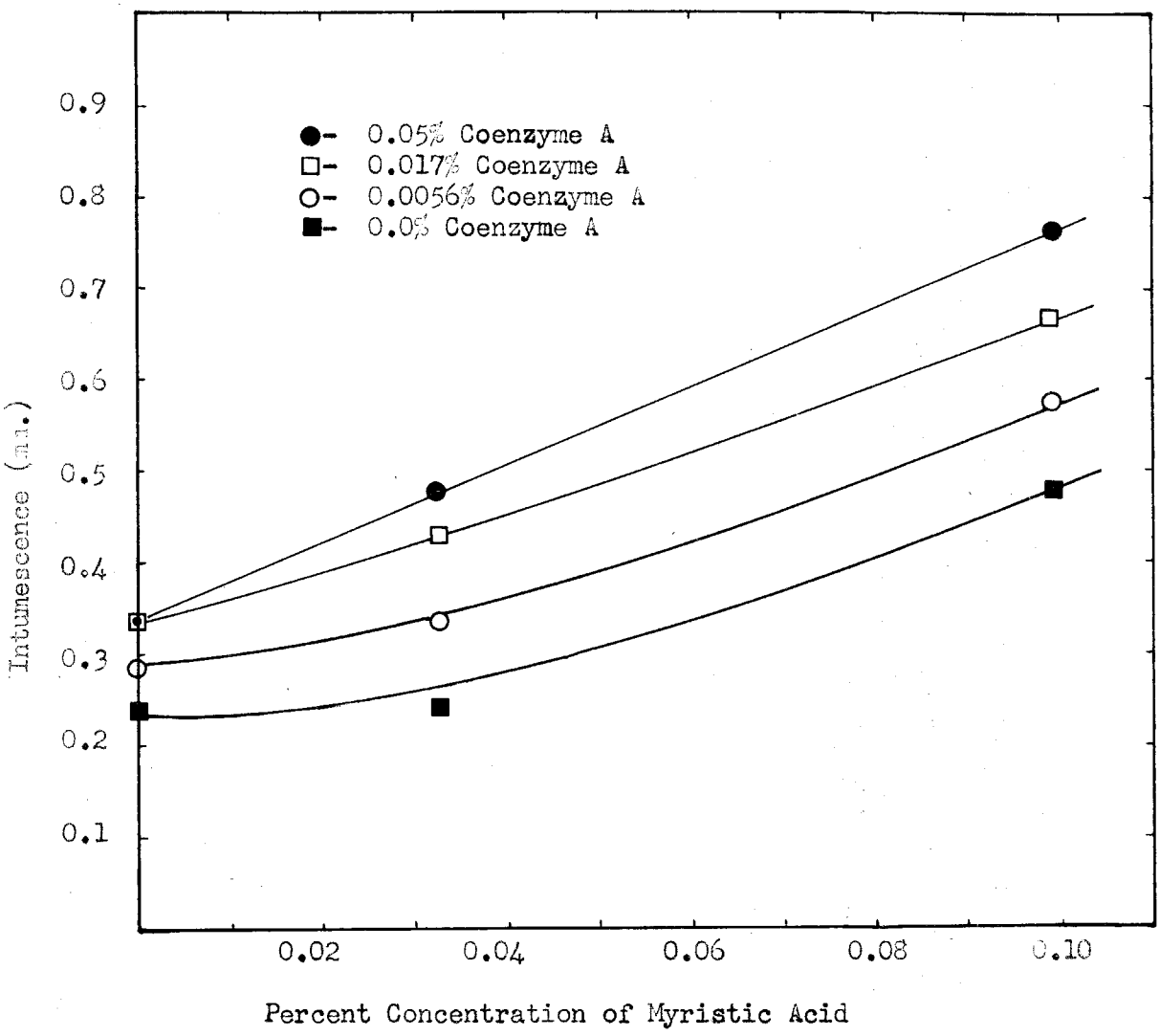


Figure 31. Coenzyme A Effect on the Growth Response to Myristic Acid.

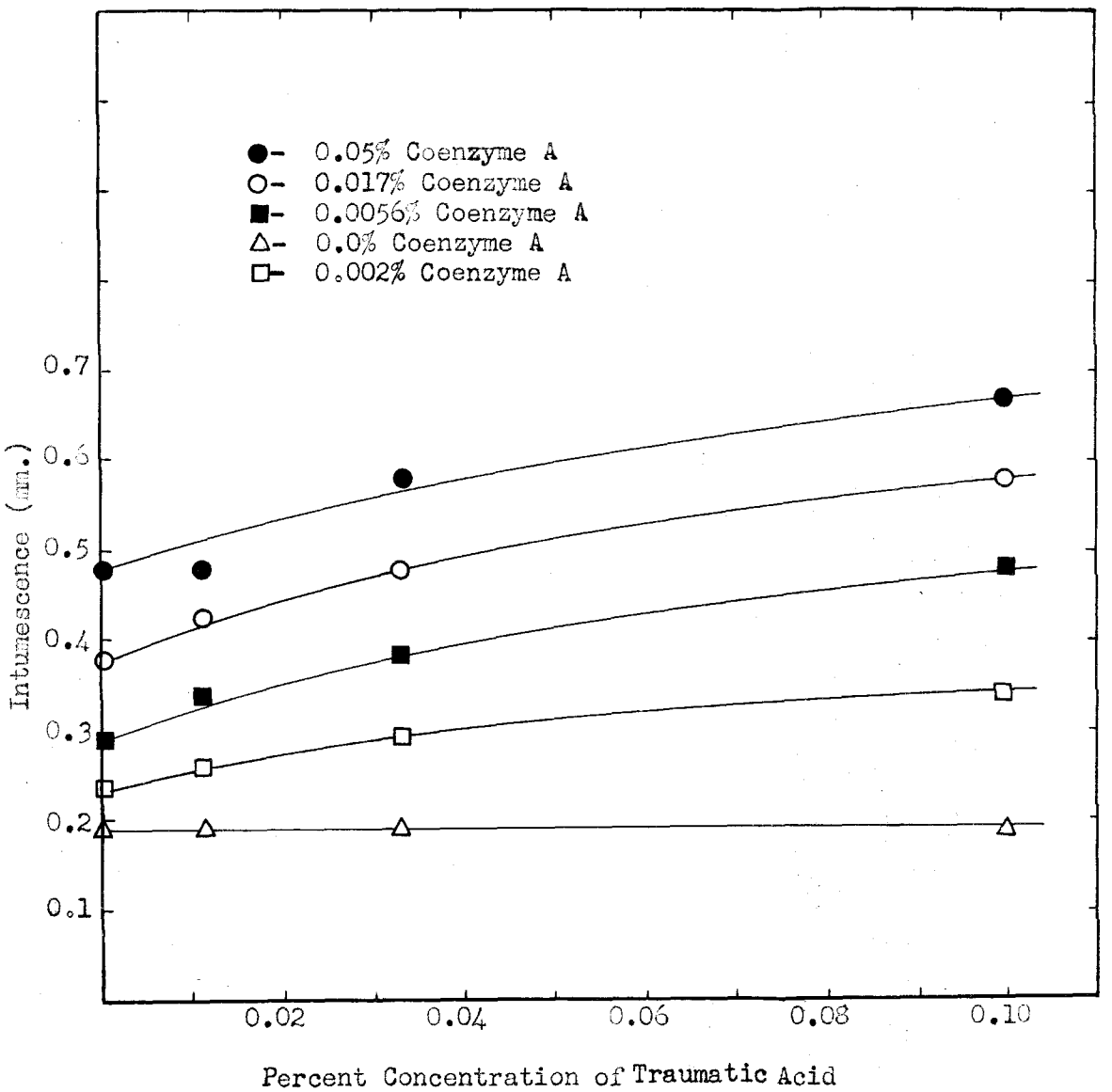


Figure 32. Coenzyme A Effect on the Growth Response to trans-Traumatic Acid.

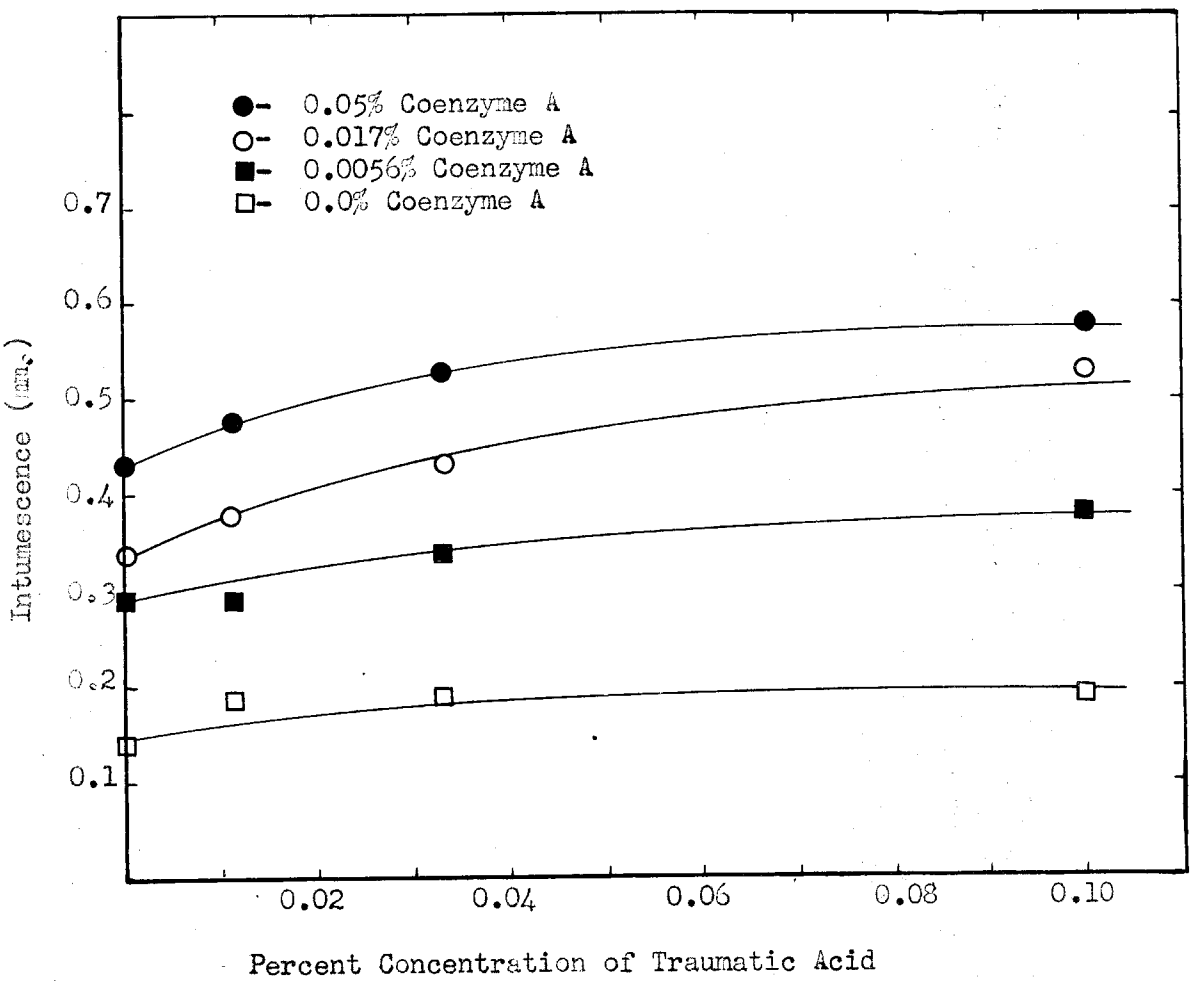


Figure 33. Coenzyme A Effect on the Growth Response to cis-Traumatic Acid.

acids less active (Fig. 34,35) and the other acids inactive with Coenzyme A system.

TABLE 29

Dicarboxylic Acids Tested for Wound Hormone Activity with Coenzyme A.

Sebacic Acid	Fumaric Acid
Traumatic Acid (cis and trans)	Malonic Acid
Decane-1,10-dicarboxylic Acid	Glutaric Acid

E) EFFECT OF STRUCTURE AND TYPE OF UNSATURATION IN WOUND RESPONSE

Since unsaturation increases the activity of a potential wound hormone (13)(29)(Fig. 26), a number of closely related compounds were prepared and their activities compared in the presence of Coenzyme A.

Stearic acid and purified oleic acid show no activity whereas the doubly unsaturated linoleic acid and the triply unsaturated linolenic acids do. Any isomerization arising in the debrominating step of the bromination procedure for purification of linoleic or linolenic acid is of little importance with respect to wound activity (Fig. 27,28,36,37).

The effect of converting linolenic acid (all cis 9,12,15-octadecatrienoic acid) to the all trans form, elaidolinolenic acid, is quite marked. The all trans isomer is much less active than the cis isomer and its activity is enhanced only slightly by the addition of Coenzyme A (Fig. 38).

Conversion of the isolated double bonds of linolenic acid to conjugated positions as in cis,trans,trans- α-eleostearic acid

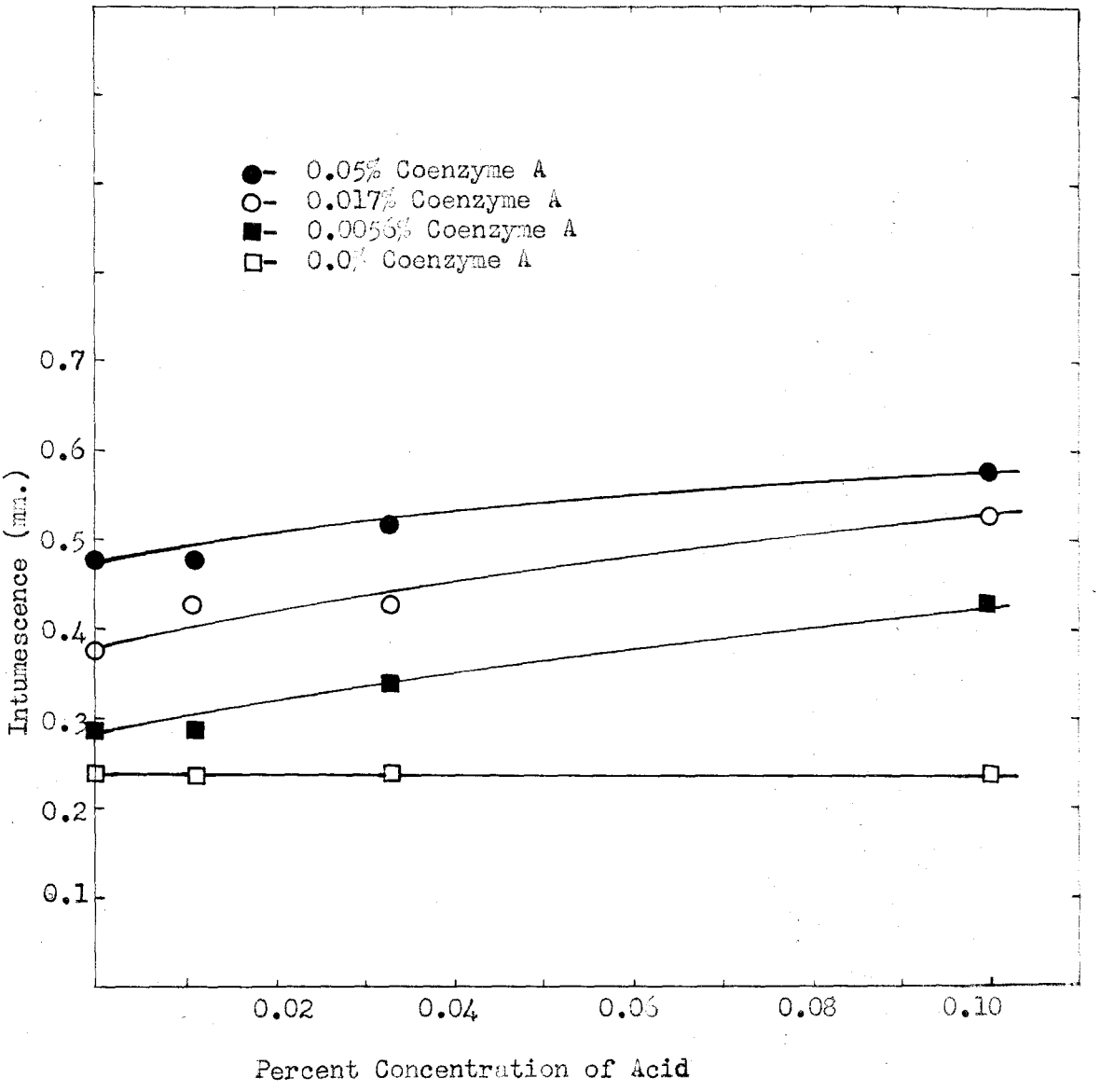


Figure 34. Coenzyme A Effect on the Growth Response to Decane-1,10-dicarboxylic Acid.

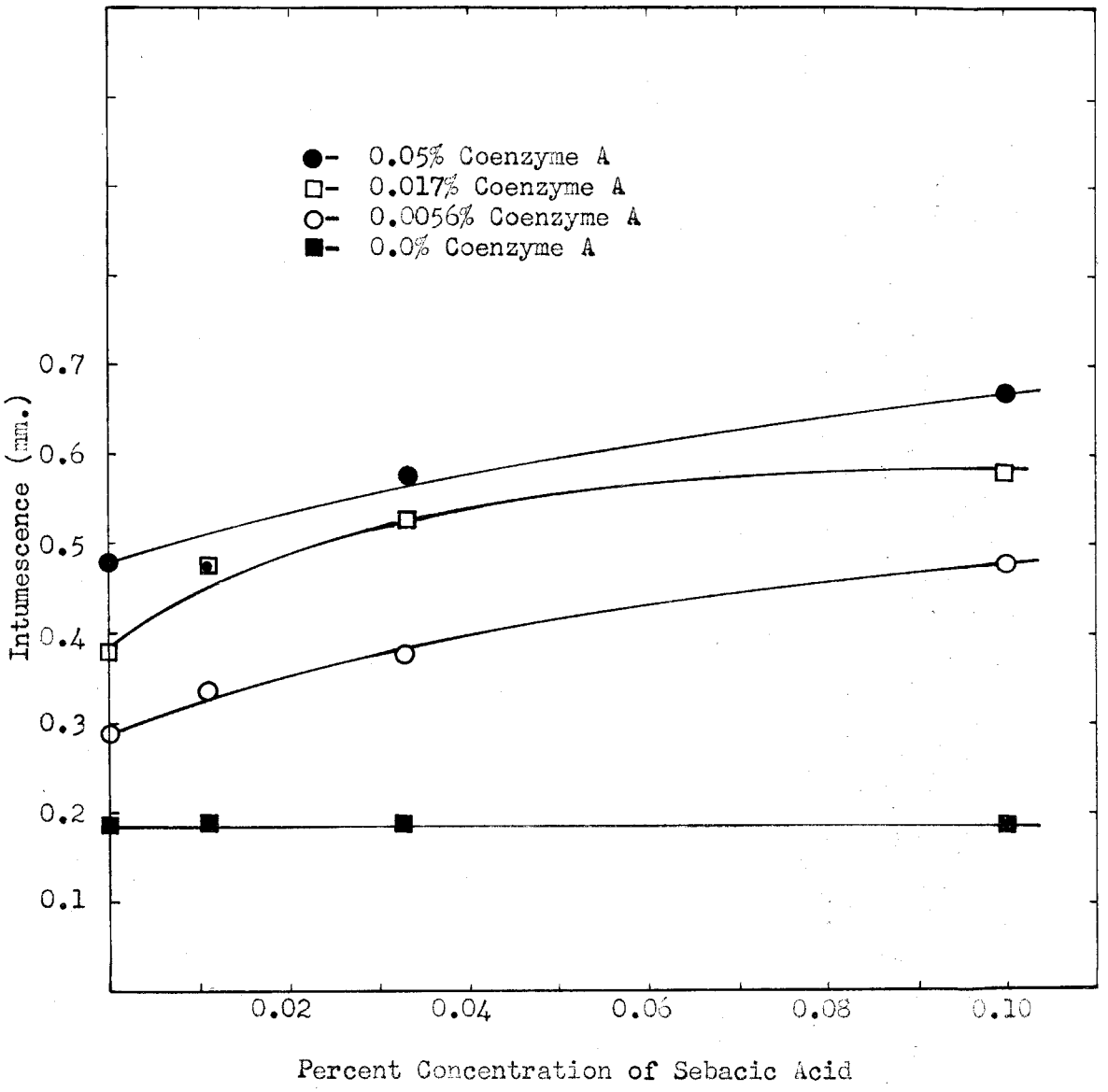


Figure 35. Coenzyme A Effect on the Growth Response to Sebacic Acid.

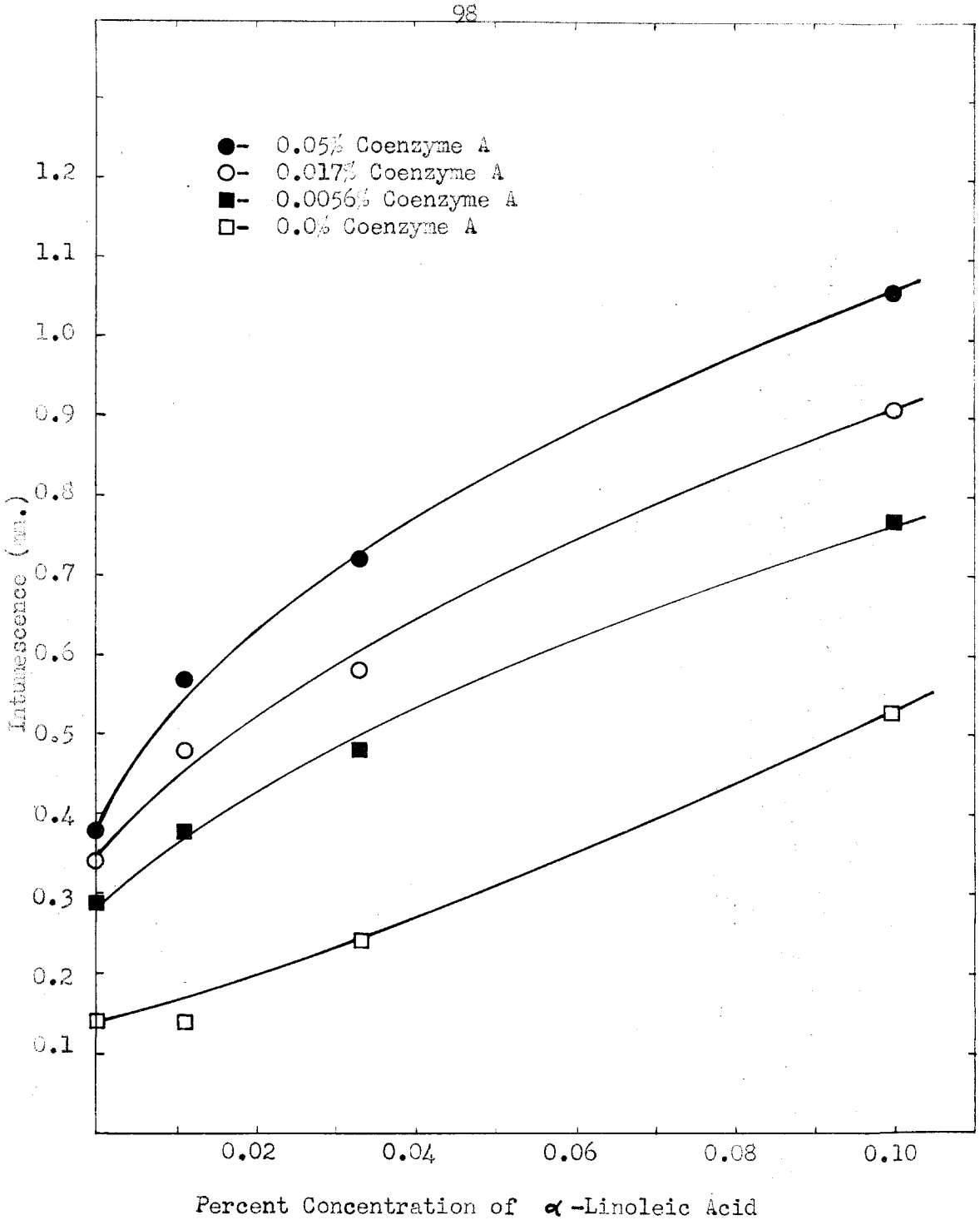


Figure 36. Coenzyme A Effect on the Growth Response to ∝-Linoleic Acid.

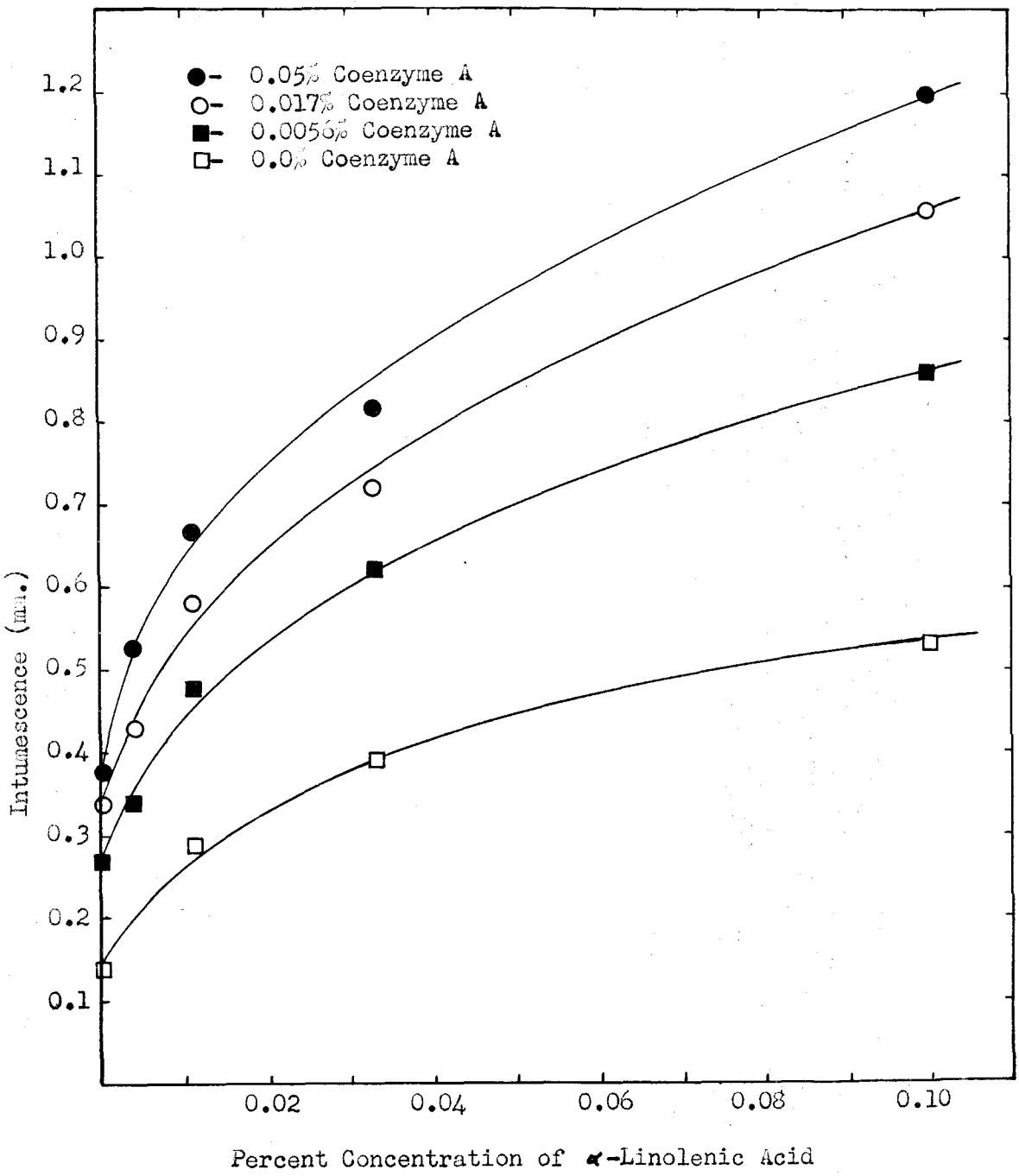


Figure 37. Coenzyme A Effect on the Growth Response to α-Linolenic Acid.

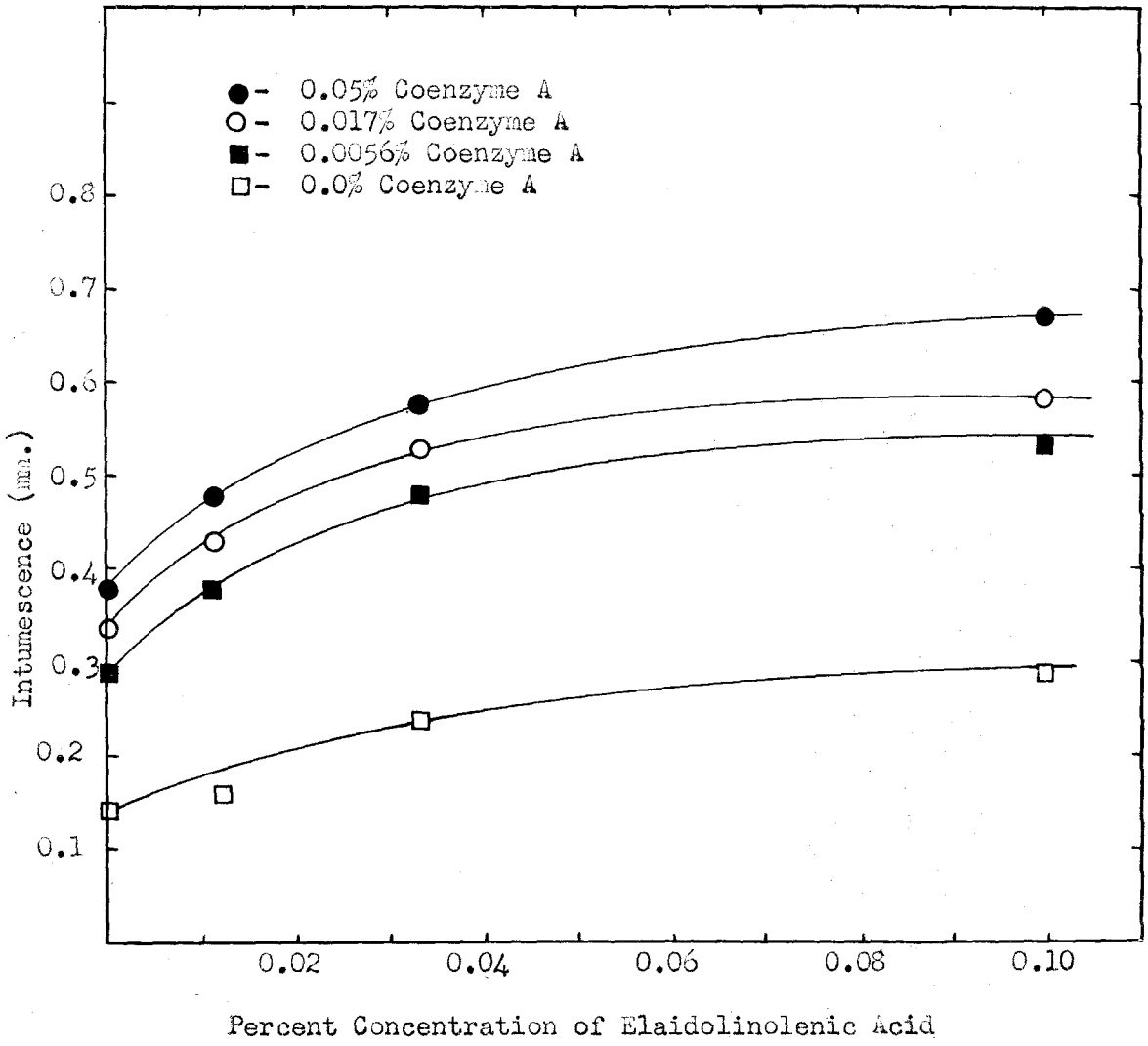


Figure 38. Coenzyme A Effect on the Growth Response of Elaidolinolenic Acid.

(9,11,13-octadecatrienoic acid) or the all trans isomer, β -eleostearic acid, again results in a decrease of activity (Fig. 39,40). In this respect it is interesting to note that if a sample of linolenic acid is autoxidized in air after the method of Farmer and coworkers (30), and then prepared for testing in the usual manner, there is no loss of activity even though spectroscopic evidence, by Farmer (30), Holman (31), Bolland (32) and Bergström (33), has indicated that in the initial phases, autoxidation is associated with an isomerization of the double bonds to a conjugated system.

F) SUMMARY

The relative activities of the most active organic acids considered in the previous section are shown in graphic form in Fig. 41 and 42 and in tabular form in Table 30. Linolenic acid is shown to be very active, at least 8 times as active as traumatic acid. Linoleic acid is also active, on an average about 40% as active as linolenic acid. This is also seen in Fig. 44 where the response curves of linolenic and linoleic acids coincide if the concentration of the latter is adjusted to 40%. In Fig. 43 at Coenzyme A concentration 0.005%, the concentrations of linolenic acid for 0.4, 0.6 and 0.7 mm. intumescence are 0.009%, 0.026% and 0.037%. At a 10 fold Coenzyme A concentration the respective linolenic acid concentrations are 0.001%, 0.008% and 0.012% or reduced by factors of 9, 3.3 and 3. This indicates that something else besides Coenzyme A is needed for the fullest response of linolenic acid.

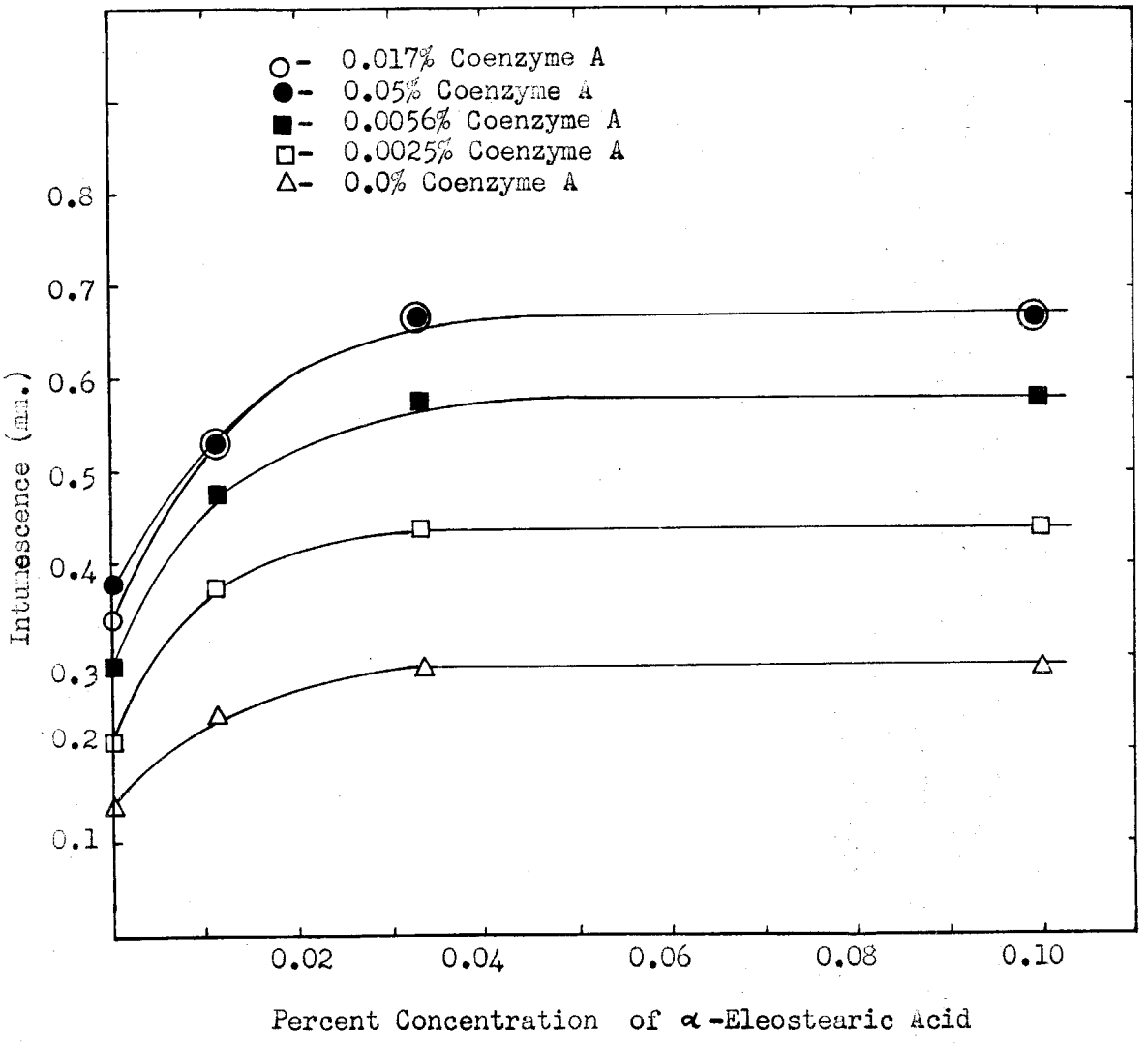


Figure 39. Coenzyme A Effect on the Growth Response to α-Eleostearic Acid.

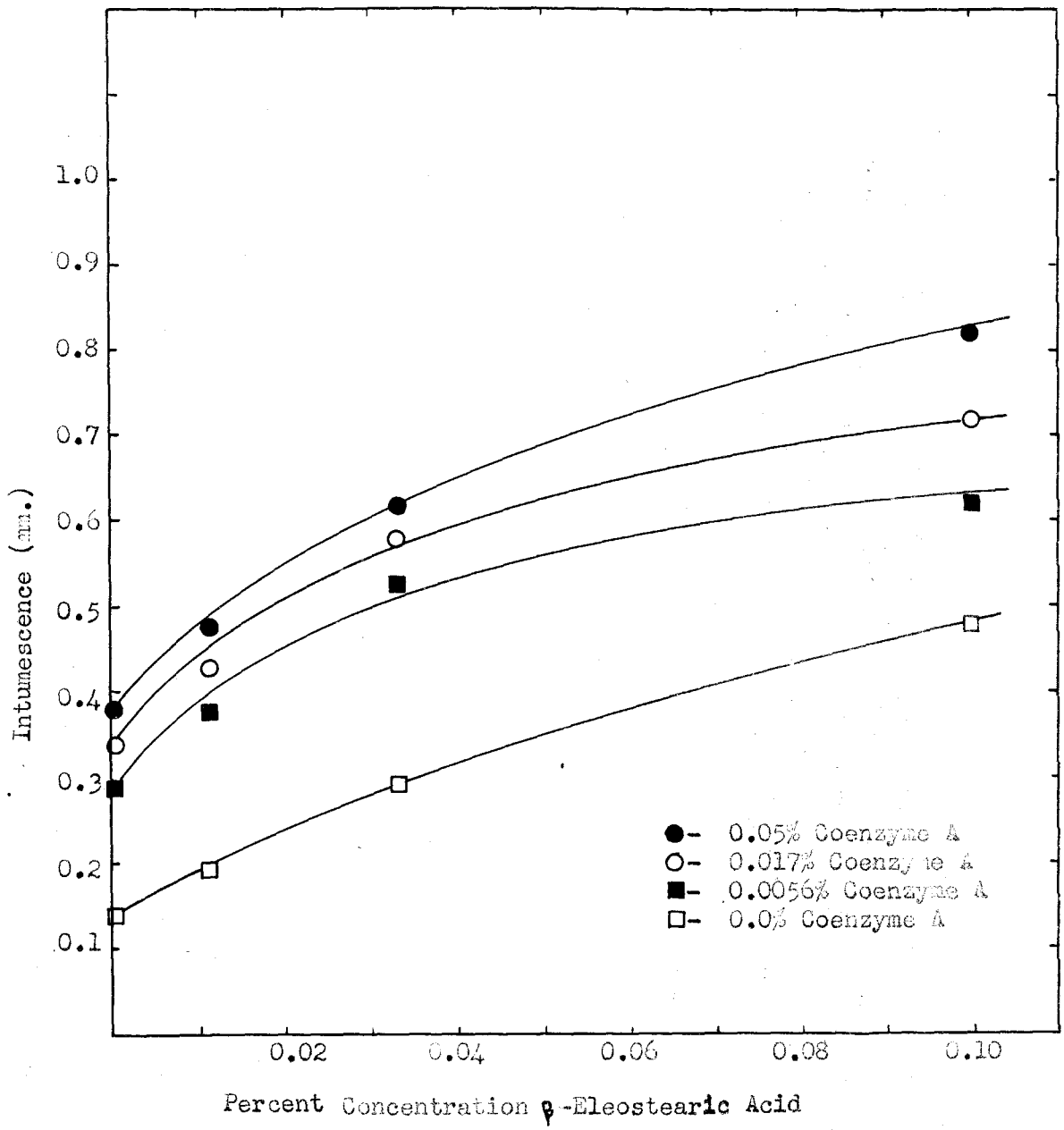


Figure 40. Coenzyme A Effect on the Growth Response to β-Eleostearic Acid

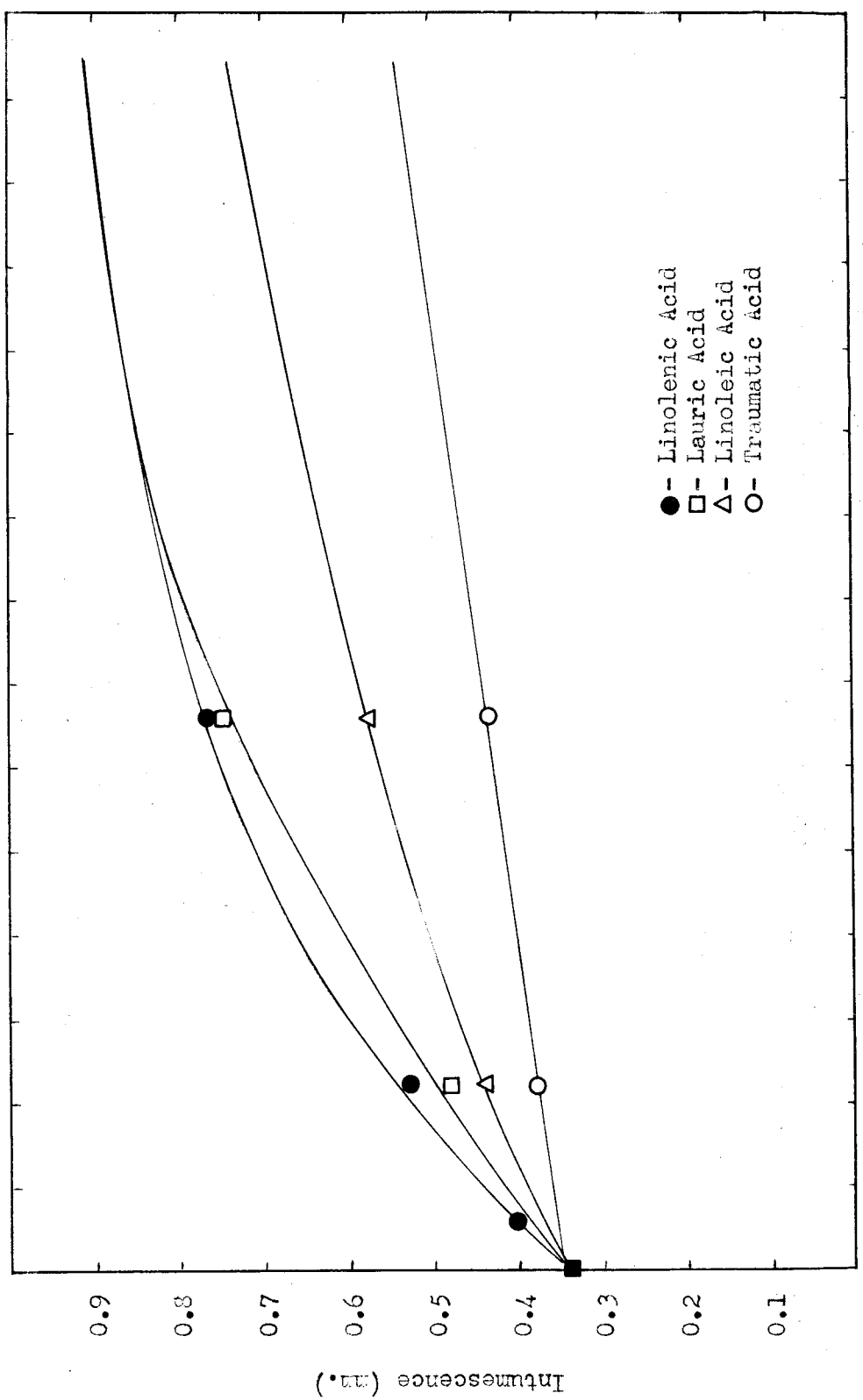


Figure 41. Relative Activities of Different Acids with Concentration of Coenzyme A 0.017%.

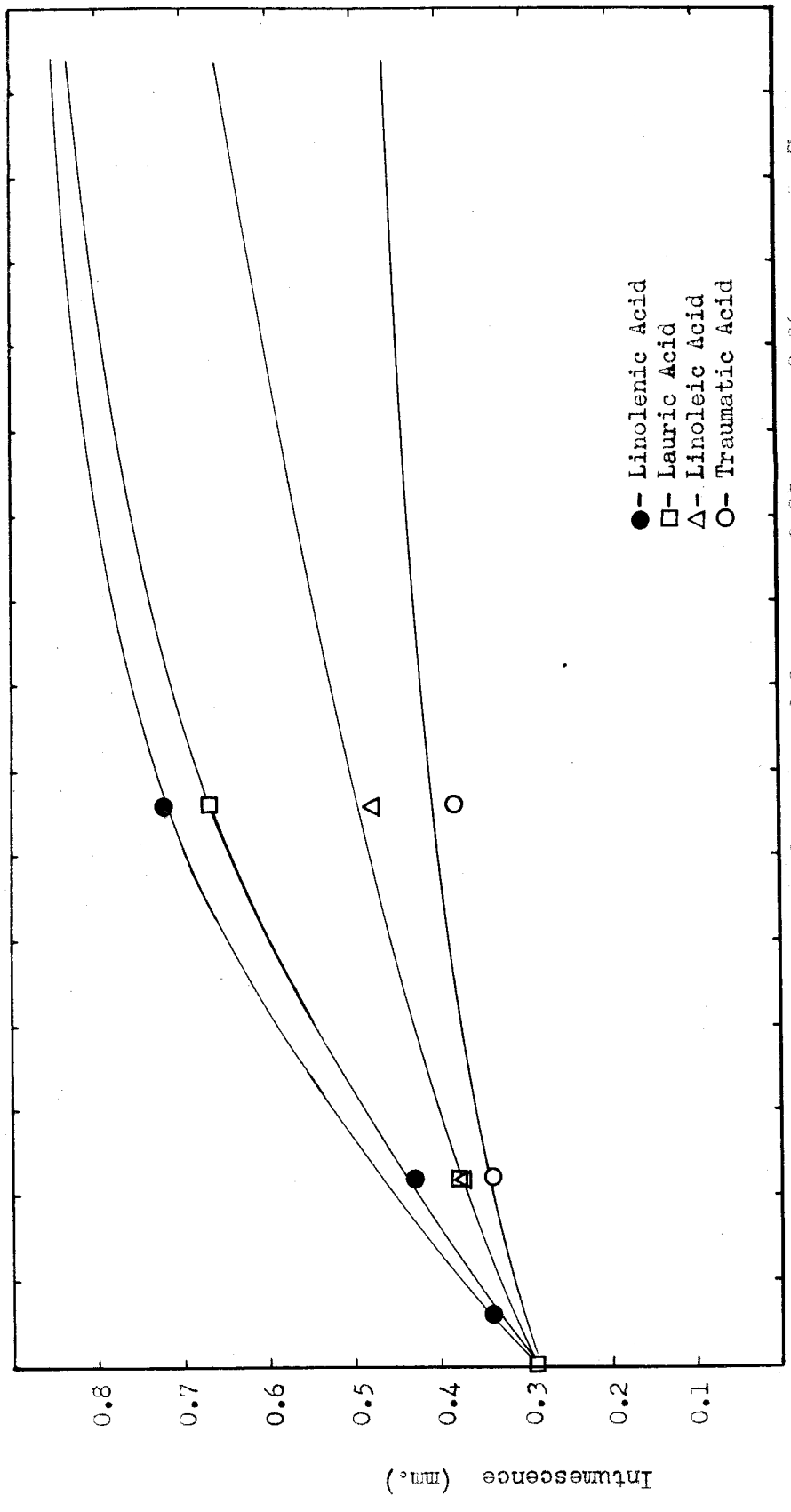


Figure 42. Relative Activities of Different Acids with Coenzyme A Concentration 0.0056%.

TABLE 30

Relative Activities of Known Acids for the Wound Hormone Response Shown by Phaseolus when Combined with Coenzyme A. (Intumescence is 0.5 mm.)

	Relative Activity	
	Concentration Coenzyme A	
Saturated Fatty Acids	0.017 %	0.005 %
Acetic	0	0
Propionic	0	0
Butyric	0	0
Caproic	0	0
Caprylic	0	0
Capric	1	1
Lauric	6	7
Myristic	1	1
Palmitic	0	0
Stearic	0	0
Unsaturated Fatty Acids		
Oleic	0	0
Linoleic	3.5	3.5
α-Linoleic	3.5	3.5
Linolenic	8	9
α-Linolenic	8	9
Elaidolinolenic	1	1
α-Eleostearic	1	1
β-Eleostearic	1	1
Dicarboxylic Acids		
Sebacic	1	1
Traumatic (cis and trans)	1	1
Decane-1,10-dicarboxylic	1	1

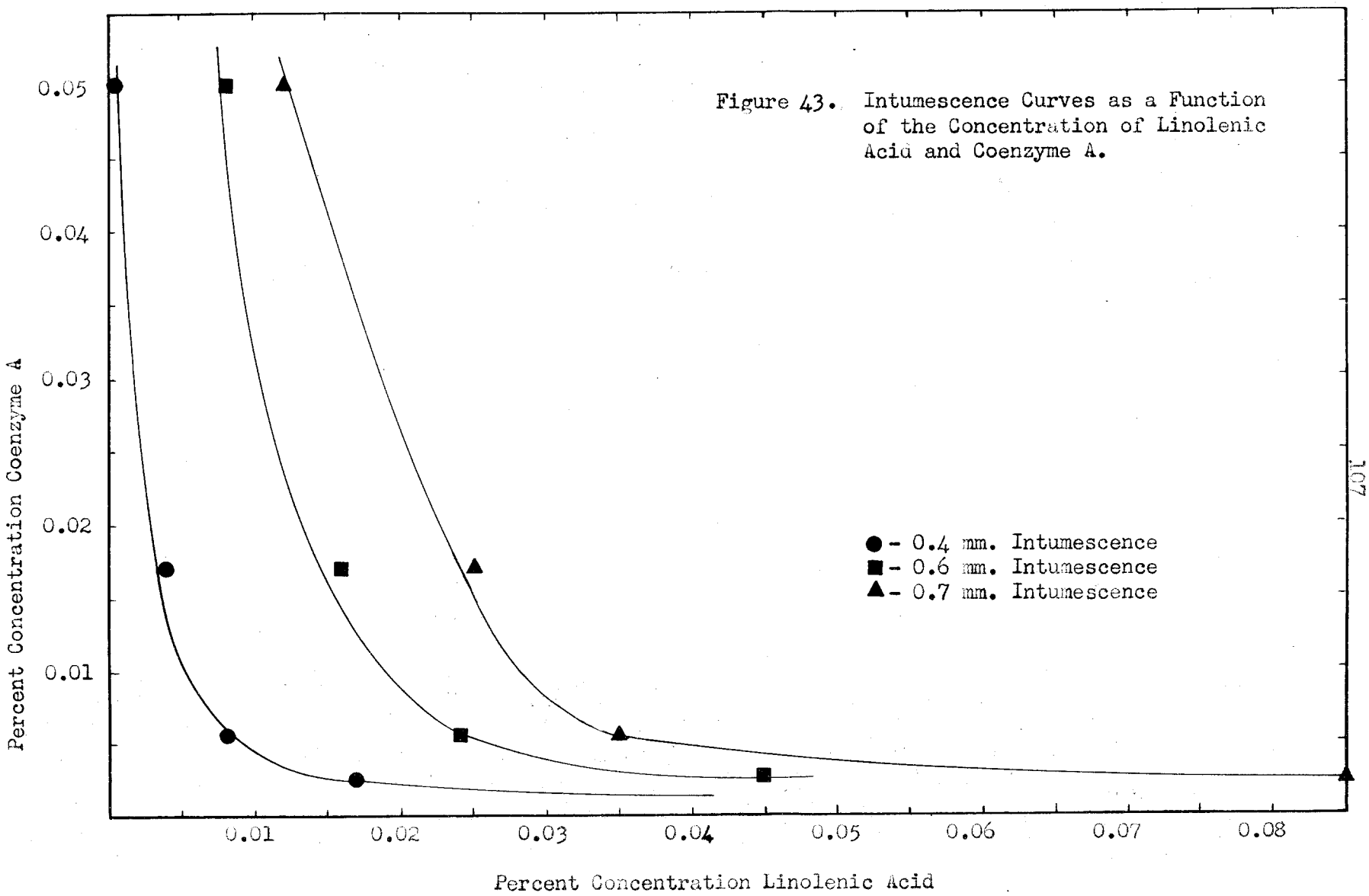
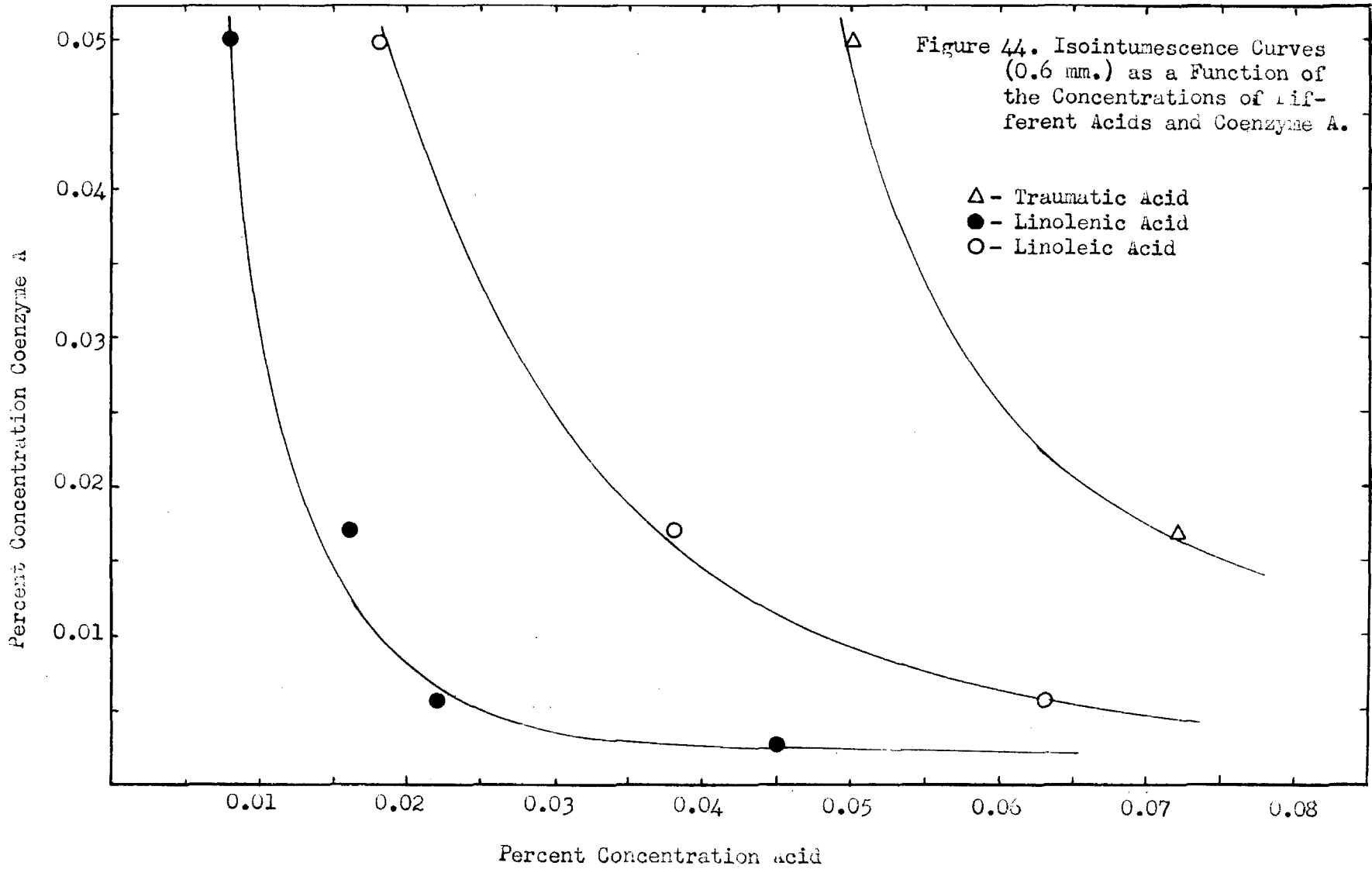


Figure 43. Intumescence Curves as a Function of the Concentration of Linolenic Acid and Coenzyme A.


0.05
0.04
0.03
0.02
0.01
Percent Concentration Coenzyme A
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
Percent Concentration Acid
Figure 44. Isointumescence Curves (0.6 mm.) as a Function of the Concentrations of Different Acids and Coenzyme A.
Δ - Traumatic Acid
● - Linolenic Acid
○ - Linoleic Acid

G) EXPERIMENTAL

1/ Purified Oleic Acid (cis-9-Octadecenoic Acid)

Commercial oleic acid, a yellow viscous oil, was purified by the low temperature crystallization method of Shinowara and Brown (34). Thirty-two grams of commercial oleic acid was dissolved in 500 ml. of acetone and cooled by means of a methanol-dry ice bath to -30°C. as measured by a -100°C. to 50°C. pentane thermometer. The saturated acids precipitated at this temperature were removed by filtration and the filtrate cooled further to -60°C. The crude crystal fraction of oleic acid was collected by filtration and recrystallized 4 times from 240 ml. of acetone at the same temperature. The last crystal fraction was then dissolved in 150 ml. of acetone and cooled to -30°C. again to remove final traces of saturated acids. The acetone was removed from the filtrate under reduced pressure and the residual oil distilled at less than 10 μ vapor pressure. The distillate, 8.2 grams of colorless, water-clear oleic acid gave an iodine number (Wijs, one hour) of 87 (Theory 90).

2/ Purified Linoleic Acid (cis,cis-9,12-Octadecadienoic Acid)

Natural linoleic acid was prepared by the method of Brown (35)(36), low temperature fractional crystallization of the fatty acids of corn oil. The crude fatty acids were obtained by saponification of commercial corn oil after the method of Beal (37). Corn oil (500 ml.) was heated with one liter of 10% NaOH to emulsification then refluxed for several hours until clear. The hot soap solution was poured into 3 liters of hot 3% HCl solution. The clumps of free acid and salt were broken up with a current of steam. The dark

colored oil was collected with the aid of a separatory funnel, dried with anhydrous sodium sulfate and distilled at less than 10μ pressure to give 370 ml. of a clear colorless oil.

Thirty-eight grams of the fatty acid mixture were dissolved in 500 ml. of acetone and cooled by means of a methanol-dry ice bath then filtered to give crystal fractions at -30°C., -50°C., and -70°C. The -70°C. crystal fraction (9.3 gm.) was then dissolved in 140 ml. of petroleum ether (30-60°C.), cooled and the crystal fraction (5.6 gm.) at -48°C. collected. This fraction in turn was dissolved in 900 ml. of petroleum ether (30-60°C.), cooled and the crystal fraction (4.9 gm.) at -62°C. collected. The solvent was removed from the crystal fraction and the residual oil distilled at 10μ pressure to give a colorless distillate, linoleic acid. Iodine number (Wijs, one hour) 175, theory 181 .

3/ Purified α -Linoleic Acid

Purified α -Linoleic acid was prepared after the method of McCutcheon (38). One hundred grams of the fatty acids of corn oil were dissolved in 2 liters of petroleum ether (30-60°C.), chilled to 0°-10°C. for 30 minutes then filtered. The small amount of precipitate formed was discarded and the filtrate brominated at 10°-15°C. with 31 ml. of liquid bromine, than allowed to stand 30 minutes. The crystalline precipitate formed was collected on a suction filter then washed with fresh petroleum ether and recrystallized from ethylene dichloride. The pure crystalline tetrabromide (9.2 gm., m.p. 115°C.) was then treated with 10 grams of granulated zinc in 40 ml. of ethanol and refluxed for one hour. The solution was separated from residual

metallic zinc by decantation, then refluxed 3 minutes with 15 ml. of 15% NaOH, and poured into one liter of 3% sulfuric acid. The oil was collected with the aid of petroleum ether and dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residual oil distilled at less than 10μ pressure to give 4.2 grams of clear, colorless α-linoleic acid, iodine number (Wijs, one hour) 180, theory 181.

4/ Purified Linolenic Acid (cis,cis,cis-9,12,15-Octadecatrienoic Acid)

Purified natural linolenic acid was prepared after the method of Shinowara and Brown (39) from the fatty acids of raw linseed oil. The crude fatty acids were prepared by the method of Beal (37) then distilled at 10μ pressure to give a pale yellow distillate. Fatty acids from the distillate were dissolved in acetone (1 gm./12 ml.) and cooled by the usual dry-ice-methanol bath. Successive crystal fractions were filtered off at -17°C., -45°C., -55°C. and -65°C. and the filtrate fraction, richest in linolenic acid, collected. The residual pale colored oil remaining after removing the acetone at reduced pressure was dissolved in petroleum ether (30-60°C.) (1 gm./16 ml.) and re-crystallized 6-10 times. The colorless oil after distillation in vacuo (pressure about 10μ) gave an iodine number (Wijs, one hour) 260, theory 273.5 .

5/ Purified α-Linolenic Acid

The preparation of α-linolenic acid was accomplished by treating linseed oil fatty acids after the method of Brown (39). The mixture of distilled fatty acids (50 gm.) was dissolved in 950 gm. of ethyl ether and reacted in the cold (0°-10°C.) with liquid bromine

until a reddish coloration persisted. After remaining below 10°C. for 3 hours, the insoluble material was collected on a pre-cooled suction filter and the crystals washed several times with fresh solvent. The reaction yielded 24 grams of white crystalline hexabromide, m.p. 180°C. The hexabromide was suspended in 60 ml. of hot ethanol then refluxed one hour with 24 gm. of zinc dust with occasional addition of a few drops of concentrated HCl. The alcoholic solution was separated from the excess zinc by decantation, acidified to decompose zinc linolenate, evaporated to half-volume under reduced pressure and diluted with one liter of water. The oily layer (acid plus ethyl ester) was separated with the aid of ether then saponified by the method of Beal (37). The free acid distilled at 10 μ to give a clear colorless oil (iodine number (Wijs, one hour) 268, theory 273.5).

6/ Purified Elaidolinolenic Acid (trans,trans,trans-9,12,15-Octadecatrienoic Acid)

Elaidolinolenic Acid was prepared by selenium isomerization of natural linolenic acid after the method of Kass, Nichols and Burr (40). Fatty acids from linseed oil were esterified by refluxing with dry alcoholic HCl according to the method of McCutcheon (41). Two grams of selenium powder, kept in suspension with vigorous mixing, were heated with 200 grams of esters to 205°C.-215°C. in a nitrogen atmosphere. After 17 hours the esters were distilled(10 μ pressure)and saponified in the usual fashion. The saponified acids (145 gm.) were dissolved in 400 ml. of ether, cooled to 0°C. and brominated after the method of McCutcheon (41). The crude bromo-acid (19 gm.) was collected on a suction filter, washed with ether and recrystallized from xylene (13 gm., m.p. 168-170°C.). The purified

hexabromide was debrominated in the usual manner by refluxing with an equal weight of zinc and a trace of HCl in ethanol. The mixture of acid and ester was collected with the aid of petroleum ether upon addition of a dilute acid to the reaction mixture. The residual oil remaining after removal of the solvent at reduced pressure was saponified in a nitrogen atmosphere. The free acid was collected with petroleum ether after acidification of the soap mixture. Elaidolinolenic acid (4.5 gm., m.p. 29-30°C.) was obtained after 3 recrystallizations at -60°C. from a 5% petroleum ether solution.

7/ Purified α-Eleostearic Acid (cis,trans,trans-9,11,13-Octadecatrienoic Acid)

The α isomer of Eleostearic Acid was prepared from fresh Argentine Tung Oil after the method of Meyers, Kass and Burr (42). One hundred grams of fresh tung oil was saponified by refluxing with a mixture of 40 grams KOH, 30 ml. of water and 200 ml. of 95% ethanol under nitrogen. The petroleum ether layer was separated and diluted to give a 10-15% solution of crude acids. The solution was then cooled to give crystal fractions at 0°C. and -20°C. The α-Eleostearic acid crystal fraction (-20°C.), recrystalized twice at -20°C. from a 10% petroleum ether solution melted at 48-49°C., literature m.p. 49°C.

8/ Purified β-Eleostearic Acid (trans,trans,trans-9,11,13-Octadecatrienoic Acid)

The β isomer of Eleostearic Acid (m.p. 69°C.) was prepared by the method of Meyers, Kass and Burr (42). Fresh tung oil was isomerized to tung butter with flowers of sulfur, then saponified and purified by the procedure used for α-Eleostearic acid.

9/ Purified Lauric Acid (Dodecanoic Acid)

Commercial lauric acid (m.p. 40-41°C.) was purified by dissolving 5.0 grams in 25 ml. of absolute ethyl alcohol and cooling to -10°C. The resulting cake was put on a thick pad of filter paper at -10°C. and drained for several hours. After this process was repeated with purified crystals, the acid was distilled at less than 50 μ pressure to give a clear colorless oil which crystallized on cooling (m.p. 42-43°C., reported 43-44°C.).

PART X

DISCUSSION

The earlier investigations into the wound hormones of bean tissue led Bonner, English and Haagen-Smit to the isolation of traumatic acid from lipoid soluble substances. The compound, by itself, was found to vary markedly in activity on successive daily tests; whereas if an inactive standard mixture, prepared from water soluble, lipoid insoluble substances, was added,the response in the bean test was found to be enhanced and less subject to fluctuation. These investigators did not examine the water soluble mixture in detail but found that glutamic acid and sucrose possessed similar properties and could be substituted for it. From these investigations it was concluded that traumatic acid accounted for at most 10% of the activity present in the initial extract. The difference was ascribed to unrecognized enhancing effects of other water soluble substances as well as other wound substances. This contention was supported by the observations that one of the discarded fractions contained a water soluble unstable material capable of a large enhancement of the growth response to traumatic acid and that glutamic acid at higher concentrations possessed some wound hormone activity. Furthermore, by a different procedure Bonner and English had previously prepared a concentrate containing an active compound called traumatin. This water soluble oil contained nitrogen and possessed activity independent of other substances. It was not likely to contain traumatic acid. In view of these findings one can conclude that there are in beans, at least two wound response inducing systems, one involving lipoid

soluble as well as water soluble factors and the other involving just one water soluble factor.

Water Soluble, Lipoid Soluble Factors

The purpose of this investigation was to establish whether the wound activity of other plant products as determined by the bean test was attributable to the same wound hormones found in beans. Tests of various natural source materials indicated that citrus products would be a satisfactory starting material. For practical considerations a commercial preparation, lemon peel infusion, was chosen for the study of the water soluble factors. Preliminary purification procedures established that at least two factors were involved in the promotion of wound response. These factors could be separated by Norite treatment whereby one factor was adsorbed. This factor which could be eluted from the charcoal by an acetone-ammonia-water mixture was active by itself. However, its activity was considerably enhanced by the filtrate from charcoal adsorption. This enhancing effect was also observed in the chloroform extraction; the extract was active while the inextractable matter was slightly active, but proportional recombination restored the original activity. It was possible to purify a water soluble, lipoid soluble, active preparation by: chloroform extraction of an acidic aqueous solution, petroleum ether extraction of impurities, high vacuum distillation and extraction from chloroform by aqueous bicarbonate. This preparation, containing nitrogen (43), forming water soluble barium and lead salts and giving an inactive ethyl ester, showed many characteristics similar to traumatin but differed in several respects as shown in Table 31. Although the unknown stage of purification of

TABLE 31

Traumatin	Lipoid Soluble Factor from Lemon Peel Infusion	Traumatic Acid
Similarities:		
1. Soluble: water, acetone, alcohol. Insoluble: ether, ethyl acetate.	1. Soluble: water, acetone, alcohol. Insoluble: petroleum ether.	1. Soluble: alcohol, acetone, petroleum ether. Slightly soluble: water.
2. Stable to heat and alkali.	2. Stable to heat and alkali.	2. Stable to heat and alkali.
3. Forms water soluble barium salt and less soluble mercury salt.	3. Forms water soluble barium and lead salts.	3. Forms water insoluble barium salt.
4. Takes up hydrogen catalytically. (possibly 2 double bonds)	4. Takes up hydrogen catalytically.	4. Takes up hydrogen catalytically.
5. Contains nitrogen.	5. Contains nitrogen.	5. Contains no nitrogen ($C_{12}H_{22}O_4$).
6. Adsorbs on Norite and elutes with pyridine.	6. Adsorbs on Norite and elutes with acetone-water-ammonia.	6. - - - - - - - - - - - - - - -
7. Forms inactive methyl ester.	7. Forms inactive ethyl ester.	7. Forms inactive dimethyl ester.
8. Methyl ester distills at 165 to 185°C at 0.007 mm.	8. Free acid distills at 185 to 210°C at less than 0.010 mm.	8. Dimethyl ester distills at 140°C at less than 0.1 mm.
Dis-similarities:		
1. Insoluble chloroform.	1. Soluble chloroform.	1. Soluble chloroform.
2. Must be esterified with diazomethane. Cannot be esterified with alcohol and acid.	2. Easily esterified with alcohol and acid.	2. Easily esterified with diazomethane.
3. Equivalent weight by titration is about 220.	3. Equivalent weight by titration is about 155.	3. Equivalent weight by titration is about 118.
4. Active per se; no cofactor effect found.	4. Active per se; but activity enhanced by water soluble factors.	4. Active per se; but activity enhanced by cofactors.

both preparations makes a definite conclusion regarding the chemical and physical properties of the pure compound uncertain, there is reason to believe that these factors are not identical.

Lipoid Soluble Factor

Tests of slightly milky water extracts of the outer colored portion of orange peels indicated considerable activity. Active fractions could also be obtained from the alkali extracts of pressed oil of the peel. Since the oil would not contain the water soluble factors of type W, orange oil was used in the isolation of lipoid soluble factors. As in the case of the purification of the factor from lemon peel infusion, enhancement was found in the presence of factor W. Therefore, during the purification, this factor was added in the tests.

An enriched active preparation from orange oil was obtained by: fractional distillation of the oil, bicarbonate extraction of the high boiling distillate, removal of impurities by crystallization from methanol and petroleum ether and a second fractional distillation. The active material was found in the highest boiling fraction. Elemental analysis, iodine number and equivalent weight determination (by titration) of the inactive crystalline impurities indicated saturated fatty acids; iodine number and equivalent weight determination (by titration) of the active oily high boiling distillate indicated the presence of unsaturated fatty acids. These results are supported by the work of Matlack (44,45) who identified palmitic, stearic, oleic, linoleic and linolenic acids comprising the bulk of the free fatty acids of the California Valencia Orange, and of Markley and co-workers

(46) who found linoleic and linolenic acids comprising the bulk of the free fatty acids of the peel oil of the Florida Grapefruit. Activity tests with preparations of linoleic and linolenic acids purified from other sources were found strongly positive. It therefore seems quite likely that the wound activity of the oil of orange is due to the essential fatty acids present.

Character of Known Growth Promoting Agents in the Bean Test

In the dicarboxylic acid series, the presence of a double bond gives rise to a two fold increase in activity over the saturated analogue (13,29). There is no apparent difference in activity between α or β unsaturation. Unsaturation at both ends of the chain making the compound symmetrical with respect to functional groups shows no greater activity than the singly unsaturated compound.

Activity in this series begins with a carbon chain length of 8 carbon atoms and continues to a chain length of at least 15 carbon atoms. From an α unsaturated C_{10} acid to an α unsaturated C_{15} acid the activity remains the same.

The effect of cis-trans isomerism in dicarboxylic acids was studied in the case of traumatic acid and no difference in activity was found. The other unsaturated members of the series have not been examined.

The activity of saturated dicarboxylic acids is increased by the presence of oxygen substituents such as hydroxyl or carbonyl groups. Several oxygen derivatives of the saturated members of the series are just as active as the unsaturated compounds even though the oxygen substituent is several carbons removed from the position

of the double bond. For example in nonane-1,9-dicarboxylic acid a hydroxyl or carbonyl group in the 5 position is just as effective as the double bond in the 1 or 2 position.

In the fatty acid series the presence of a single double bond in the alkyl chain does not appear sufficient for activity; oleic acid is inactive.* If a second double bond is introduced as in linoleic acid, the compound becomes active, while a third double bond as in linolenic acid makes the compound very active (Table 30).

---

* Oleic Acid (9-Octadecenoic Acid)

$$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\underset{\text{cis}}{CH = CH}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

Linoleic Acid (9,12-Octadecadionic Acid)

$$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\underset{\text{cis}}{CH = CH}\text{-}CH_2\text{-}\underset{\text{cis}}{CH = CH}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

Linolenic Acid (9,12,15-Octadecatrienoic Acid)

$$CH_3\text{-}CH_2\text{-}\underset{\text{cis}}{CH = CH}\text{-}CH_2\text{-}\underset{\text{cis}}{CH = CH}\text{-}CH_2\text{-}\underset{\text{cis}}{CH = CH}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

Elaidolinolenic Acid (9,12,15-Octadecatrienoic Acid)

$$CH_3\text{-}CH_2\text{-}\underset{\text{trans}}{CH = CH}\text{-}CH_2\text{-}\underset{\text{trans}}{CH = CH}\text{-}CH_2\text{-}\underset{\text{trans}}{CH = CH}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

α-Eleostearic Acid (9,11,13-Octadecatrienoic Acid)

$$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\underset{\text{cis}}{CH = CH}\text{-}\underset{\text{trans}}{CH = CH}\text{-}\underset{\text{trans}}{CH = CH}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

β-Eleostearic Acid (9,11,13-Octadecatrienoic Acid)

$$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\underset{\text{trans}}{CH = CH}\text{-}\underset{\text{trans}}{CH = CH}\text{-}\underset{\text{trans}}{CH = CH}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

The effect of chain length in the fatty acid series is very interesting in that activity first appears in the saturated C_{10} acid, becomes strong in the C_{12} acid, decreases in the C_{14} acid and then disappears. It shows up again very strongly in the higher unsaturated C_{18} acids (Table 30).

The marked effect of geometrical isomerism is seen in the great difference in activity of active all cis-linolenic acid as compared to slightly active all trans-elaidolinolenic acid. In a further attempt to correlate the presence of double bonds and the effect of cis-trans isomerism on activity, α- and β-eleostearic acids were tested and found to be slightly active. Both compounds show less activity than the corresponding C_{18} acids with isolated double bonds. The decrease in activity may be caused by the trans configuration of the double bonds or the presence of conjugation. A definite conclusion must await the testing of conjugated acids of the cis configuration.

The results of activity tests with unsaturated fatty acids indicated a correlation between activity in the bean test and effectiveness as a substrate for the enzyme lipoxidase (47,48). This enzyme first known as the carotene-destroying enzyme is able to use gaseous oxygen to oxidize linoleic, linolenic and arachidonic acids (49). It occurs in highest concentration in soybeans but is generally present in Leguminosae as well as other plants and animal tissues (50,51,52,53,54,55,56). Balls and co-workers (57) as well as Sumner (58) have found that oleic acid is not attacked and Sumner concluded that the substrate must contain:

$$-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH.$$

This however, is not consistent with lipoxidase action on arachidonic acid (5,8,11,14-eicosatetraenoic acid). Both Strain (53) and Sumner found that trans isomers were not attacked. Kies (59) observed that fatty acids containing the conjugated grouping:

$$-CH = CH-CH = CH-$$

were not affected by lipoxidase. It is striking that modifications which render compounds inactive as substrates also render them inactive in the bean test.

In view of the parallel action on essential fatty acids by lipoxidase and bean tissue one might suspect that lipoxidase in the bean cells catalyzes the oxidation to products affecting metabolic processes. The initial step of wound response could be visualized as involving an oxidative attack on the unsaturated fatty acid by lipoxidase. Since current belief is that lipoxidase acts as a free radical initiator for oxidation similar to the scheme suggested for autoxidation, this concept receives support from the observation that linolenic acid, autoxidized in air after the method of Farmer (30), is able to adsorb up to 20% of its weight in oxygen during a period of 4 days with no loss of activity.

The Function of Other Water Soluble Factors

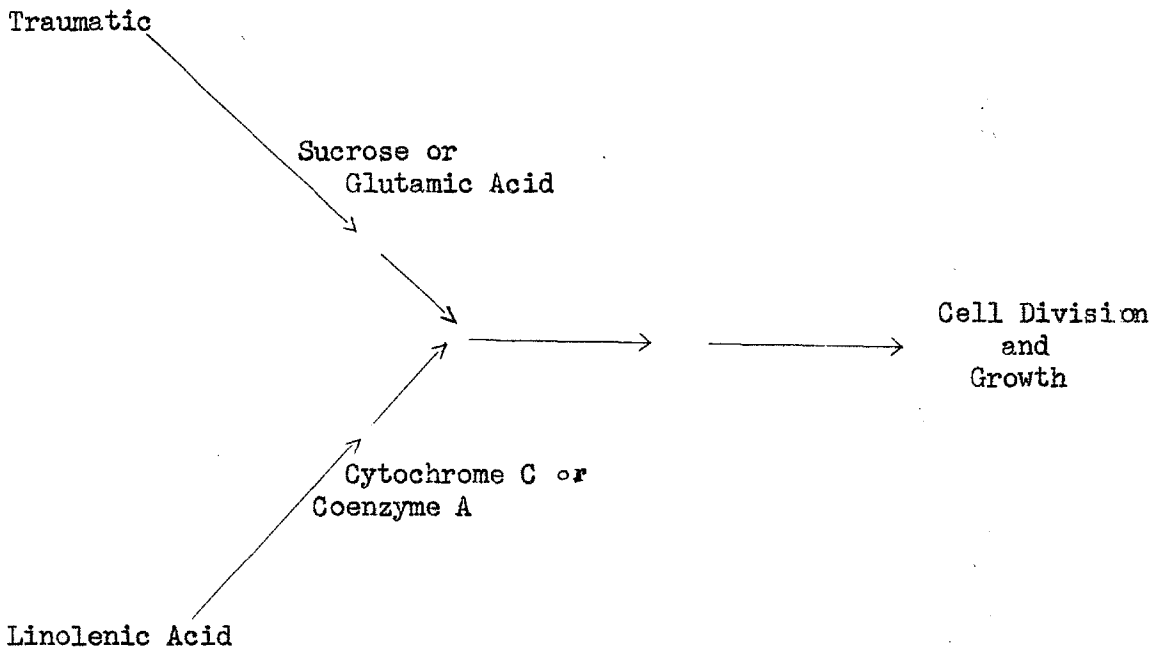
The role of other water soluble factors in wound response is not completely clear. Bonner, English and Haagen-Smit (11) have suggested that substances which they call cofactors are present in variable amounts in test beans themselves. The diversity of water soluble substances which are able to augment the response of the lipoid soluble factors would indicate that each substance supplements

a deficiency in the cells at a different step in the complex wound reaction. It may be expected that a combination of water soluble factors would be more effective in enhancing the activity of lipoid soluble substances; there is some indication of this in the combinations of Adenosine Triphosphate-Ascorbic Acid and Cytochrome C-Ascorbic Acid which are more effective than the individual compounds (Table 26).

In work on traumatic acid Bonner, English and Haagen-Smit found that several pure substances, sucrose or glutamic acid, inactive themselves, could augment the response to the isolated hormone. In the present investigation several other naturally occuring substances, linoleic and linolenic acids, were found capable of wound hormone activity especially when combined with water soluble Cytochrome C, Coenzyme A, etc. If traumatic acid is the active agent in the wound response, formed in the degradation of linolenic acid as some authors believe (60,61,62), then at comparable concentrations it could be expected that at best, linolenic acid would be as active as traumatic. Results show however, that linolenic acid is several times more active than traumatic acid. This would indicate therefore, that traumatic and linolenic acids independently give rise to wound active substances as shown in Fig. 45.

Figure 45

Pathway of Activity



Some 25 years have passed since linolenic and linoleic acids were recognized as an essential part of the diet of animals and since then, questions have been raised as to their function in the growth process. From existing literature it is evident that no specific responses can be attributed to the presence of these fatty acids and no mechanism for their action proposed. The finding that linoleic and linolenic acids cause an easily reproducible, localized response in readily available material could be of importance for the study of the mechanism of action of the essential fatty acids.

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APPENDIX

Statistical Evaluations of Observed Intumescence for Figures 27-40.

ACID	Concentration Acid %	Concentration Coenzyme A %	Average Intumescence mm.	Average Deviation mm.	Variance $mm.^2$	Standard Deviation mm.
Linolenic	0.10	0.05	1.08	0.09	0.0124	0.11
	0.03		0.87	0.11	0.0187	0.14
	0.01		0.65	0.07	0.0075	0.09
	0.004		0.46	0.06	0.0064	0.08
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.017	0.95	0.06	0.0063	0.08
	0.03		0.78	0.06	0.0052	0.07
	0.01		0.49	0.08	0.0092	0.10
	0.004		0.40	0.05	0.0034	0.06
	0.00		0.34	0.05	0.0031	0.06
	0.10	0.0056	0.86	0.05	0.0049	0.07
	0.03		0.72	0.05	0.0045	0.07
	0.01		0.46	0.04	0.0034	0.06
	0.004		0.33	0.05	0.0039	0.06
	0.00		0.30	0.06	0.0046	0.07
	0.10	0.0025	0.72	0.05	0.0050	0.07
	0.03		0.56	0.06	0.0050	0.07
	0.01		0.35	0.05	0.0041	0.06
	0.004		0.24	0.04	0.0032	0.06
	0.00		0.20	0.04	0.0022	0.05
	0.10	0.00	0.38	0.07	0.0060	0.08
	0.03		0.26	0.04	0.0032	0.06
	0.01		0.20	0.04	0.0022	0.05
	0.004		0.14	0.03	0.0019	0.04
	0.00		0.14	0.03	0.0022	0.05
Linoleic	0.10	0.05	1.02	0.09	0.0121	0.11
	0.03		0.71	0.08	0.0092	0.10
	0.01		0.51	0.05	0.0037	0.06
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.017	0.82	0.06	0.0066	0.08
	0.03		0.56	0.05	0.0045	0.07
	0.01		0.46	0.07	0.0068	0.08
	0.00		0.34	0.05	0.0031	0.06
	0.10	0.0056	0.72	0.07	0.0083	0.09
	0.03		0.49	0.05	0.0036	0.06
	0.01		0.38	0.05	0.0052	0.07
	0.00		0.30	0.06	0.0046	0.07
	0.10	0.0025	0.57	0.07	0.0067	0.08
	0.03		0.38	0.06	0.0075	0.09
	0.01		0.29	0.04	0.0026	0.05
	0.00		0.20	0.04	0.0022	0.05
	0.10	0.00	0.38	0.06	0.0055	0.07
	0.03		0.24	0.06	0.0053	0.07

ACID	Concentration Acid %	Concentration Coenzyme A %	Average Intumescence mm.	Average Deviation mm.	Variance mm.^{2}	Standard Deviation mm.
Linoleic (cont.)	0.01		0.14	0.05	0.0033	0.06
	0.00		0.14	0.03	0.0022	0.05
Capric	0.10	0.05	0.77	0.07	0.0084	0.09
	0.03		0.53	0.11	0.0184	0.14
	0.00		0.34	0.05	0.0058	0.08
	0.10	0.017	0.67	0.07	0.0084	0.09
	0.03		0.54	0.07	0.0083	0.09
	0.00		0.33	0.06	0.0044	0.07
	0.10	0.0056	0.58	0.06	0.0070	0.08
	0.03		0.49	0.06	0.0066	0.08
	0.00		0.29	0.06	0.0053	0.07
	0.10	0.00	0.48	0.05	0.0039	0.06
	0.03		0.38	0.06	0.0054	0.07
	0.00		0.23	0.06	0.0045	0.07
Lauric	0.10	0.05	1.10	0.11	0.0201	0.14
	0.03		0.81	0.08	0.0098	0.10
	0.01		0.49	0.12	0.0177	0.13
	0.00		0.34	0.05	0.0058	0.08
	0.10	0.017	0.95	0.08	0.0107	0.10
	0.03		0.76	0.09	0.0125	0.11
	0.01		0.44	0.08	0.0096	0.10
	0.00		0.33	0.06	0.0044	0.07
	0.10	0.0056	0.87	0.09	0.0123	0.11
	0.03		0.66	0.10	0.0145	0.12
	0.01		0.41	0.11	0.0172	0.13
	0.00		0.29	0.06	0.0053	0.07
	0.10	0.00	0.48	0.08	0.0120	0.11
	0.03		0.40	0.07	0.0074	0.09
	0.01		0.24	0.07	0.0081	0.09
	0.00		0.23	0.06	0.0045	0.07
Myristic	0.10	0.05	0.76	0.08	0.0095	0.10
	0.03		0.48	0.07	0.0068	0.08
	0.00		0.34	0.05	0.0058	0.08
	0.10	0.017	0.68	0.07	0.0079	0.09
	0.03		0.43	0.09	0.0122	0.11
	0.00		0.33	0.06	0.0044	0.07
	0.10	0.0056	0.57	0.08	0.0100	0.10
	0.03		0.36	0.06	0.0060	0.08
	0.00		0.29	0.06	0.0053	0.07
	0.10	0.00	0.48	0.07	0.0085	0.09
	0.03		0.24	0.06	0.0057	0.08
	0.00		0.23	0.06	0.0045	0.07

ACID	Concentration Acid %	Concentration Coenzyme A %	Average Intumescence mm.	Average Deviation mm.	Variance $mm.^2$	Standard Deviation mm.
trans-Traumatic	0.10	0.05	0.67	0.10	0.0164	0.13
	0.03		0.57	0.06	0.0068	0.08
	0.01		0.48	0.04	0.0030	0.05
	0.00		0.48	0.08	0.0098	0.10
	0.10	0.017	0.58	0.10	0.0136	0.12
	0.03		0.48	0.07	0.0079	0.09
	0.10		0.42	0.07	0.0056	0.08
	0.00		0.38	0.07	0.0070	0.08
	0.10	0.0056	0.49	0.07	0.0077	0.09
	0.03		0.39	0.08	0.0093	0.10
	0.01		0.34	0.05	0.0040	0.06
	0.00		0.29	0.04	0.0040	0.06
	0.10	0.002	0.33	0.07	0.0077	0.09
	0.03		0.28	0.05	0.0046	0.07
	0.01		0.26	0.07	0.0060	0.08
	0.00		0.19	0.06	0.0042	0.07
	0.10	0.00	0.19	0.08	0.0090	0.10
	0.03		0.20	0.05	0.0041	0.06
	0.01		0.19	0.06	0.0050	0.07
	0.00		0.19	0.06	0.0042	0.07
cis-Traumatic	0.10	0.05	0.58	0.08	0.0122	0.11
	0.03		0.52	0.09	0.0128	0.11
	0.01		0.47	0.07	0.0084	0.09
	0.00		0.48	0.08	0.0098	0.10
	0.10	0.017	0.52	0.07	0.0064	0.08
	0.03		0.43	0.07	0.0079	0.09
	0.01		0.39	0.07	0.0070	0.08
	0.00		0.38	0.07	0.0070	0.08
	0.10	0.0056	0.39	0.07	0.0068	0.08
	0.03		0.33	0.04	0.0025	0.05
	0.01		0.29	0.07	0.0070	0.08
	0.00		0.29	0.04	0.0040	0.06
	0.10	0.00	0.20	0.04	0.0034	0.06
	0.03		0.19	0.04	0.0035	0.06
	0.01		0.20	0.05	0.0045	0.07
	0.00		0.19	0.06	0.0042	0.07
Decane-1,10-dicarboxylic	0.10	0.05	0.57	0.13	0.0205	0.14
	0.03		0.53	0.06	0.0076	0.09
	0.01		0.48	0.08	0.0097	0.10
	0.00		0.48	0.08	0.0098	0.10
	0.10	0.017	0.53	0.09	0.0105	0.10

ACID	Concentration Acid %	Concentration Coenzyme A %	Average Intumescence mm.	Average Deviation mm.	Variance $mm.^2$	Standard Deviation mm.
Decane-1,10-dicarboxylic (cont.)	0.03		0.42	0.06	0.0058	0.08
	0.01		0.42	0.09	0.0130	0.11
	0.00		0.38	0.07	0.0070	0.08
	0.10	0.0056	0.43	0.09	0.0104	0.10
	0.03		0.34	0.05	0.0040	0.06
	0.01		0.29	0.06	0.0061	0.08
	0.00		0.29	0.04	0.0040	0.06
	0.10	0.00	0.24	0.03	0.0020	0.04
	0.03		0.22	0.07	0.0061	0.08
	0.01		0.25	0.05	0.0047	0.07
	0.00		0.19	0.06	0.0042	0.07
Sebacic	0.10	0.05	0.67	0.04	0.0033	0.06
	0.03		0.58	0.11	0.0177	0.13
	0.01		0.48	0.09	0.0129	0.11
	0.00		0.48	0.08	0.0098	0.10
	0.10	0.017	0.58	0.10	0.0142	0.12
	0.03		0.53	0.08	0.0107	0.10
	0.01		0.48	0.07	0.0074	0.09
	0.00		0.38	0.07	0.0070	0.08
	0.10	0.0056	0.48	0.07	0.0093	0.10
	0.03		0.38	0.07	0.0094	0.10
	0.01		0.33	0.06	0.0056	0.07
	0.00		0.29	0.04	0.0040	0.06
	0.10	0.00	0.20	0.04	0.0031	0.06
	0.03		0.19	0.05	0.0041	0.06
	0.01		0.19	0.03	0.0014	0.04
	0.00		0.19	0.06	0.0042	0.07
α-Linoleic	0.10	0.05	1.05	0.08	0.0096	0.10
	0.03		0.72	0.05	0.0064	0.08
	0.01		0.58	0.08	0.0088	0.09
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.017	0.91	0.07	0.0067	0.08
	0.03		0.58	0.06	0.0059	0.08
	0.01		0.48	0.04	0.0033	0.06
	0.00		0.34	0.05	0.0031	0.06
	0.10	0.0056	0.77	0.05	0.0037	0.06
	0.03		0.48	0.07	0.0077	0.09
	0.01		0.39	0.05	0.0040	0.06
	0.00		0.30	0.06	0.0046	0.07
	0.10	0.00	0.54	0.10	0.0134	0.12
	0.03		0.23	0.03	0.0014	0.04
	0.01		0.14	0.04	0.0023	0.05
	0.00		0.14	0.03	0.0022	0.05

ACID	Concentration Acid %	Concentration Coenzyme A %	Average Intumescence mm.	Average Deviation mm.	Variance mm.^{2}	Standard Deviation mm.
α-Linolenic						
	0.10	0.05	1.20	0.06	0.0053	0.07
	0.03		0.81	0.08	0.0098	0.10
	0.01		0.68	0.06	0.0064	0.08
	0.004		0.52	0.08	0.0099	0.10
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.017	1.05	0.12	0.0178	0.13
	0.03		0.72	0.07	0.0077	0.09
	0.01		0.58	0.05	0.0053	0.07
	0.004		0.43	0.05	0.0031	0.06
	0.00		0.34	0.05	0.0031	0.06
	0.10	0.0056	0.87	0.07	0.0070	0.08
	0.03		0.62	0.06	0.0068	0.08
	0.01		0.48	0.06	0.0050	0.07
	0.004		0.34	0.07	0.0062	0.08
	0.00		0.30	0.06	0.0046	0.07
	0.10	0.00	0.53	0.10	0.0140	0.12
	0.03		0.39	0.06	0.0059	0.08
	0.01		0.30	0.06	0.0041	0.07
	0.00		0.14	0.03	0.0022	0.05
Elaidolin-olenic	0.10	0.05	0.68	0.07	0.0074	0.09
	0.03		0.58	0.07	0.0070	0.08
	0.01		0.48	0.07	0.0070	0.08
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.017	0.58	0.08	0.0080	0.09
	0.03		0.52	0.08	0.0103	0.10
	0.01		0.43	0.08	0.0107	0.10
	0.00		0.30	0.05	0.0031	0.06
	0.10	0.0056	0.52	0.09	0.0120	0.11
	0.03		0.48	0.04	0.0045	0.07
	0.01		0.38	0.07	0.0091	0.10
	0.00		0.30	0.06	0.0046	0.07
	0.10	0.00	0.24	0.06	0.0068	0.08
	0.03		0.23	0.06	0.0051	0.07
	0.01		0.15	0.04	0.0024	0.05
	0.00		0.14	0.03	0.0022	0.05
α-Eleostearic						
	0.10	0.05	0.67	0.06	0.0080	0.09
	0.03		0.67	0.09	0.0112	0.11
	0.01		0.53	0.09	0.0099	0.10
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.017	0.68	0.08	0.0089	0.09
	0.03		0.67	0.07	0.0072	0.09
	0.01		0.54	0.08	0.0091	0.10
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.0056	0.57	0.07	0.0080	0.09

ACID	Concentration Acid %	Concentration Coenzyme A %	Average Intumescence mm.	Average Deviation mm.	Variance $mm.^2$	Standard Deviation mm.
α-Eleostearic (cont.)						
	0.03		0.57	0.09	0.0096	0.10
	0.01		0.48	0.06	0.0054	0.07
	0.00		0.30	0.06	0.0046	0.07
	0.10	0.0025	0.43	0.06	0.0067	0.08
	0.03		0.43	0.04	0.0044	0.07
	0.01		0.38	0.04	0.0047	0.07
	0.00		0.19	0.05	0.0041	0.06
	0.10	0.00	0.29	0.05	0.0043	0.07
	0.03		0.24	0.07	0.0079	0.09
	0.01		0.24	0.06	0.0062	0.08
	0.00		0.14	0.03	0.0022	0.05
β-Eleostearic						
	0.10	0.05	0.81	0.09	0.0120	0.11
	0.03		0.61	0.06	0.0063	0.08
	0.01		0.48	0.07	0.0084	0.09
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.017	0.71	0.06	0.0072	0.09
	0.03		0.57	0.09	0.0112	0.11
	0.01		0.44	0.07	0.0065	0.08
	0.00		0.37	0.06	0.0068	0.08
	0.10	0.0056	0.63	0.07	0.0076	0.09
	0.03		0.53	0.06	0.0055	0.07
	0.01		0.39	0.07	0.0061	0.08
	0.00		0.30	0.06	0.0046	0.07
	0.10	0.00	0.48	0.07	0.0059	0.08
	0.03		0.28	0.04	0.0023	0.05
	0.01		0.19	0.03	0.0018	0.04
	0.00		0.14	0.03	0.0022	0.05

PROPOSITIONS

1. The mechanism of action of the essential fatty acids in the Wehnelt test involves an oxidative process.

2. Meites (1) has postulated that the wound hormone, traumatic acid is formed by degradation of the higher saturated fatty acids. Nye and Spoehr (2) have suggested that traumatic acid is formed by degradation of linolenic acid. These postulates are inadequate in explaining the activity of the parent compounds.

(1) Meites, M. Bull. Soc. Chim. biol. (1945) 27 438
(2) Nye, W. and Spoehr, H.A. Arch. Biochem (1943) 2 23

3. Several investigators (1,2) have found that sugars are necessary for the bio-synthesis of anthocyanidins, but the mechanism involved is uncertain. It has been suggested that C_6 and C_6-C_3 fragments are intermediates (3). This interpretation is considered correct.

(1) Stadler, L.J. Amer. Jour. Bot. (1942) 29 17
(2) Thimann, K.V. and Edmondsen, Y. Arch. Biochem (1949) 22 33
(3) Geissman, T.A. and Hinreiner, E. Bot. Rev. (1952) 18 77,165

4. Golombic (1), Calkins (2) and Privett and Quackenbush (3) have postulated mechanisms by which the synergist functions in synergist-antioxidant combinations for the preservation of fats.

a) It is unlikely that all compounds capable of synergistic action function by the same mechanism and,

b) An insight into the mechanism of action of organic acid synergists may be obtained by investigating their fate in an inhibition reaction.

(1) Golombic, C. J. Amer. Oil Chemists Soc. (1946) 23 184
(2) Calkins, V.P. J. Amer. Chem. Soc. (1947) 69 384
(3) Privett, O.S. and Quackenbush, F.W. J. Amer. Oil Chemists Soc. (1954) 31 321

5. Satisfactory data for the physical constants of many chemical compounds are not existent. It is proposed that the American Chemical Society enlist the aid of other interested groups and actively support an organization for procuring such data.

6. The cysts of the parasite Heterodera rostochiensis, hatch upon stimulation by root excretion of the host plant (1,2). This hatching factor is an intermediate utilized in normal cellular metabolism by the host plant.

(1) Calam, C.T., Raistrick, H. and Todd, A.R. Biochem. J. (1949) 45 513
(2) Massey, L.M., Jr. and Neal, A.L. Jour. Wash. Acad. Sci. (1953) 43 396

7. Tissue extracts from the potato, Solanum tuberosum, have been found effective in the promotion of cell division (1,2,3). This activity of potato extracts can be explained by the presence of the higher fatty acids (4).

(1) Haberlandt, G. Beitr. allg. Bot. (1921) 2 1
(2) Bonner, J. and English, J. Jr. Plant Physiol. (1938) 13 331
(3) This Thesis page 19
(4) Völksen, Wilhelm Arch. Pharm. (1950) 283 203

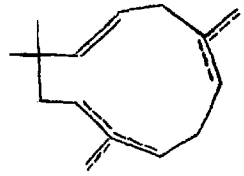
8. After autoxidation fats containing linolenic acid, give a color reaction with thiobarbituric acid (1). The color reaction involves the condensation of thiobarbituric acid with Δ^3-hexen-1,6-dial.

(1) Progress In The Chemistry of Fats and Other Lipids (1954) 2 87

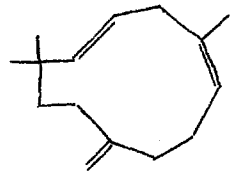
9. Excised flowers cultured *in vitro* develop into miniature fruits(1). The inability of the fruits to develop normally may be due to inadequate culture techniques besides possible requirement of special fruit growth factors.

(1) Nitsch, J.P. Ann. Rev. of Plant Physiol. (1953) 4 201

10. The structure of humulene has been shown to be



with the position of two double bonds uncertain (1). It is proposed that the structure



is to be preferred.

(1) Faucett, R.W. and Harris, J.O. J. Chem. Soc. (1954) 2673