THE INVESTIGATION OF CERTAIN ANTIBACTERIALS IN POPULUS TACAMAHACA MILL. AND HYPERICUM PROLIFICUM

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Gerald Gean Dull

1956

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A THESIS

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1956

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. James L. Fairley for the encouragement and stimulating guidance throughout the duration of this work. The author would also like to thank Dr. E. H. Lucas, who initiated this problem, for his help and suggestions.

The author would also like to express his thanks to the National Institute of Health for providing funds in support of this work.

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VITA

The author was born in Laurel, Montana on the 20th of August, 1930. He received his secondary schooling at Glasgow High School, Glasgow, Montana.

The author graduated from Montana State College in Bozeman, Montana, June 1952 with a Bachelor of Science degree in chemistry. He enrolled at Michigan State University in September 1952. During his time at Michigan State University, he was a Graduate Teaching Assistant for eight quarters and a Special Graduate Research Assistant for seven quarters. He also worked for the E. I. duPont deNemours Company during the summers of 1952 and 1953.

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AN ABSTRACT

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Approved James L Fairley

ABSTRACT

Two sesquiterpene alcohols, one of which was identified as an isomer of bisabolol, were isolated from the buds of Populus tacamahaca Mill, by means of solvent extraction, fractional distillation and chromatographic techniques. Both substances showed in vitro activity against Mycobacterium tuberculosis at the level of five micrograms per milliliter.

An ethyl acetate extract of the flowers of H. prolificum yielded, after purification by adsorption chromatography, a highly antibacterial substance. The structure of this principle has not been established as yet but the substance has been shown to contain an aromatic nucleus, a hydroxyl group and a carbonyl group. The absence of nitrogen, sulfur and halogens has been established and it appears that the molecule is composed of only carbon, hydrogen and oxygen.

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HISTORICAL

From the start of recorded history man has been engaged in a fight against infectious diseases, and the plant world has provided a bountiful source of materials used in this struggle. Ancient Chinese literature records that Shên-nung (2738-2698 B. C.) investigated the pharmacological effect of some plant materials. In the many centuries following his work additional information was collected and in 1597 A. D. Li Shih-chên produced a 52-volume series on Chinese medicinal materials (including many plant sources) entitled Pên-ts'ao kang-mu. An interesting discussion of Chinese medicinal materials has been prepared by Mosig and Schramm (1). The Chinese were not alone in their endeavors to find plant sources of medicines, as the Egyptians were also enlightened in this area. The Papyrus Ebers (1500 B. C.) (2) represents another example of ancient records which include descriptions of the use of plants for medicinal purposes. The art of using plants as sources of medications for human ailments was kept alive by Hippocrates (460-361 B. C) and Galen (103-193 A. D.). These two men helped bridge the gap from the time of the Chinese herbists to the seventeenth century herbists such as John Gerard. Gerard, an Englishman, compiled a large amount of information on herbs. This information may be found in the revised edition of The Herball of General Historie of Plants (3). Gerard reported that an extract of the cyperus plant could affect miraculous cures of illnesses ranging from dropsy and snake bites to mental tension.

Although much of the early work was done with crude plant extracts, more recent chemotherapeutic investigations concern the isolation and identification of specific compounds from both high and low forms of plants. The isolation of penicillin (4) from Penicillium notatum is a classic example of a lower plant being the source of a chemotherapeutic agent.

Similar isolations have been done with higher plants but results have been somewhat less spectacular. The red and yellow varieties of the common onion (Allium cepa) are not susceptible to the fungal disease Colletotrichum circinans (Berk) Vogl, while the white varieties are susceptible. In 1933 Link et al. (5) isolated 3,5-dihydroxybenzoic acid (protocatechuic acid) from the scales of the pigmented variety. The compound was found to be toxic to C. circinans in an aqueous dilution of 1:3000. Partial purification of extracts of sweet potato (6), banana (7), and Indian carrot (8) has produced materials with weak antibacterial activity. Lucas and co-workers have been conducting an extensive screening program (9) with the intent of finding seed plant sources of potent antibacterials. They found (10) that extracts of many plants demonstrated appreciable antibacterial activity. Of these, Populus tacamahaca Mill. and Hypericum prolificum extracts exhibited extremely high antibacterial activities. On the basis of these findings this research project was initiated with the purpose of isolating and identifying the main active principles of these two plants.

PART I

INVESTIGATION OF SOME ANTIBACTERIAL PRINCIPLES IN BUDS OF *POPULUS TACAMAHACA* MILL.

INTRODUCTION

A commercially available oil (Balm of Gilead) from the buds of Populus candicans Ait. and Populus tacamahaca Mill. (also known as Populus balsamifera L.) has been used in the perfume industry for many years. However, the first interest in the antibacterial properties of poplar bud oil arose when Lucas and co-workers found (10) that ethanolic and hot aqueous extracts of the leaves and buds of P. tacamahaca were active against Mycobacterium tuberculosis and Micrococcus pyogenes, var. aureus. On the basis of the findings of Lucas et al., William Houff and George Levitt carried out some preliminary isolation experiments (unpublished work) under the guidance of Dr. H. M. Sell. These experiments were unsuccessful with respect to the isolation of the active principle involved.

Prior to the interest in the antibacterial properties, several groups did considerable work on the isolation and identification of constituents of poplar bud oil. In 1924, Nakao (11) isolated the sesquiterpene "populene" from the buds of Populus nigra. He also mentioned the possibility of the presence of sesquiterpene alcohols in the oil. In 1936, Goris and Canal (12) isolated and identified many constituents of poplar bud oil from P. balsamifera, one of which was a sesquiterpene alcohol. Šorm et al., (13), in 1952, identified the major terpenic constituents in poplar bud oil from P. balsamifera as cineol, d-cadinene, ar-curcumen, farnesene and α-d-bisabolol. Using this

previous work as a basis, the isolation and identification of the antibacterial substances in poplar bud oil from P. tacamahaca was undertaken.

EXPERIMENTAL AND RESULTS

Bioassay

The purification procedures were followed by biological assay* against Mycobacterium tuberculosis and Micrococcus pyogenes var. aureus. The assays were designed and directed by Dr. R. Y. Gottshall at the Division of Laboratories, Michigan Department of Health. A discussion of the methods of assay will be found in Appendix II. The activities are expressed as the least number of micrograms (γ) per milliliter of culture broth which inhibits bacterial growth.

Source of Crude Material

The poplar buds used in this research were obtained from two sources. A mixture of buds of P. tacamahaca and P. candicans was procured from the S. B. Penick Company. Buds from P. tacamahaca were made available through the courtesy of Professor M. W. Day, Superintendent of the Dunbar Forest Experiment Station of Michigan State University at Sault Ste. Marie, Michigan.

Preparation of Crude Extract

A fifteen kilogram quantity of poplar buds was extracted in a Barnstead extractor using diethyl ether as a solvent. After five hours the buds were removed, ground and then extracted for an additional five hours. The main portion of the ether was removed by distillation,

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*The author would like to thank Mrs. Shirley Geiss for conducting the bioassays on samples from this project.

leaving a dark, greenish-brown, viscous residue. The extract had an activity of 12.5 γ/ml. against M. tuberculosis. Portions of this extract were used in the following purification procedures.

Alkaline Extraction

Three hundred grams of the crude extract was dissolved in 300 milliliters of diethyl ether. The ether solution was then extracted with five percent potassium hydroxide solution. An emulsion formed and was broken, only with difficulty, by manipulation. It was found that by the use of additional ether, three fractions could be obtained: a five percent potassium hydroxide extract, an ether solution, and a gelatinous precipitate which was insoluble in both ether and base. Two additional quantities of crude extract (200 grams and 427 grams) were subjected to the initial alkaline extraction. The three partially extracted ether solutions were combined and extracted further until the potassium hydroxide solution exhibited only a faint yellow tinge. A total volume of six liters of potassium hydroxide solution was necessary for the extraction.

The base-extracted ether solution was washed with distilled water until the washings were neutral to litmus.

Acidic Extraction

The neutral ether solution from the alkaline extraction was then washed with five percent hydrochloric acid solution until the wash was colorless (volume of acid solution required was approximately two liters). The ether solution was washed with distilled water to remove hydrochloric

acid and then dried over calcium chloride. The ether solution of the neutral oil was then concentrated by simple distillation. The 183 grams of neutral oil obtained had an activity of 12.5 γ/ml. against M. tuberculosis.

Simple Distillation

The neutral oil had a pale green color. The color remained in the pot when the oil was subjected to a simple distillation at reduced pressure (1 mm.). The simple distillate had an activity of 12.5 γ/ml. against M. tuberculosis. A summary of the biological activities for the various fractions in these preliminary procedures are given in Table I.

TABLE I

BIOLOGICAL ACTIVITIES OF FRACTIONS FROM PRELIMINARY PROCEDURES

Sample	Test Concentration in Percent (w/v)	γ/ml. to Inhibit Growth of M. tuberculosis
Crude extract	0.1	12.5
Neutral oil	0.1	12.5
Simple distillate	0.1	12.5

Fractional Distillation

An adiabatic jacketed column, 1.2 cm. x 57 cm., packed with glass helices was used for a vacuum fractional distillation of the neutral oil simple distillate (pressure, 1 mm. ± 0.5 mm.). The major portion of the active materials was found to be present in the higher boiling

fractions. The data for a typical fractionation are given in Table II.

TABLE II

DATA FOR FRACTIONATION OF NEUTRAL OIL

Fraction	Boiling Range in °C	Test Concentration in Percent (w/v)	γ/ml. to Inhibit Growth of M. tuberculosis
1	50-71	0.1	Inactive
2	71-87	0.1	Inactive
3	87-92	0.1	Inactive
4	92-99	0.1	12.5
5	99-105	0.1	6.3
6	105-114	0.1	Inactive
7	114-116	0.1	12.5
8	Pot	0.1	12.5

Chromatographic Purification

Since the antibacterial activity appeared to be concentrated in the high boiling fractions of the neutral oil, efforts were turned to the purification of these samples. The isolation methods and the infrared spectra indicated that the high boiling fractions were terpenoid in nature. Sorn and co-workers have done a large amount of terpene isolation and identification and have found chromatography on alumina to be very helpful in their work. For these reasons chromatography seemed a logical method of isolating the active principle in poplar buds.

Numerous preliminary chromatograms were prepared but results were unsuccessful owing to technical difficulties, spreading or blurring or even loss of activity. However, this work indicated, as is also borne out in Table II, that the low boiling fractions (all fractions boiling below 90°C at 1 mm. pressure) are inactive; that medium boiling fractions (fractions boiling in the range of 90°C to 110°C at 1 mm. pressure) contain an antibacterial substance; that high boiling fractions (boiling above 110°C at 1 mm. pressure) contain an antibacterial substance different from that one in the medium boiling fractions.

An aliquant of a medium boiling fraction (boiling range of 104°C to 114°C at 2 mm. pressure; n_D^{24} = 1.4930; activity = 31.3 γ/ml.) was taken up in a minimum amount of petroleum ether and applied to a 200 gram Alcoa alumina column (for a description of column preparation see Appendix I and for the exact nature of the adsorbent see Appendix III). The column was developed with petroleum ether. When the effluent solvent began absorbing in the ultraviolet region, as determined with a Beckman model DU spectrophotometer, development was stopped and the column was extruded. The column was examined under an ultraviolet light and divided into four sections based on zones of differing fluorescence. The top section, one-eighth of the column, was comprised of a reddish-purple zone and a yellow zone. The second section, one-half of the column, exhibited a purple fluorescence. The third section, three-eighths of the column, was a greenish-yellow fluorescent zone. The fourth section was composed of the remainder of the column and did not fluoresce. The sections were then extracted with ethyl alcohol.

A fraction with an activity of 7.8 γ/ml. was obtained from the center of the column as is shown in Table III.

TABLE III

CHROMATOGRAPHIC DATA FOR MEDIUM BOILING FRACTION

Sample	Sample Origin	Sample Weight in Grams	γ/ml. to Inhibit Growth of M. tuberculosis
D-127	Petroleum ether washings	2.019	Inactive
D-128	Ethyl alcohol eluate of fraction 1	0.692	62.5
D-129	Ethyl alcohol eluate of fraction 2	3.280	7.8
D-130	Ethyl alcohol eluate of fraction 3	0.392	62.5
D-131	Ethyl alcohol eluate of fraction 4	0.223	Inactive

Sample D-129 from this column was applied on a 100 gram alumina (Alcoa) column, in a petroleum ether solution. The column was washed with petroleum ether until the washings began to absorb in the ultraviolet region, at which time the washing was stopped. The column was extruded and divided into three sections. The top one-eighth of the column was section one, the next one-half of the column was section two and the lower three-eighths of the column formed section three. The sections were then extracted with ethyl alcohol. The second section produced the largest fraction but its activity was 12.5 γ/ml. which was lower

than the activity of the parent sample, D-129. The data for this column are given in Table IV.

TABLE IV

CHROMATOGRAPHIC DATA FOR D-129

Sample	Sample Origin	Sample Weight in Grams	γ /ml. to Inhibit Growth of *M. tuberculosis*
D-133	Petroleum ether washings	0.006	Inactive
D-134	Ethyl alcohol eluate of fraction 1	0.095	Inactive
D-135	Ethyl alcohol eluate of fraction 2	2.641	12.5
D-136	Ethyl alcohol eluate of fraction 3	0.025	Inactive

Sample D-135 from this column was subjected to a simple distillation to obtain a clear viscous oil, D-138, which had an activity of 12.5 γ/ml. The molecular weight of D-138 was found to be 228 as determined by the Rast method. A 0.0315 gram sample of D-138 was dissolved in 0.3496 grams of camphor to give a melting point depression of 15.0°. The molar freezing point depression constant for camphor in this concentration range was taken as 38. The specific rotation was found to be +41.85 (in chloreform). The refractive index was 1.4910. The carbon-hydrogen analysis (by the Geller Laboratories, Hackensack, New Jersey) was found to be 80.76% carbon; 11.45% hydrogen. A summary of the physical properties of D-138 are listed in Table V. The ultraviolet spectrum

TABLE V

PHYSICAL PROPERTIES OF D-138

$[\alpha]_D^{24}$ (in $CHCl_3$)	+ 41.85
Molecular weight (by Rast method)	228
n_D^{24}	1.4910
Empirical formula	$C_{15}H_{26}O_{1.3}$

is shown in Figure 1 and the infrared spectrum is shown in Figure 2. On standing for a number of weeks, the biological activity of D-138 decreased. The infrared spectrum and the decolorisation of a potassium permanganate solution indicated that the sample was an unsaturated compound. The empirical formula ($C_{15}H_{26}O_{1.3}$), as calculated from analytical data, approaches that of a monocyclic sesquiterpene alcohol ($C_{15}H_{26}O$). It seemed possible that the excess oxygen content of the isolated material was due to peroxide formation, a well known reaction in this type of compound.

A peroxide test on D-138 (four months after the initial isolation) was found to be positive. A small amount of ethyl alcohol was layered on top of a dilute aqueous solution of ferric chloride and potassium ferricyanide in a small test tube. One drop of the sample to be tested was placed on the inside of the test tube just above the alcohol layer. By tilting and rotating the tube the sample was mixed with the alcohol. A green to blue color at the interface of the ferric chloride-potassium ferricyanide solution and the alcohol layer was indicative of a peroxide.

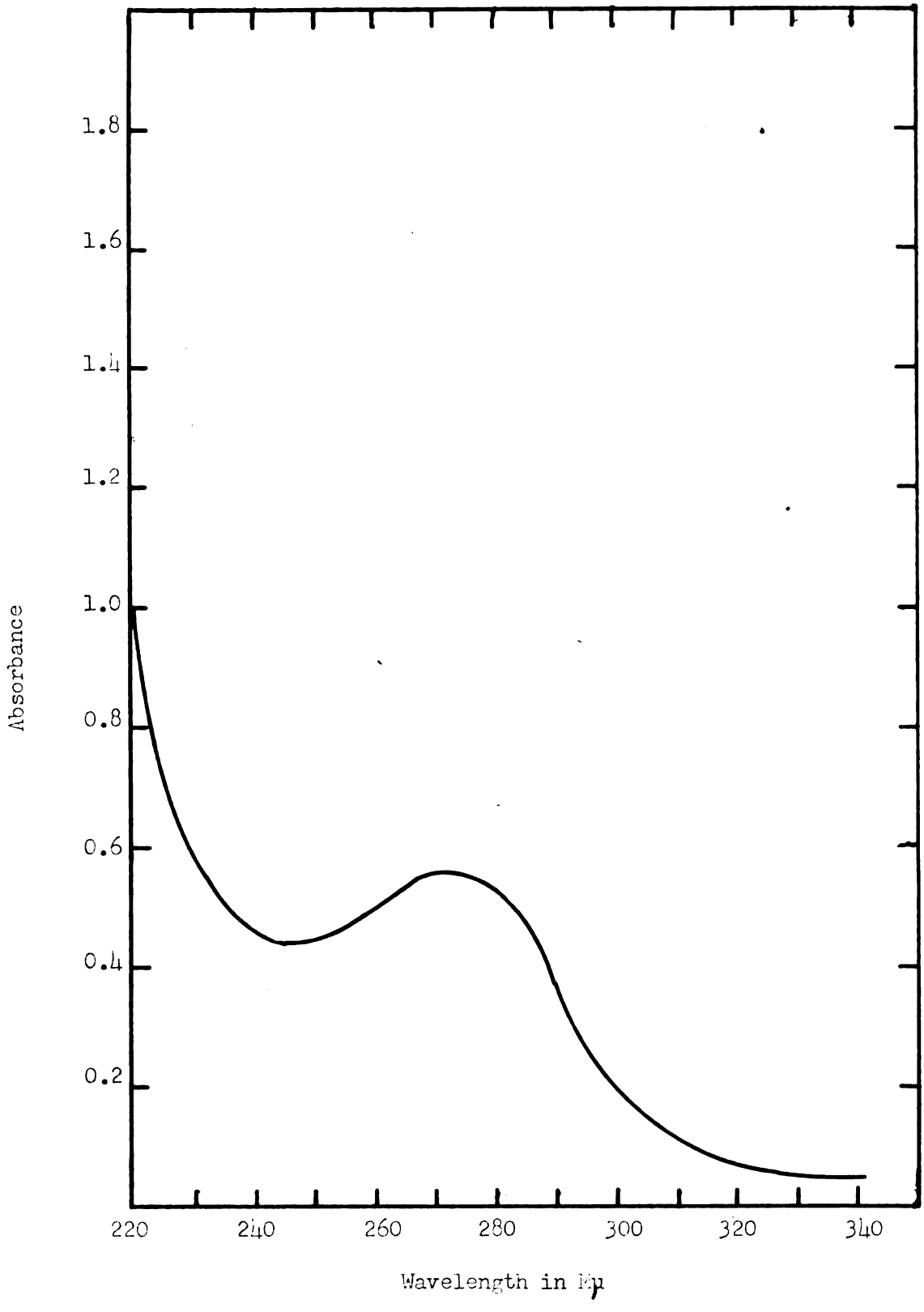


Figure 1. Ultraviolet Spectrum of D-138.

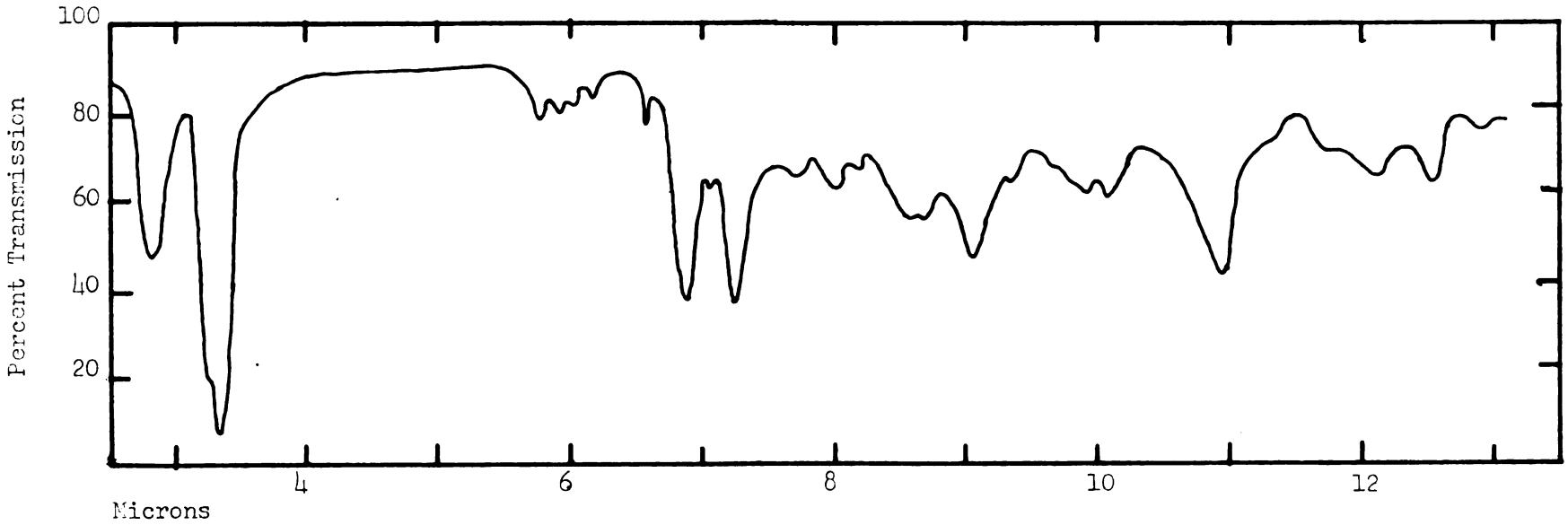


Figure 2. Infrared Spectrum of D-138 (Pure Liquid).

The loss of biological activity seemed to be associated with an increased oxygen content in the sample. This was borne out by the following experiment. After standing in the refrigerator for at least four months, sample B-138 was dried *in vacuo* for 48 hours at 45°C to remove any moisture present. The sample was then sent out for bioassay and carbon-hydrogen analysis. The sample contained 78.28% carbon and 11.01% hydrogen. As is shown in Table VI, the oxygen content was increased and the antibacterial activity was decreased. Because of these results identification work on this sample was discontinued.

TABLE VI

CORRELATION OF ACTIVITY AND OXYGEN CONTENT OF D-138

Fraction	Empirical Formula	γ/ml. to Inhibit Growth of *M. tuberculosis*
D-138 (initial)	$C_{15}H_{26}O_{1.3}$	12.5
D-138 (after four months)	$C_{15}H_{26}O_{1.73}$	37

Another medium boiling fraction (D-123) had the following properties: boiling range of 99°C-105°C at 1 mm. pressure; activity of 6.3 γ/ml. A portion of this fraction was applied on a 100 gram alumina (Alcoa) column in a petroleum ether solution. The column was developed with petroleum ether until the effluent solvent began to absorb strongly in the ultraviolet region. The development was stopped at this point and the column was extruded. The column was divided into four sections.

The first section was the top one-eighth of the column. The second section was the next one-eighth of the column. The third section was the following three-eighths of the column and the fourth section was composed of the remainder of the column. The individual sections were extracted in a Soxhlet extractor using ethyl alcohol as the solvent. A fraction with an activity of 3.9 γ/ml. was obtained from the center of the column as is shown in Table VII.

TABLE VII

CHROMATOGRAPHIC DATA FOR D-123

Sample	Sample Origin	Sample Weight in Grams	γ/ml. to Inhibit Growth of *M. tuberculosis*
D-139	Petroleum ether washings	0.172	62.5
D-140	Alcohol eluate of fraction 1	0.519	15.6
D-141	Alcohol eluate of fraction 2	0.353	31.2
D-142	Alcohol eluate of fraction 3	1.740	3.9
D-143	Alcohol eluate of fraction 4	1.021	7.8

Fractions D-142 and D-143 from this column were combined and a second chromatogram was prepared. However, the activity was lost and work was not continued on this fraction. The infrared spectrum of D-142 is given in Figure 3 and the ultraviolet spectrum in Figure 4.

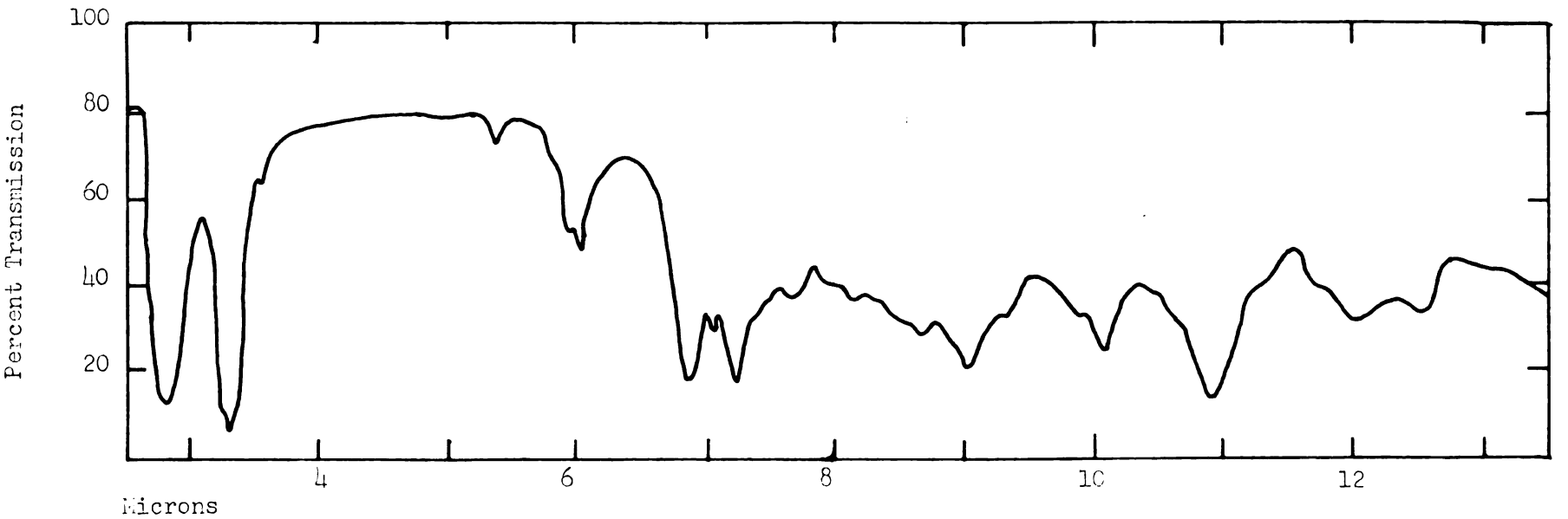


Figure 3. Infrared Spectrum of D-1142 (Pure Liquid).

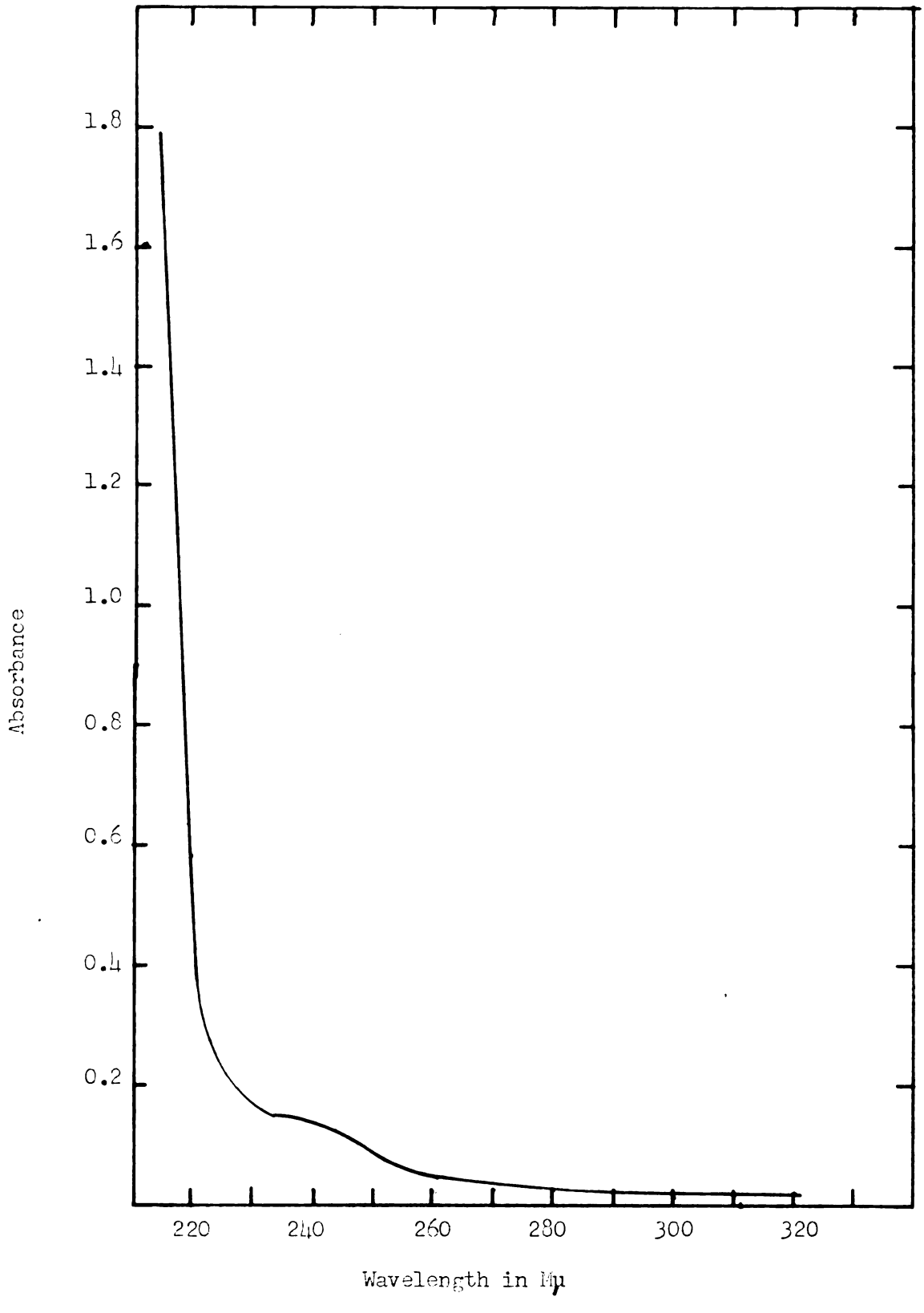


Figure 4. Ultraviolet Spectrum of D-142.

An 11.6 gram sample of a high boiling fraction (boiling range of 111°C to 114°C at 1 mm. pressure, $n_D^{20} = 1.4945$, infrared spectrum given in Figure 5) was applied on a 350 gram alumina (Alcoa) column in petroleum ether. The column was washed with petroleum ether and then was eluted with ethyl alcohol. No fractionation was obtained as is indicated by the refractive indices in Table VIII.

TABLE VIII

CHROMATOGRAPHIC DATA FOR HIGH BOILING FRACTION

Sample Origin	Sample Weight in Grams	n_D^{20}
2 1/2 liters of petroleum ether washings	0.146	--
210 ml. of ethyl alcohol-petroleum ether overlap	0.015	1.4941
20 ml. ethyl alcohol	4.058	1.4945
30 ml. ethyl alcohol	3.721	1.4941
50 ml. ethyl alcohol	1.353	1.4945
100 ml. ethyl alcohol	0.687	1.4945

The last four fractions from this column were combined and a petroleum ether solution of these was applied on a 300 gram alumina column (Woelm, neutral, grade II). The eluant was changed from petroleum ether to diethyl ether by gradient elution. Three principle fractions were obtained as is shown in Table IX.

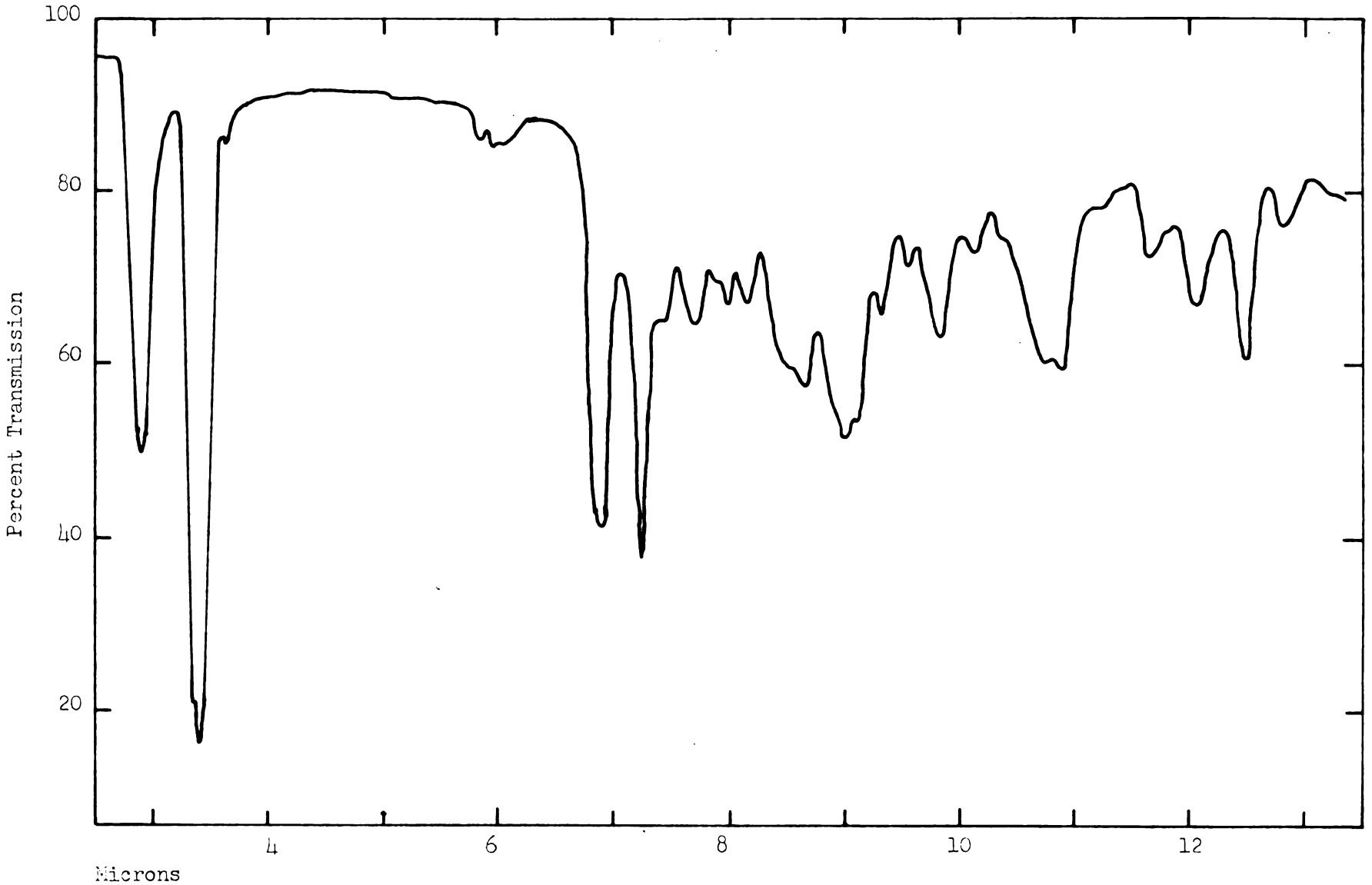


Figure 5. Infrared Spectrum of High Boiling Fraction (Pure Liquid).

TABLE IX

CHROMATOGRAPHIC DATA FOR HIGH BOILING FRACTION

Fraction	Sample Origin	Sample Weight in Grams	n_D^{20}	γ/ml. to Inhibit Growth of M. tuberculosis
1-4	60 ml. petroleum ether	0.006	-	
5	70 ml. petroleum ether	0.939	1.4975	
6 (D-206)	50 ml. petroleum ether	0.900	1.4961	12.5
7	60 ml. petroleum ether	0.186	1.4954	
8	50 ml. petroleum ether	0.180	1.4954	
9 and 10	130 ml. petroleum ether	0.338	1.4953	
11-14	300 ml. ether-petroleum ether mixture	0.641	1.4952	
15 and 16	130 ml. ether-petroleum ether mixture	0.481	1.4955	
17	70 ml. ether-petroleum ether mixture	0.716	1.4955	
18	100 ml. ether-petroleum ether mixture	1.637	1.4950	
19 (D-207)	100 ml. ether-petroleum ether mixture	2.035	1.4948	12.5
20-21 (D-208)	220 ml. ether-petroleum ether mixture	0.953	1.4969	2.5
22 (D-209)	110 ml. ether-petroleum ether mixture	0.176	-	Inactive
23-24	220 ml. ether-petroleum ether mixture	0.063	-	
25-27	300 ml. ether-petroleum ether mixture	0.044	-	
28-30	290 ml. ether-petroleum ether mixture	0.006	-	

Fractions 5 and 6 were combined to give sample D-210, fractions 7 to 16 to give sample D-211, and fractions 17 to 19 to give sample D-212. All three samples were then subjected to a simple distillation to obtain the materials which were used for the following work. The hydrochloride derivatives of D-210, 211 and 212 were prepared in the following manner, according to the method of Šorm (13). A 0.2 gram sample of the oil was dissolved in ten ml. of absolute diethyl ether. The solution was cooled to less than 0°C in a salt icebath and then saturated with anhydrous hydrogen chloride. The solution turned from light yellow to dark red-brown. The solution was kept at ice bath temperature for 36 hours, then the ether and hydrogen chloride were removed in vacuo (making sure that the temperature never rose above 0°C). The resulting crystals were washed with a minimum amount of cold methyl alcohol and then recrystallized for methyl alcohol. The yields were between 50 and 75 percent of theoretical. The melting points of the hydrochlorides of samples D-210, 211, 212 were 78°C (uncorrected). The specific rotation, refractive index and activity of these fractions were also determined. The carbon-hydrogen analyses were as follows: D-210, 80.82% carbon, 11.83% hydrogen; D-211, 80.57% carbon, 11.61% hydrogen; D-212, 80.50% carbon, 11.71% hydrogen. From this information it appeared that all three samples, D-210, 211 and 212, were primarily the same compound. The infrared spectra of these three samples, given in Figures 6, 7 and 8, also support this conclusion. A summary of the physical properties of these fractions is given in Table X.

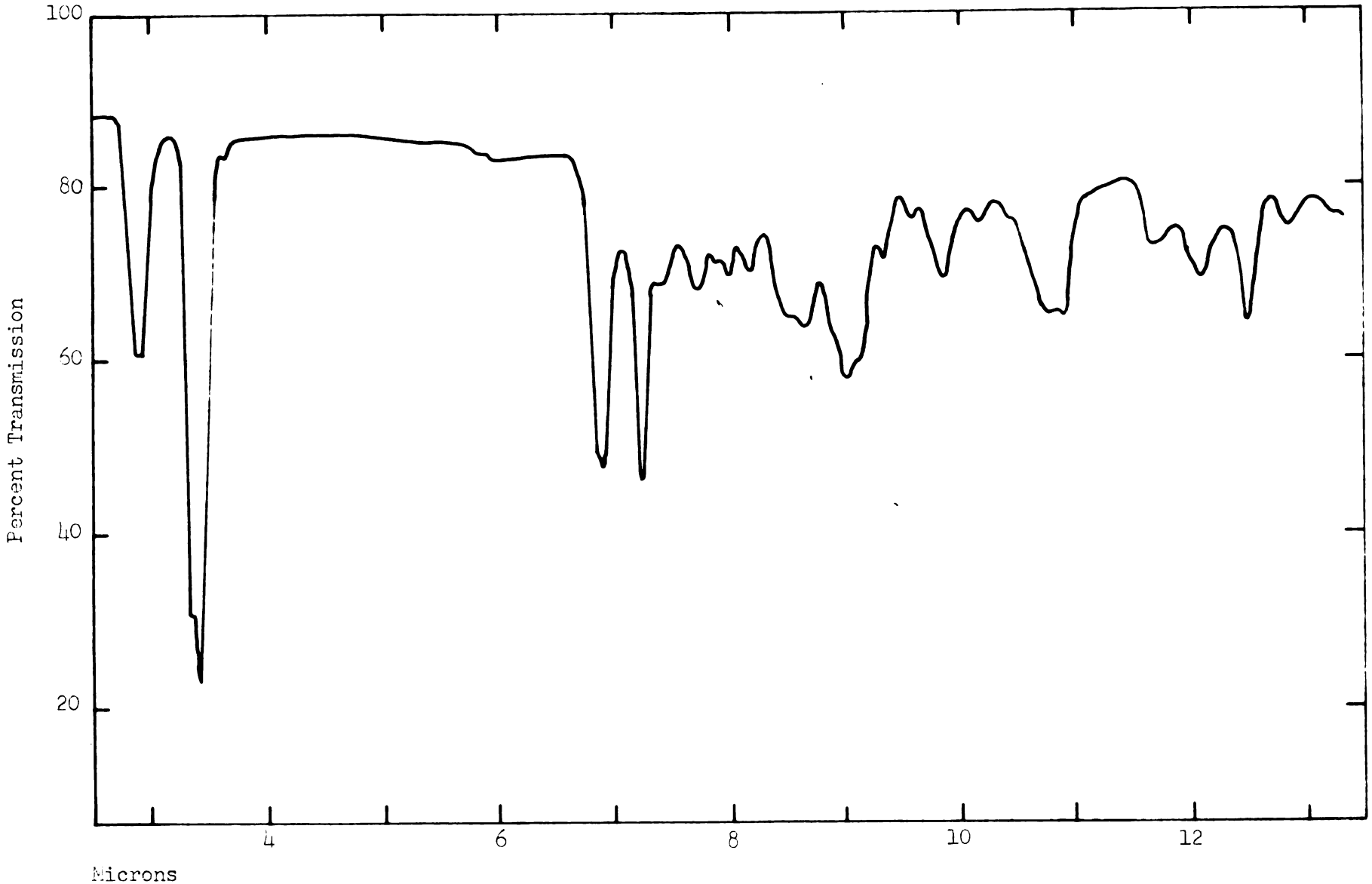


Figure 6. Infrared Spectrum of D-210 (Pure Liquid).

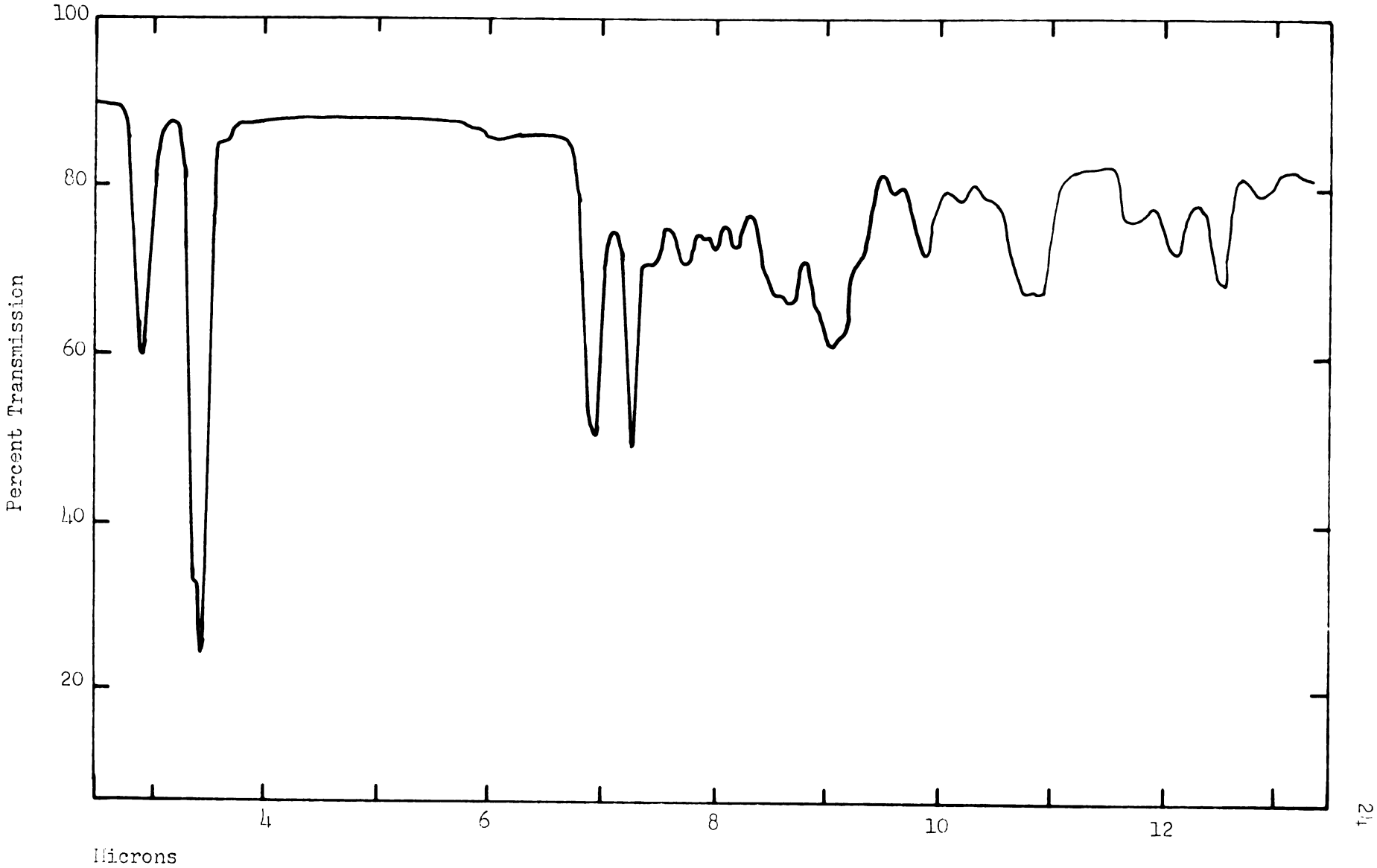


Figure 7. Infrared Spectrum of D-211 (Pure Liquid).

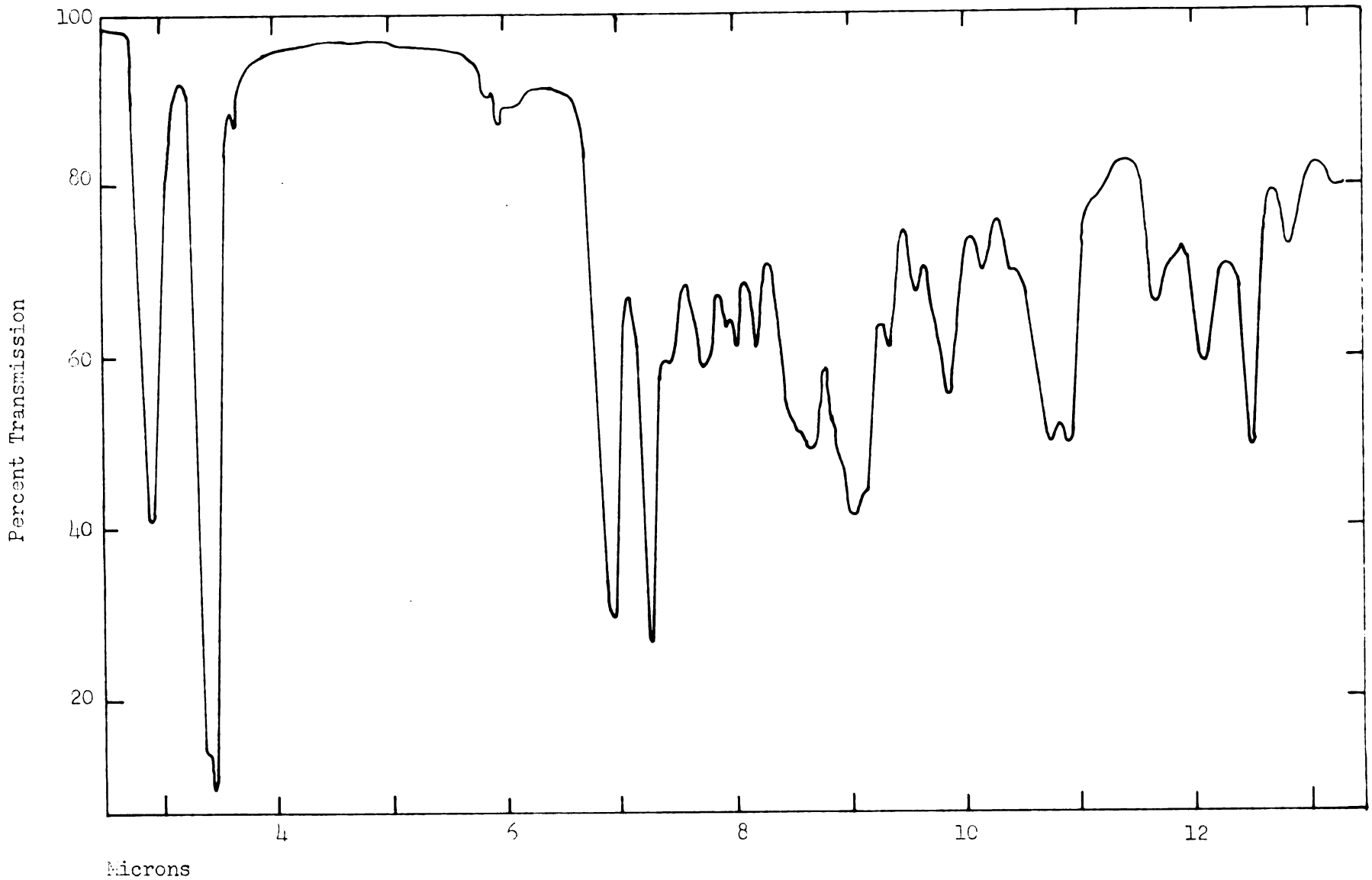


Figure 8. Infrared Spectrum of D-212 (Pure Liquid).

TABLE I

PHYSICAL PROPERTIES AND ACTIVITIES OF D-210, 211, 212

Sample	n_D^{20}	$[\alpha]_D^{24}$	M.P. of Hydrochloride	γ/ml. to Inhibit Growth of *M. tuberculosis*	Empirical Formula
D-210	1.4969	+50.9	78°C	6.3	$C_{15}H_{26}O_{1.16}$
D-211	1.4952	+53.2	78°C	6.3	$C_{15}H_{26}O_{1.22}$
D-212	1.4960	+51.8	78°C	6.3	$C_{15}H_{26}O_{1.22}$

Sample D-210 was rechromatographed under conditions comparable to the parent column. The sample was analogous to D-212 in chromatographic behavior. One may conclude from this that the initial column was overloaded, sample D-210 being an excess of the applied sample, and that Sample D-212 is a relatively pure compound.

Since the experimental results indicated the active principles in the neutral fraction of poplar bud oil were terpenic in nature, it seemed of interest to determine whether terpenes in general possess such antibacterial activity. Accordingly, all readily available terpenoid compounds were tested for activity against a group of bacteria, *Micrococcus pyogenes*, var. *aureus*, *Salmonella typhimurium*, *Xanthomonas phaseoli* and *Mycobacterium tuberculosis*. The results of these tests are recorded in Table II.

TABLE XI

ACTIVITY OF SEVERAL TERPENES AGAINST BACTERIA

Terpene	Micrococcus pyogenes, aureus γ/ml.	Salmonella typhimurium γ/ml.	Xanthomonas phaseoli γ/ml.	Mycobacterium tuberculosis γ/ml.
Cineol	Inactive	Inactive	Inactive	Inactive
Citronellal	310	Inactive	310	62
Citronellol	310	620	160	62
Geraniol	310	620	310	62
Linalool	Inactive	620	620	Inactive
d-Limonene	Inactive	Inactive	Inactive	Inactive
dl-Menthol	310	620	620	62
l-Menthol	620	Inactive	620	62
Pinene	620	Inactive	Inactive	Inactive
Pulegone	Inactive	Inactive	620	Inactive
Squalene	Inactive	Inactive	Inactive	Inactive
α-Terpineol	Inactive	Inactive	Inactive	125
Terpinol	Inactive	Inactive	Inactive	Inactive
Farnesol	Not tested	Not tested	Not tested	31

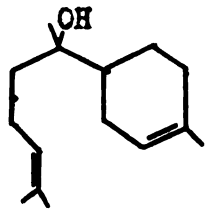
DISCUSSION

An examination of the data in Table I could cause one to wonder if any purification or fractionation had been effected by the alkaline and acidic extractions. However, Goris and Canal (12) isolated and identified some cinnamic acid derivatives from an alkaline extract of poplar bud oil from P. balsamifera. A number of cinnamate derivatives were tested by Lucas (unpublished work) and some were found to have strong antibacterial activity. In all probability, therefore, active cinnamates were separated from the terpenoid compounds on which emphasis has been placed in this work.

Fractional distillation of the neutral oil indicated the existence of two different antibacterial substances. Experiments with the material from the fractional distillation with a boiling range of 104°C to 114°C at 1 mm. pressure did not yield results which were conclusive as to the exact nature of the active principle. The infrared spectrum (14), ultraviolet spectrum, optical rotation, index of refraction (15) and carbon-hydrogen analysis did indicate that the material was a sesquiterpene alcohol. Since the material was ascertained to be terpenic in nature, it was logical to assume that oxygen could react with the unsaturated carbon atoms of the molecule to form peroxides. This point was borne out by a positive peroxide test. A change of this type in the molecular structure or composition might be expected to alter the antibacterial properties of a material. In the present case a decrease in activity did occur, as is shown in Table VI. Because of these

difficulties, work on the active compound in the medium boiling fraction was discontinued.

A purified high boiling fraction (D-212) is believed to be an isomer of bisabolol (I). The preparation of a hydrochloride (II) which was identical in melting point with the hydrochloride obtained by Sorm (13) from an authentic sample of bisabolol is good evidence for this conclusion.



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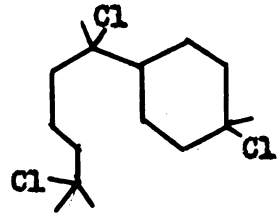

II

The refractive index, specific rotation, and carbon-hydrogen analysis of D-212 compare favorably with those values for bisabolol, as is shown in Table XII.

TABLE XII

PHYSICAL PROPERTIES OF D-212 AND BISABOLOL

Sample	n_D^{20}	$[\alpha]_D^{24}$	Percent Carbon	Percent Hydrogen
D-212	1.4959	+51.8	80.50	11.71
Bisabolol	1.4919 (Šorm) (12) 1.4936 (Ruzicka) (16)	+51.7	80.68	11.58

The infrared spectrum of D-212 (Figure 9) also compares favorably with the infrared spectrum of bisabolol (Figure 10) (13). In view of these findings it was concluded that bisabolol is one of the major active principles in the neutral fraction of poplar bud oil from P. tacamahaca.

It is also possible however, that a small amount of impurity could account for the biological activity of this fraction. Final and unequivocal proof would require several steps. First, isolation of bisabolol from other plant sources and then testing the compounds for activity against M. tuberculosis. One source of bisabolol, according to Naves (17), is Cabreuva oil.. Sorm and co-workers (18) isolated l-bisabolol from oil of chamomile. A sample of the latter oil was tested for antibacterial activity and found to be negative. This brings to light the possibility that the "d" form of bisabolol is active while the "l" form is inactive. However, the actual isolation and testing of the "l" form is necessary to establish this point.

A second step in proof of the antibacterial activity of bisabolol would be the synthesis of the compound. This synthesis has been described by Ruzicka and Liguori (16). However, this product would be a mixture of four isomers in an unknown proportion since there are two asymmetric centers in the molecule. Therefore, the synthesis alone would not constitute full proof for the antibacterial activity of any one specific isomer of bisabolol.

The testing of a variety of terpenoid compounds for antibacterial activity demonstrated that although many of these possessed activity, a considerable number were inactive. The terpene hydrocarbons tested

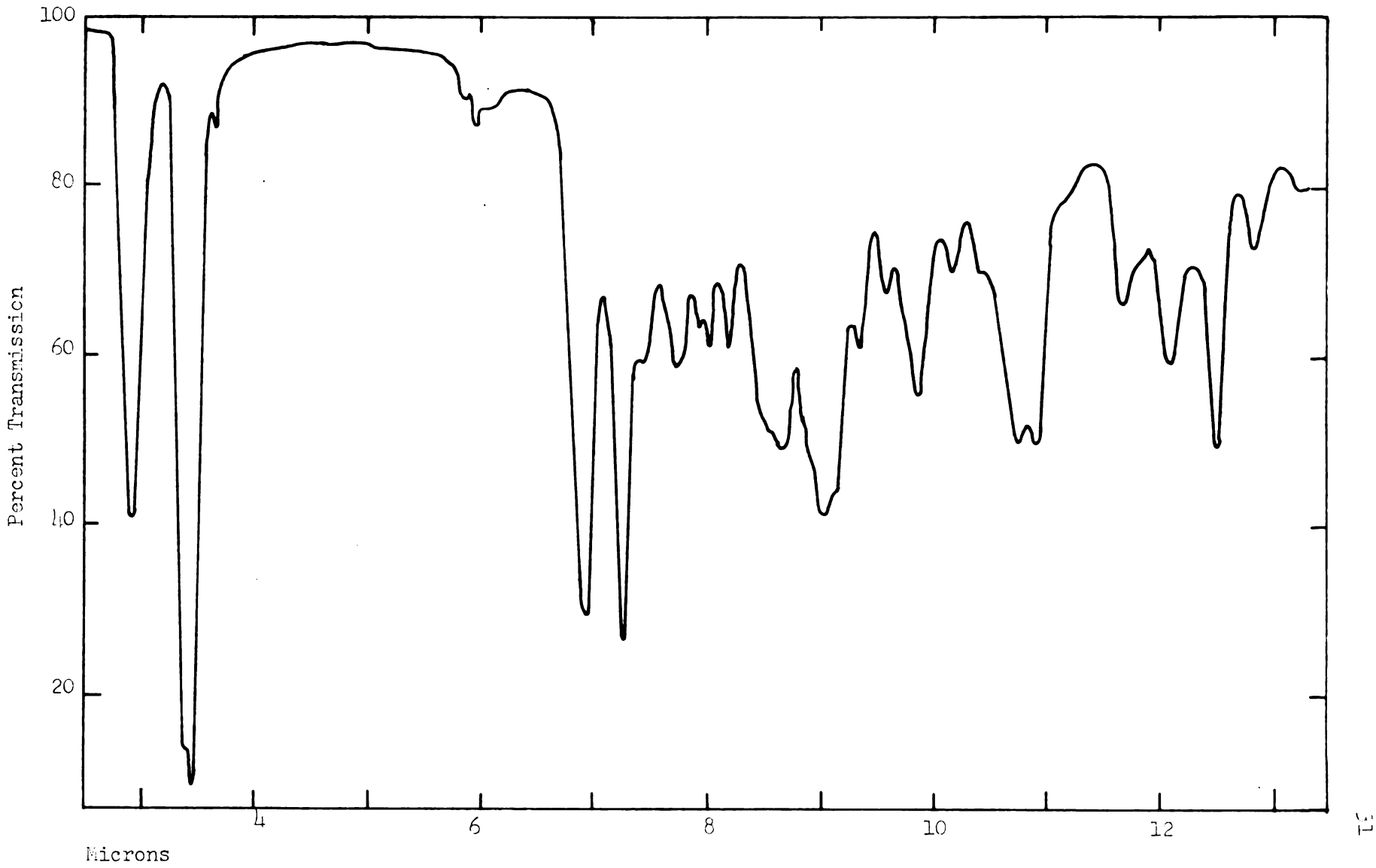

Figure 9. Infrared Spectrum of D-212. (Pure Liquid)

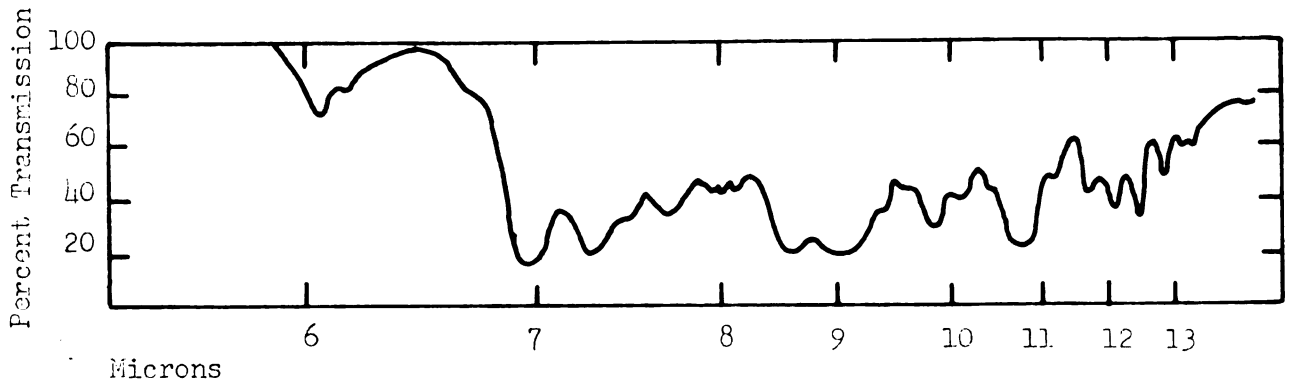

Figure 10. Infrared Spectrum of Bisabolol.

were inactive whereas the terpenoids containing oxygen, in general, possessed activity against one or more of the organisms used in the test. Whether or not this conclusion is generally valid requires further work. None of the samples tested, however, approached the activity of the sample of bisabolol isolated in the present work.

PART II

INVESTIGATION OF ANTIBACTERIAL PROPERTIES OF THE FLOWER OF *HYPERICUM PROLIFICUM*

INTRODUCTION

As far back as the time of Galen and Dioscorides curative powers were attributed to the plant Hypericum. During the middle ages this plant was mentioned time and again, mostly as a protective against witchcraft, but also by German and Arabic physicians as a remedy against hemorrhages, kidney stones and intestinal worms, as well as a treatment of wounds. English herbalists have also used olive oil extracts of Hypericum to cure deep wounds (3).

Next to nothing was known about the nature of the curative agents of this plant when Černy (19), in 1911, published his account about a red pigment which he called hypericin. The biological interest in this compound was aroused in particular by the fact that it sensitises light-skinned animals to irradiation (20). It appeared that this biologically active substance might also be the agent which was responsible for the medicinal properties. The structure of this compound was not known until 1951 when Brockman et al. (21) characterised hypericin as hexahydroxydimethylnaphthodianthrone.

However, it became obvious that other constituents of this plant also had biological properties, although clear distinction that the various effects were due to different constituents was not made at first. The first public report about the antibacterial activity of crude extracts of this plant came from Jensen and Miller (22), who reported activity against Staphylococcus aureus in dilutions up to 1:2000. These workers dealt with rather crude preparations which, in

addition to an antibacterial principle, also contained other substances, as for instance hypericin. While all of these literature sources dealt with the species H. perforatum (a weed, commonly called St. John's Wort), Lucas and co-workers investigated several species of the genus Hypericum for antibacterial properties and first reported in 1949 (23) considerable activity of leaf extracts of H. calycinum against M. tuberculosis. Later, the same group reported strong antibacterial properties of extracts of leaves and particularly of flowers of H. perforatum and H. prolificum (10) and of flowers of H. moserianum and H. polyphyllum (24). At the same time, Hagenström (25) found an unidentified antibacterial agent in capsules of H. perforatum.

In view of the foregoing information, a project was initiated to isolate and identify the antibacterial principle(s) in H. prolificum.

EXPERIMENTAL AND RESULTS

Bioassay

The principal test organism in the work with Hypericum prolificum was Micrococcus pyogenes var. aureus. However, a gram-negative organism, Xanthomonas phaseoli, was also used in the testing. The activity against X. phaseoli was generally higher than that against M. pyogenes, but in order to simplify procedures only the activities against the gram-positive M. pyogenes were used in the following experimental work, results and discussion. The bioassay was accomplished by the serial dilution method (Appendix II). A discussion of the methods of expressing and calculating the activities may be found in Appendix II.

Source of Crude Material

The blossoms of H. prolificum which were used in this work were obtained by Dr. E. H. Lucas from cultivated shrubs grown in the Horticultural Gardens of Michigan State University. The shrub produces an abundance of flowers during the month of July. The harvested flowers were stored at -10°C until needed.

Preparation of the Crude Extract

A study was first undertaken to investigate the efficiency of various extraction methods and solvents. Preliminary unpublished work by Lucas indicated that the three most suitable solvents were ether, petroleum ether and ethyl acetate. To put these preliminary results on a quantitative basis the following procedures were followed.

A 15-gram quantity of flowers was macerated in a Waring Blendor, using 150 ml. of the appropriate solvent. The extract was then filtered and a portion was sent for bioassay. A Soxhlet apparatus was used for a second method of extraction. The 15-gram flower sample was placed in the extractor along with 150 ml. of the solvent and refluxed for two and one-half hours. The Waring Blendor-ethyl acetate combination gave the highest amount of activity as well as the lowest amount of total solids and therefore the extract of the highest specific activity. These data are given in Table XIII,

TABLE XIII

RESULTS OF VARIOUS EXTRACTION PROCEDURES WITH HYPERICUM FLOWERS

Extraction Method	Extraction Solvent	Total Solids in Grams	γ / ml. to Inhibit Growth of M. pyogenes
Soxhlet	ethyl acetate	0.975	5
Soxhlet	ether	0.870	2.5
Soxhlet	petroleum ether	0.750	8
Waring Blendor	ethyl acetate	0.435	0.6
Waring Blendor	ether	0.750	4
Waring Blendor	petroleum ether	0.535	6

For large scale preparations the use of a Waring Blendor was impracticable; thus the procedure actually used in the experiments to be described involved maceration of the flowers in a food chopper

followed by extraction of the macerate with ethyl acetate. The following is an example of a typical preparation:

An 840-gram quantity of flowers was macerated, covered with two liters of ethyl acetate and allowed to stand for one hour with frequent, thorough agitation. The ethyl acetate was drained from the macerate and the extraction was repeated two additional times, using one liter of ethyl acetate for each extraction. The individual solutions were combined to give the crude extract. The activity in this particular case was 2.0 γ/ml.; however, the value varied with different preparations, between 2 and 10 γ/ml. Some variation in the degree of activity of the crude extract was noticed, depending upon the year of harvest, the stage of flowering at the time of harvest and, of course, the method of preparation.

Preparation of Stock Solution

The crude extract was taken to dryness in a flash evaporator, leaving a residue of approximately 100 grams. Attempts to purify this residue by use of different solvents were unsuccessful. For example, the residue from a crude extract was dissolved in a quantity of ethyl acetate equal to 0.05 of the volume of the original extraction solvent. This solution was decanted from the residue, diluted with diethyl ether until precipitation ceased, filtered, taken to dryness in a flash evaporator, redissolved in ether and then filtered again to give the final stock solution. However, it was found that activity was lost at each step in the procedure and that all samples when exposed to air

were subject to an intense browning reaction. As a result, the final method of preparing the stock solution consisted of simply adding 100 ml. of ether to the residue from the crude extract and letting the solution stand at 8°C overnight. The solution was filtered and the residue washed with 100 ml. of ether. The washings were added to the filtrate. The biologically inactive residue was a green powder which gave a positive Molisch test, indicating the presence of a carbohydrate. The filtrate prepared in this manner was designated as the stock solution and had an activity of 1.25 γ/ml. However, the activity of different stock solutions varied from 1.25 γ/ml. to 10 γ/ml.

Acidic and Alkaline Extraction of the Stock Solution

A 20-ml. aliquant of a stock solution was extracted consecutively with three 50 ml. portions of 0.1 normal acetic acid and three 50-ml. portions of 0.1 normal ammonium hydroxide. The acidic and alkaline extracts were taken to dryness and dissolved in ethyl alcohol for bioassay purposes. The activity of the original stock solution was not changed by acidic and alkaline extraction. The acidic extract was inactive and the alkaline extract did not exhibit appreciable antibacterial activity (Table XIV).

In another experiment using 0.1 normal hydrochloric acid and potassium hydroxide, some activity appeared in the alkaline extract. The acidic extract was inactive, and the residual ether solution showed a loss of activity (Table XV). It was concluded that the active principle could not be satisfactorily isolated from the stock solution by acidic or alkaline extraction under the conditions of the experiment.

TABLE XIV

BIOASSAY DATA ON ACIDIC AND ALKALINE EXTRACTION OF STOCK SOLUTION USING CH_3COOH AND NH_4OH

Sample	γ/ml. to Inhibit Growth of *M. pyogenes*
Stock solution	1.25
Acidic extract	Inactive
Alkaline extract	320
Stock solution after acidic and alkaline extraction	1.25

TABLE XV

BIOASSAY DATA ON ACIDIC AND ALKALINE EXTRACTION OF STOCK SOLUTION USING HCl AND KOH

Sample	γ/ml. to Inhibit Growth of *M. pyogenes*
Stock solution	2.5
Neutralised alkaline extract	10
Acid extract	Inactive
Stock solution after acidic and alkaline extraction	7

Selection of Adsorbent

Since further solvent extraction did not aid in purifying the stock solution, it was thought that adsorption chromatography could be helpful. Using several different adsorbents (Appendix III) columns were prepared in the manner described in Appendix I. The results of these trials were such that the adsorbents could be grouped in three classes. Some, such as Alcoa alumina, Woelm neutral alumina, and magnesia, adsorbed the active principle so strongly (Table XVI) that it could not be removed, even by prolonged treatment in a Soxhlet extractor. On the other hand, another class of adsorbents composed of calcium carbonate, cellulose, silica gel and starch, had so little affinity for the entire sample that it passed through the column in the first few milliliters of the developing solvent (Table XVII). The third class of adsorbents consisted of Celite, pH 2 alumina and pH 4 alumina. It was possible to apply the sample on columns prepared from these adsorbents and then successfully elute the antibacterial activity from the column.

As might be expected, all adsorbents did not fall into just one group. Work with charcoal produced varied results. In one case, the sample was applied on the column in ether and then eluted with ether. The sample had an activity of 0.01 γ/ml. Efforts were made to reproduce this sample but all attempts failed. In all of the following chromatograms prepared using charcoal as the adsorbent, the activity either could not be removed from the column or it was lost when the sample was removed by extraction in a Soxhlet apparatus. The conditions for

TABLE XVI

ADSORBENTS WHICH RETAIN ACTIVITY FROM HYPERICUM STOCK SOLUTION

Column Number	Adsorbent	Method of Preparing Chromatogram	Solvent Used to Remove Activity
I	Alcoa alumina	extrusion	ethyl alcohol
II	Alcoa alumina	elution	ether ethyl alcohol
XIV	Woelmn neutral alumina	elution and extrusion	ether ethyl alcohol ethyl acetate acetic acid in ethyl alcohol ammonia in ethyl alcohol
XV	Magnesia	elution	ether ethyl alcohol

TABLE XVII

ADSORBENTS WHICH DO NOT RETAIN ACTIVITY FROM HYPERICUM STOCK SOLUTION

Column Number	Adsorbent	Method of Preparing Chromatogram	Solvent Used to Remove Activity
III	Calcium carbonate	elution	ether
VII	Cellulose	elution	ether
XX	Starch	elution	ether
XXI	Silica gel	elution	ether

purification of the stock solution on charcoal must be quite specific and sufficient time for further investigation of these conditions was not available. Consequently, work with charcoal was discontinued.

Chromatographic Purification by Elution

Preliminary work indicated that purification of the stock solution by two step adsorption chromatography, using pH 2 alumina followed by pH 4 alumina, was very effective. Both elution and extrusion chromatography were used in order to see which method would give the best resolution of the stock solution. A 23.4-gram sample of a stock solution (activity = 10 γ/ml.) was applied to a 615 gram alumina (Woelmn, pH 2) column. The initial eluant was ether and the fraction volume was approximately 300 ml. The ether removed a yellow-green zone containing about 1.5 grams of sample. The most active fraction in this zone inhibited bacterial growth at a concentration of 31 γ/ml. After allowing 1500 ml. of ether to pass through the column, the solvent was changed to two percent ethyl alcohol in ether and elution of a dark-green zone followed. The total weight of solids in this zone was about 9 grams. The peak fraction had an activity of 1.7 γ/ml. One liter of two percent ethyl alcohol in ether was followed by two liters of ethyl alcohol, eluting a green fraction (three grams). The activity of this last fraction was over 20 γ/ml. The data for this chromatogram are given in Table XVIII

Fractions 9 and 10 from this chromatogram were combined and applied to a 345 gram alumina (pH 4) column in ether. The column was developed

TABLE XVIII

DATA FOR ELUTION CHROMATOGRAM FROM pH 2 ALUMINA

Fraction	Volume in Ml.	Solvent	Total Weight in Grams	γ /ml. to Inhibit Growth of M. pyogenes
Stock solution		ether	23.4	10
1	300	ether	0.026	Inactive
2	206	ether	0.050	Inactive
3	230	ether	0.247	Inactive
4	285	ether	0.765	220
5	300	ether	0.207	31
6	280	ether	0.126	41
7	310	ether	0.100	5
8	310	2% ethyl alcohol in ether	0.108	4.5
9	320	2% ethyl alcohol in ether	5.075	3
10	140	2% ethyl alcohol in ether	2.027	1.7
11	320	2% ethyl alcohol in ether	1.432	3.6
12	140	ethyl alcohol	0.340	8.4
13	300	ethyl alcohol	2.063	22
14	300	ethyl alcohol	0.574	31
15	1500	ethyl alcohol	0.545	210

and eluted with ether which removed one zone, the most active fraction having an activity of 7.8 γ/ml. The zone containing the major portion of the active principle was eluted with a one percent solution of ethyl alcohol in ether. The most active fraction was number 13 which had an activity of 0.25 γ/ml. The data for this column are given in Table XIX.

TABLE XIX

DATA FOR ELUTION CHROMATOGRAM FROM pH 4 ALUMINA

Fraction	Volume in Ml.	Solvent	Total Weight in Grams	γ/ml. to Inhibit Growth of M. pyogenes
1	270	ether	0.084	Inactive
2	320	ether	0.143	31
3	320	ether	0.076	31
4	320	ether	0.068	15
5	290	ether	0.054	15
6	320	ether	0.063	15
7	310	ether	0.049	15
8	170	ether	0.035	7.8
9	320	½% ethyl alcohol in ether	0.071	7.8
10	320	½% ethyl alcohol in ether	0.053	3.9
11	320	½% ethyl alcohol in ether	0.047	3.9
12	300	1% ethyl alcohol in ether	0.045	3.9
13	300	1% ethyl alcohol in ether	0.162	0.25
14	300	1% ethyl alcohol in ether	1.248	0.50
15	370	1% ethyl alcohol in ether	1.610	1.0
16	500	1% ethyl alcohol in ether	0.370	3.9

An attempt was made to rechromatograph the combined sample of fractions 13, 14, and 15 on pH 4 alumina. In the process, however, the activity was lost. The ultraviolet spectrum of fraction 13 (H-282) is given in Figure 11 and the infrared spectrum in Figure 12.

Chromatographic Purification by Extrusion

The stock solution separated into a number of sharply defined zones when applied on a pH 2 alumina column and developed with ether. For this reason it was believed that extrusion and sectioning of a column of this type could produce a major purification of the stock solution. To this end 1.62 grams of stock solution (activity = 1.2 γ/ml.) was applied on a 100 gram alumina (Woelm, pH 2) column, which was then developed with ether. As development of the column progressed, some zones widened and new ones formed so that when 200 ml. of ether had passed through the column the zones were arranged in the following order from the top of the column downward: a blue-green zone, a dark-green zone, a yellow zone, a clear area which blended into a light-green zone, a narrow zone of a bright red material, a blue-green zone and a yellow zone. A yellow zone which passed through the column with the ether was found to be inactive. The column was then extruded and divided into three major sections. The first section, H-448, contained the top three zones and part of the clear area. The light-green zone, the red zone and the blue-green zone composed the second section (H-449). The yellow zone and the remainder of the column made up the third section (H-450). Each section was then extracted with both ethyl alcohol and ethyl acetate. The extracts were combined, taken to dryness,

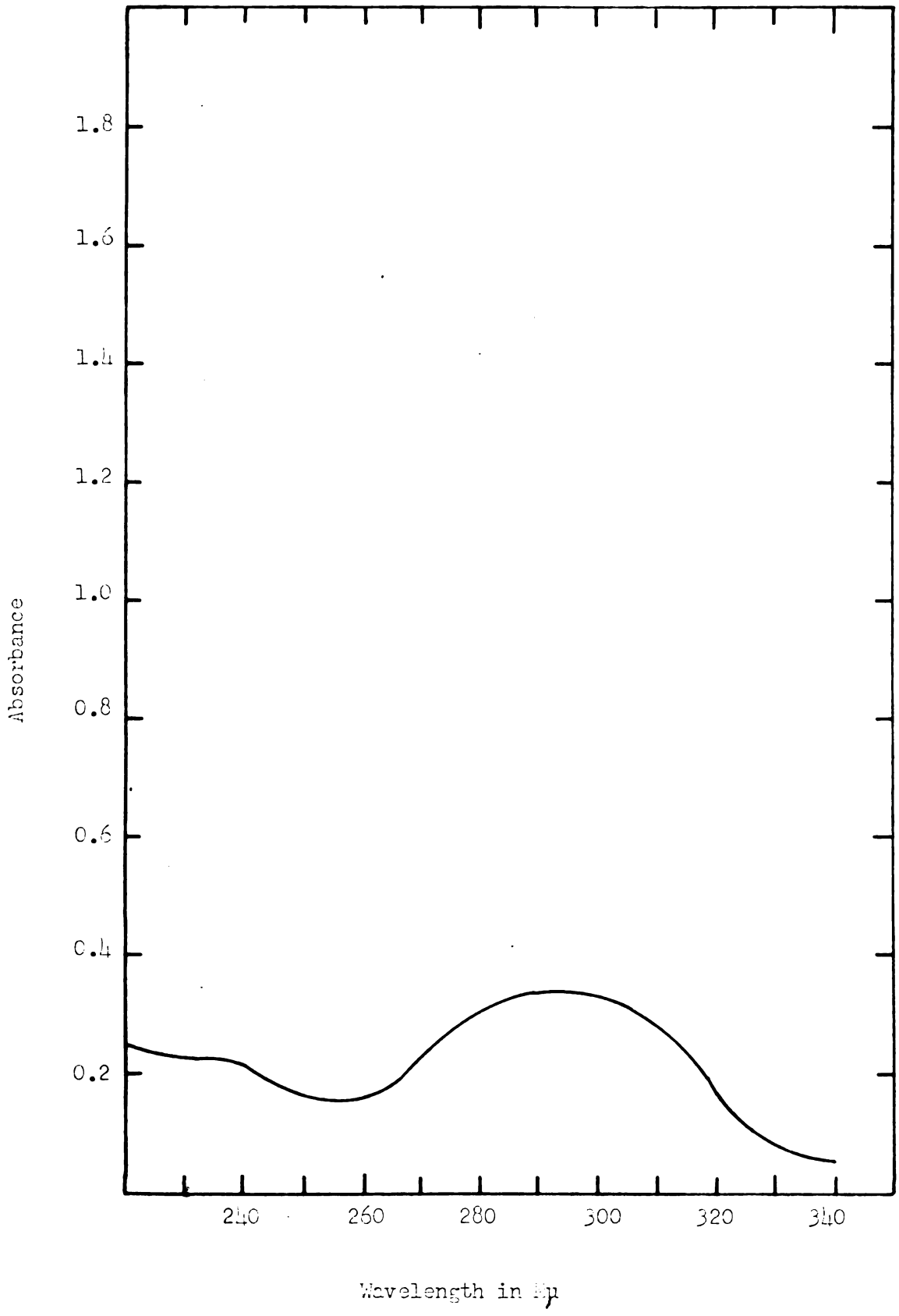

Figure 11. Ultraviolet Spectrum of H-282.

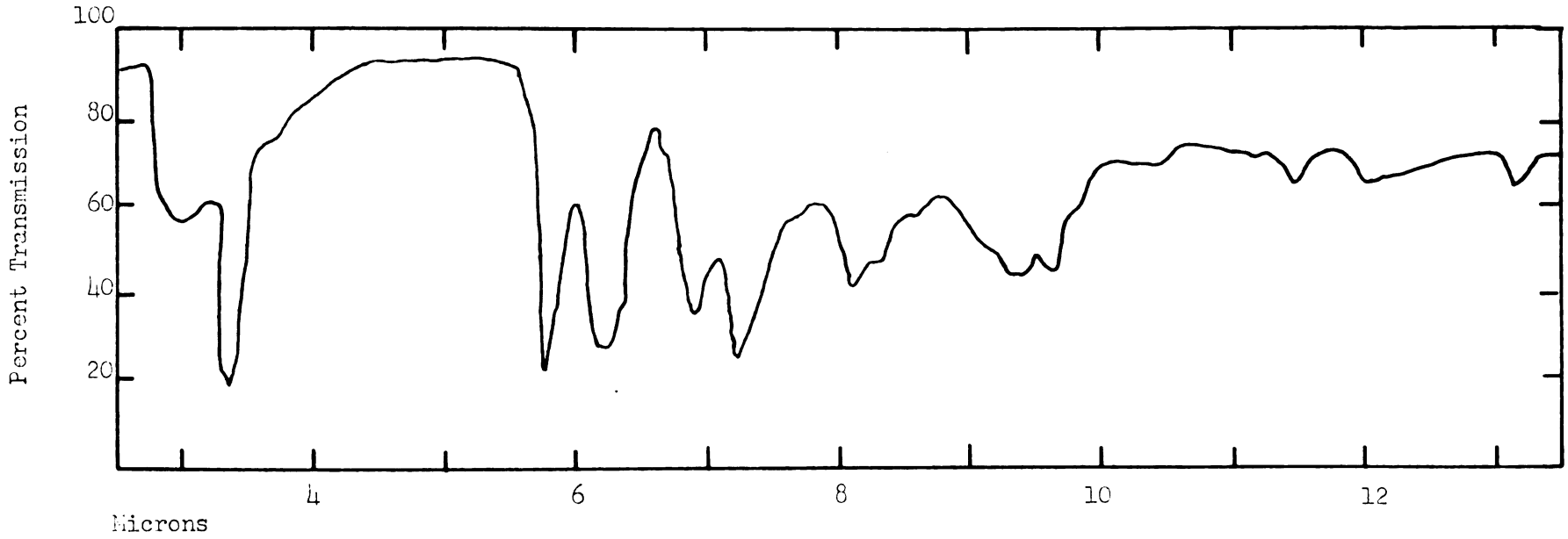

Figure 12. Infrared Spectrum of H-282. (Pure Liquid)

and redissolved in ether. The activities of the extracts of these sections were as follows: section 1, 15.6 γ/ml.; section 2, 0.8 γ/ml., section 3, 0.72 γ/ml. A summary of the data for this chromatogram is given in Table XX.

TABLE XX

DATA FOR EXTRUSION CHROMATOGRAM FROM pH 2 ALUMINA

Fraction	Origin	γ/ml. to Inhibit Growth of *M. pyogenes*
H-447	192 ml. of ether eluate	Inactive
H-448	Ethyl alcohol and ethyl acetate extract of section 1	15.6
H-449	Ethyl alcohol and ethyl acetate extract of section 2	0.80
H-450	Ethyl alcohol and ethyl acetate extract of section 3	0.72

Fraction H-450 was chromatographed on a 25-gram alumina (pH 4, grade II) column. The sample was applied from ether. The developing solvent, ether, eluted an inactive yellow zone, H-451. Previous work indicates that a pH 4 alumina column gave best results when the elution method was used, thus the eluant was changed to ethyl alcohol by the gradient method and a second yellow zone (H-452) was removed from the column. This fraction had an activity of 0.42 γ/ml. The data for this column are given in Table XXI.

TABLE XXI

DATA FOR CHROMATOGRAM OF H-450

Fraction	Eluant Solvent	Volume in Ml.	Total Weight in Grams	γ/ml. to Inhibit Growth of M. pyogenes
H-451	Ether	125	0.247	Inactive
H-452	Ethyl alcohol	100	0.080	0.42

Fraction H-449 was also chromatographed on a 25-gram alumina (pH 4, grade II) column. The sample was applied on the column from an ether solution. The elution method was also used for this column. Ether was continued as the eluant and two zones were eluted, H-453 and H-454, both of which were inactive. The eluant was changed to ethyl alcohol by the gradient technique and a yellow fraction (H-456) was thus eluted. The activity of this fraction was 0.31 γ/ml. The data for this chromatogram are given in Table XXII.

TABLE XXII

DATA FOR CHROMATOGRAM OF H-449

Fraction	Eluant Solvent	Volume in Ml.	Total Weight in Grams	γ/ml. to Inhibit Growth of M. pyogenes
H-453	Ether	54	0.008	Inactive
H-454	Ether	29	0.006	Inactive
H-455	Ether alcohol	34	0.003	Inactive
H-456	Ethyl alcohol	23	0.033	0.31
H-457	Ethyl alcohol	40	0.010	0.78
H-458	Ethyl alcohol	29	0.003	0.78

It was thought that the active principle in section 2 and section 3 of the parent pH 2 alumina column was the same material. For the purpose of comparison the ultraviolet spectra of H-452 and H-456 were prepared. The spectrum of H-452 is given in Figure 13 and that of H-456 in Figure 14.

Qualitative Information Concerning Some Active Preparations

Stability in Heat and Air

An ether solution of a sample with an activity of 3.9 γ/ml. was allowed to stand at 37°C for eight hours. The activity of the sample upon retesting was found to be 16 γ/ml. Another portion of the same sample was allowed to stand at room temperature for eight hours with air bubbling through it. The bioassay on this sample was found to be 16 γ/ml. The original sample stood at room temperature for eight hours and when retested still had an activity of 3.9 γ/ml. These findings definitely bear out results which were observed throughout all purification procedures.

Stability in Solvents

Crude extracts of the flowers were prepared using methyl alcohol, ethyl alcohol, isopropyl alcohol, butyl alcohol, and ethyl acetate. The extracts were tested for antibacterial activity when they were first prepared and then a second time 15 months later. All samples were stored in sealed containers and at a temperature of 8°C. It was found that the activity was greatly reduced in methyl and ethyl alcohol,

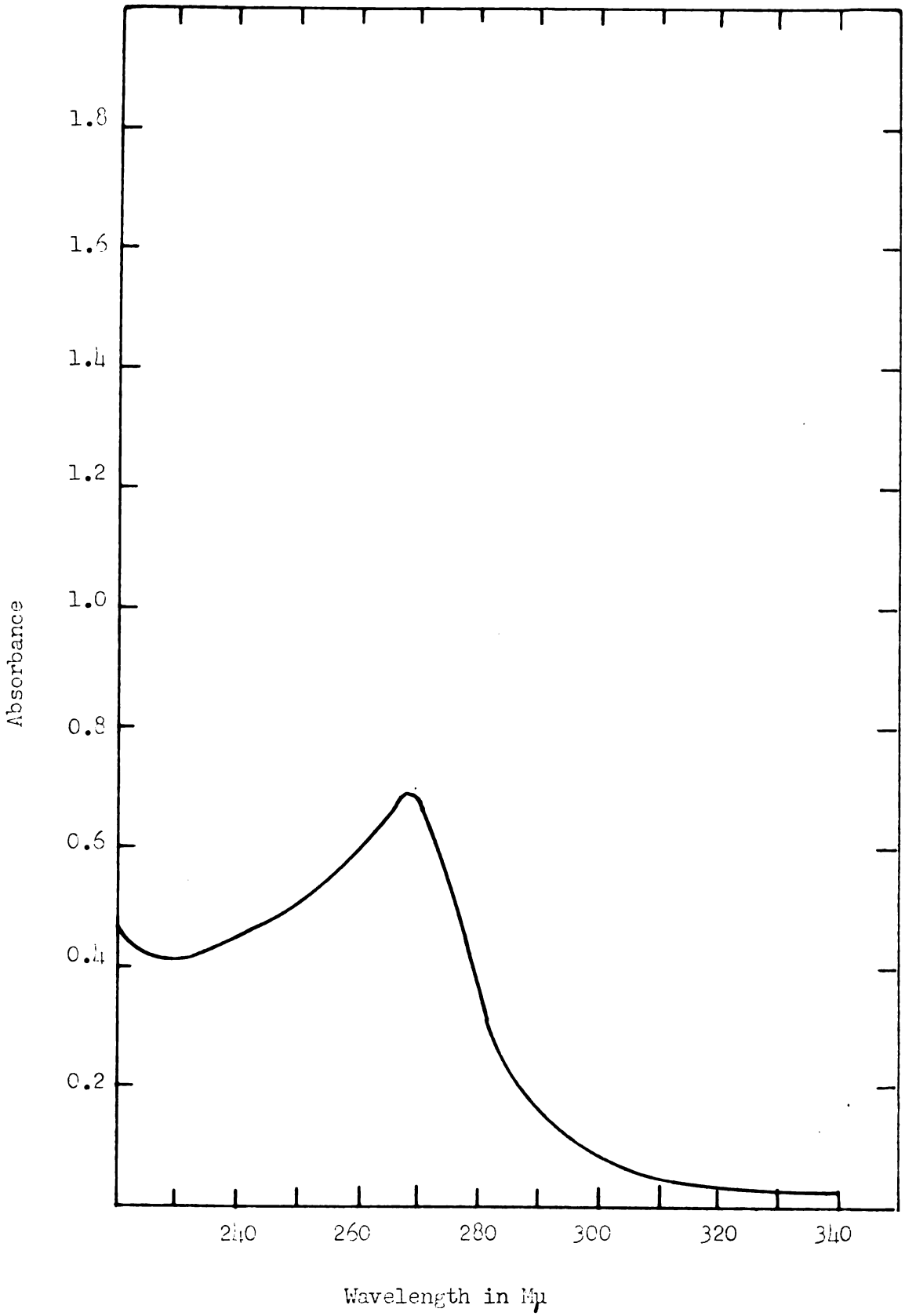

Figure 13. Ultraviolet Spectrum of H-452.

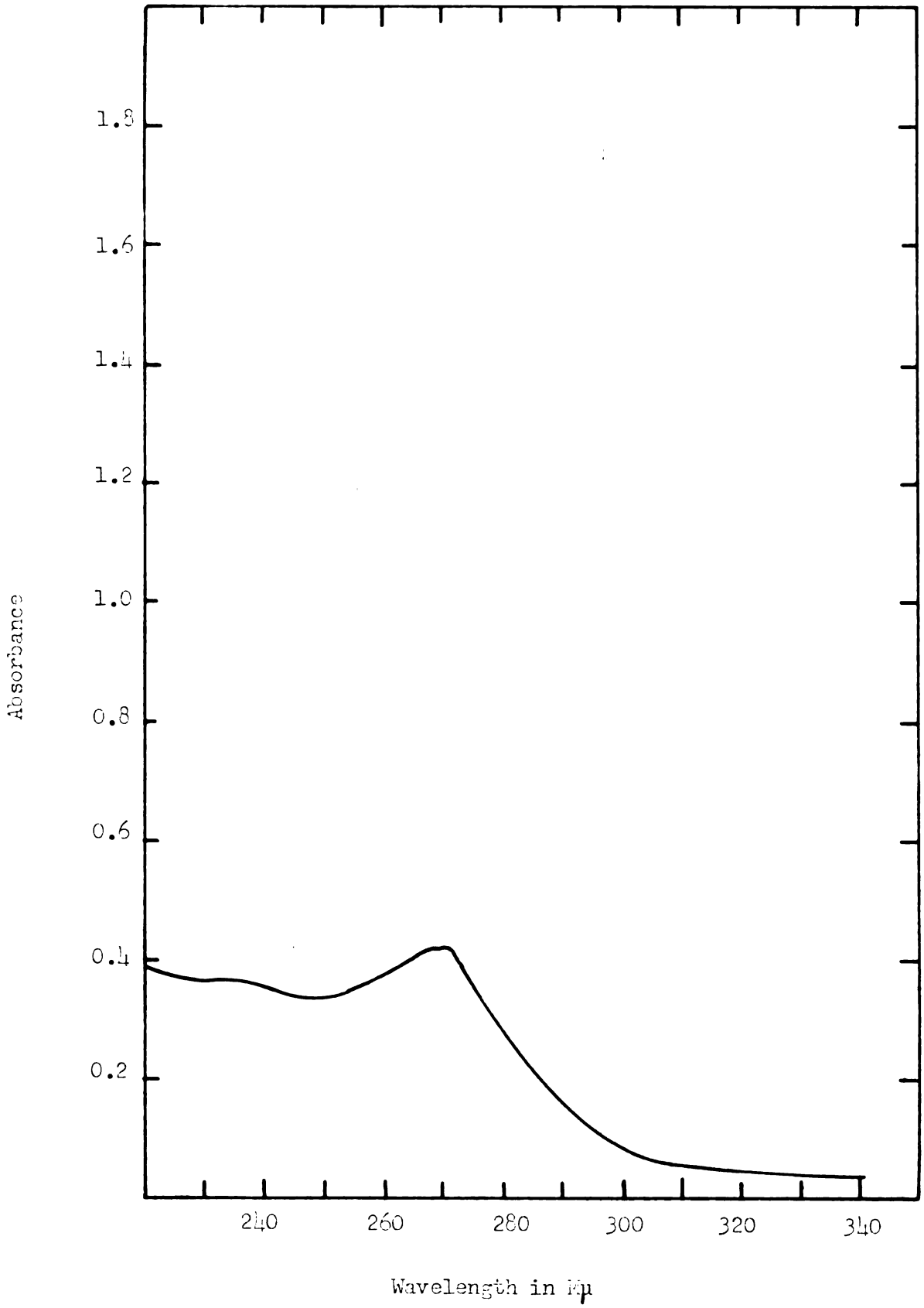

Figure 14. Ultraviolet Spectrum of H-456.

slightly reduced in isopropyl alcohol, and not altered in butyl alcohol and ethyl acetate. The data for these tests are recorded in Table XXIII.

TABLE XXIII

ANTIBACTERIAL ACTIVITIES OF CRUDE EXTRACTS AFTER LONG STANDING

Solvent	Initial Activity*	Activity* After 15 Months
Methyl alcohol	1:2000	1:60
Ethyl alcohol	1:2000	1:60
Isopropyl alcohol	1:2000	1:1000
Butyl alcohol	1:2000	1:2000
Ethyl acetate	1:2000	1:2000

*Greatest dilution exhibiting inhibition of M. pyogenes.

Although ether extracts were not tested under comparable conditions, routine stock solutions of the active material in ether solution maintained their activity for periods of at least three months.

Elemental Analysis

Several sodium fusions were prepared using portions of active fractions. The test solutions obtained from these fusions were used for elemental analysis which gave the following results: sodium nitroprusside test for nitrogen, negative; lead acetate test for sulfur, negative; silver nitrate test for halogens, negative. As a check on the fusion, a Kjeldahl nitrogen determination was also made on an active fraction with results indicating the complete absence of nitrogen.

DISCUSSION

The purpose of this research project as initially outlined was to isolate and identify the antibacterial substance(s) in the flowers of Hypericum prolificum. However, extenuating circumstances, such as the instability of the active principle, have kept the author from attaining the initial goal. It has been possible though, to obtain a fraction with a very high activity against Micrococcus pyogenes. Considerable information has been gained concerning the nature and properties of the active principle which may be of help in the future isolation of the antibacterial in this plant.

The preparation of a highly active fraction may be summarized as follows. The macerated flowers were extracted three times with ethyl acetate. The crude extract was then taken to dryness and redissolved in ether, producing the stock solution. The stock solution was then applied on a pH 2 alumina column. A two percent solution of ethyl alcohol in ether eluted an active fraction which was rechromatographed on pH 4 alumina (grade II). A highly active fraction with an activity of 0.3 γ/ml. could be eluted from this column by means of a one percent solution of ethyl alcohol in ether.

In the course of this research, some interesting qualitative information was learned about a number of active fractions. The infrared spectrum of a highly active fraction, prepared by the procedure just described, indicated the presence of a hydroxyl group (maximum at 3 μ),

an aromatic nucleus (maximum at 6.25 μ) and a carbonyl group (maximum at 5.79 μ). A phenol test with ferric chloride was found to be positive for some active fractions and negative for other active fractions. For one active fraction the ultraviolet spectrum exhibited a maximum at 290 mμ and for another active fraction the maximum was at 270 mμ. All of this information can do little more than give an approximate idea as to what the nature of the active material(s) may be. However, the phenol test and the ultraviolet spectra seem to indicate the presence of more than one antibacterial material.

The active material(s) apparently do not contain nitrogen, sulfur or halogens. It can also be stated that the active principle is somewhat acidic in nature as it can not be eluted from alkaline alumina but can easily be eluted from acid alumina.

SUMMARY

1. A neutral oil was prepared from an ether extract of the buds of P. tacamahaca. A medium boiling fraction of the neutral oil was chromatographed on alkaline alumina, yielding an unidentified sesquiterpene alcohol which was active against M. tuberculosis. A high boiling fraction of the neutral oil was chromatographed on neutral alumina. In this manner it was possible to isolate a sesquiterpene alcohol which was inhibitory towards M. tuberculosis and was identical with α-d-bisabolol in respect to the hydrochloride derivative, the infrared spectrum, the specific rotation.

2. A stock solution was prepared from an ethyl acetate extract of the flowers from H. prolificum. A fraction with strong activity against M. pyogenes and X. phaseoli was obtained by chromatographing the stock solution on pH 2 and pH 4 alumina consecutively. The exact nature of the highly active fractions was not established but they were shown to contain an aromatic nucleus, a hydroxyl group and a carbonyl group. The absence of nitrogen, sulfur and halogens was also demonstrated.

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APPENDICES

APPENDIX I

CHROMATOGRAPHIC TECHNIQUES

Column Preparation

Cassidy (26) gives a comprehensive survey of chromatographic columns, adsorbents and techniques. The choice of adsorbents, eluants and column size is difficult and is best arrived at on a basis of personal experience. In the present work two types of columns were used. A straight column reduced to a small diameter effluent outlet was used in elution work. A column with the lower end comprised of a ball and socket ground glass joint facilitated extrusion of the adsorbent. The flow rate of the eluant was controlled by a stopcock at the effluent outlet.

A plug of Pyrex glass wool was inserted into the column and taped evenly into the top of the outlet tube. The glasswool plug was then covered with a filter paper disk which was just slightly smaller than the inside diameter of the column. This provided an even base for the adsorbent. The column was mounted in a clamp and the outlet stopcock was closed. The column was then filled with the initial solvent. A funnel was inserted into a one hole stopper which was used to form an airtight seal at the top of the column thus forcing the solvent upwards into the funnel. The adsorbent, which had been covered with solvent for at least an hour, was added to the funnel and was allowed to settle by gravity with the occasional assistance of gentle tapping of the

column with a rubber mallet. When all of the adsorbent had settled, the stopcock was opened and solvent was allowed to percolate through the column to aid final packing. The stopcock was then closed, a second filter paper disk was placed on top of the adsorbent and solvent was maintained over the adsorbent until the sample was ready to be applied to the column.

Addition of Sample

The tip of a thistle tube was drawn out to 1 mm. inside diameter and bent at a right angle to the stem. The thistle tube was then inserted into a two hole rubber stopper. A small stopcock was inserted in the other hole. The stopper was then placed in the top of the column in such a manner as to produce an airtight seal and also allow the tip of the thistle tube to touch the side of the column. The stopcocks at the top and bottom of the column were opened and solvent was allowed to flow until two millimeters of solvent remained above the column. At this time the sample, dissolved in a minimal amount of solvent, was added through the thistle tube. The sample was allowed to settle into the column until only two millimeters remained above the column, then a small volume of solvent was added through the thistle tube. This again was allowed to pass into the column until two millimeters remained above the column. Then the developing solvent was added through the thistle tube until the liquid level reached the thistle tube tip at which time the stopcock at the top of the column was closed. Volatile solvents such as ether tended to "vapor lock" and this was remedied by manipulation of the stopcock at the top of the column.

Gradient Elution

Abrupt changes in the type of solvent used as an eluant often do not produce satisfactory chromatograms. In this research a change of eluant was accomplished by gradually increasing the concentration of the new eluant in the previous eluant. This is defined as gradient elution.

Fraction Collection and Treatment

For elution chromatography a Misco fraction collector was used to cut fractions. The exact size of fractions was determined by criteria such as column size and flow rate, which were specific for each type of mixture being resolved. The fractions were taken to dryness by means of a flash evaporator. Special care was taken to keep sample temperature below 35°C when working with unknown materials. The dry sample was dissolved in anhydrous, peroxide free diethyl ether and transferred to a 50 ml. tared beaker. The beaker was placed in a vacuum desiccator and taken to dryness. Complete drying of sample was indicated by a constant weight. In this manner a weight-eluant volume graph could be prepared to show the effectiveness of separation. Physical and chemical tests, such as infrared spectra and refractive indices, were applied to establish the nature of the sample. The sample was dissolved in a suitable solvent and a portion sent for bioassay. The remainder of the sample was set aside for further use. All samples were stored at a refrigerated temperature and in sealed containers.

In some cases it was necessary to determine the concentration (w/v) of the eluant directly. This was accomplished by pipetting a specific

volume of the sample directly into a tared weighing bottle. The sample was then taken to dryness in a gentle air stream, placed in a 100°C oven for five minutes, and then weighed. In this manner the weight/volume concentration of solid materials was determined.

APPENDIX II

BIOLOGICAL ACTIVITY

Bioassay

The samples from Populus tacamahaca Mill. were tested for activity against Mycobacterium tuberculosis, strain H-37, and Micrococcus pyogenes, var. aureus using the serial dilution method of Gottshall et al. (23).

Hypericum prolificum samples were tested against Micrococcus pyogenes var. aureus and Xanthomonas phaseoli by the serial dilution method of Gottshall et al. (23). In all cases, the highest dilution at which growth was inhibited was recorded as the activity of the specific sample.

Activity Expression and Calculation

The specific activity of each sample was expressed as the smallest number of micrograms (γ) per milliliter of culture broth which inhibited bacterial growth.

Example: A 1% (w/w) sample solution was sent for bioassay and was found to inhibit bacterial growth at a dilution of 1:512. The concentration at this dilution is obviously 1/512 of the original sample (which in this case was 0.01 grams per milliliter) or 19.5 micrograms (γ) per milliliter. A simple formula for calculating activity in this manner is given below.

$$\text{Specific activity (in } \gamma\text{/ml.)} = \frac{C_s \times 10^4}{D}$$

C_s = concentration of original sample in percent (w/w)

D = maximum observed dilution inhibiting bacterial growth

For the above example then;

$$\gamma/\text{ml.} = \frac{C_s \times 10^4}{D} = \frac{1 \times 10^4}{512} = 19.5$$

Interpretation of Bioassay Results

The bioassays used in this work have definite limitations. For example, solvents such as ether and alcohol inhibit the growth of M. tuberculosis at a dilution of 1:40 and M. pyogenes at a dilution of 1:8. Consequently, only dilutions larger than these values can really be considered significant. Also, a small error in the concentration of the sample, whether by weighing or dilution, may cause the end point reading to be shifted one tube higher or lower. A retest of the sample would be necessary to determine the significance of a result in this category.

Total Activity

In the process of resolving a mixture of active and inactive materials, it was necessary to have some measure of the total activity in order to follow the course of purification. A unit of activity was set up as follows:

1 unit = 1 milligram of solids/milliliter of broth inhibiting bacterial growth.

If a 1% solution of a sample (10 mg./ml.) diluted a thousand times inhibited bacterial growth, the sample was said to contain 10,000 units

of activity. A formula for calculating the total activity in a fraction is given below:

Total units of activity = W_s x D

W_s = weight of total solids in the fraction expressed in milligrams.

D = greatest dilution at which a 1% solution of the sample inhibits bacterial growth.

Example: A chromatographic sample weighed 0.5000 grams. A 1% solution of this sample inhibited bacterial growth at a dilution of 512. Then:

Total units of activity = W_s x D

= 500 x 512

= 2.56×10^5

APPENDIX III

MATERIALS

Solvents

Diethyl ether obtained from Merck and Company was washed with a solution of ferrous sulfate, dried over calcium chloride and magnesium sulfate and distilled from over sodium. The solvent was stored over sodium in a brown bottle.

Merck and Company petroleum ether was redistilled and the fraction boiling from 30°C to 60°C was used in all chromatographic work.

All ethyl alcohol used in this research was absolute ethyl alcohol obtained from the Commercial Solvents Corporation. The solvent was not purified further.

The chloroform used was Mallinckrodt C. P. grade.

The ethyl acetate used in this research was Merck and Company C. P. grade.

Adsorbents

Alumina -- Alcoa alumina, grade F-20, 80 mesh, Activity I, was used in all chromatographic columns unless otherwise specified. When dispersed in a small amount of water, this alumina produced an alkaline reaction in the range of pH 12.

An acid alumina, pH 4, was used in preparing several chromatograms. It was prepared in a manner similar to that of Kuhn and Wieland (27). A quantity of Alcoa alumina, grade F-20, 80 mesh, was slurried with one normal hydrochloric acid solution for one hour. The acid was decanted

and the alumina was washed with distilled water until the washings reached a pH of 3.9 to 4.0. It was very critical that the pH of the washing lie within the given range. The alumina was then filtered on a large Buchner funnel and air dried with the aid of a heat lamp. The alumina was placed in large evaporating dishes and dried at 225°C for a period of four hours.

Three types of Woelm alumina (acid-pH 2, neutral-pH 6, alkaline-pH 10) were obtained from Alupharm Chemicals, 54 C Street, Elmount, L. I., N. Y.

The activity of alumina is a measure of the affinity of the adsorbent for the adsorptive and is proportional to the amount of moisture in the alumina. Brockman and Schodder (28) have established a method for determining the activity of alumina based on the relative ease of elution of a series of asobenzene dyes by a benzene-petroleum ether eluant. In most cases, the activity of the alumina may be adjusted by adding water to the alumina. For example, grade I (Brockman) alumina may be changed to grade II (Brockman) alumina by addition of water in an amount equal to three percent of the weight of the alumina. In this research, the activity of all alumina was I, unless otherwise specified.

Other Adsorbents

Corn starch -- A commercial product obtained from The Carrier Stevens Company, Lansing, Michigan.

Calcium carbonate -- Mallinckrodt anhydrous analytical reagent.

Charcoal -- Nuchar-C-190 VHG, Industrial Chemical Sales, New York, New York.

Cellulose -- Solka-Floc, grade BW-200, Brown Co., Berlin, N. H.

Celite -- Analytical Filter-Aid, Johns-Manville Company.

Magnesia -- Merck and Co., U. S. P. grade.

Silica gel -- Davison PA 100 silica gel, Davison Chemical Co., Baltimore, Md.