THE NATURE OF CROTON RESIN FROM CROTON TIGLIUM (LINNÉ)

BY

JOSEPH R. SPIES

Thesis submitted to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy

1934.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

-:ACKNOWLEDGMENT:-

The author wishes to express his appreciation to Dr. Nathan L. Drake for the advice and assistance which he has given during the course of this research.

TABLE OF CONTENTS

TABLE OF CONTENTS

I. INTRODUCTION

This research was undertaken as the logical sequence in an examination of a large number of plant materials in the search for new sources of insecticides. It was only natural that the recent development of a group of plant insecticides, of which rotenone[1] is probably the most important, should serve as a stimulus to further studies of this nature. The search for new insecticides is also inspired by a demand for cheaper and more effective agents with which to combat pests which are difficult to control with known materials. The danger of toxic spray residues on food materials is well recognized[2], and more stringent food-law restrictions against the presence of arsenic, lead, and flourine residues on fruit and vegetables have created a demand for new insecticides which are non-injurious to man and animals.

Attention was first directed to the croton bean as a possible source of insecticidal material by Drake and Spies[3] (1932) and Spies[4] (1933). In the examination of the toxicity to goldfish of acetone extracts of over one hundred reputedly poisonous plants, these authors found that from the croton bean to be considerably more toxic than any of the others.

Unpublished work by Davidson[5] et al. of the U. S. Food and Drug Department showed the acetone extract of croton tiglium to be quite effective when used as a spray material against a variety of insects.

A communication from Dr. Jung of the Bureau of Entomology of China, which accompanied the first sample of croton beans studied, stated that a "croton emulsion" made from the croton bean was used as an insecticide in China. A letter to Dr. Jung requesting more information regarding this "croton emulsion" has, however, remained unanswered up to the present time, so further facts regarding this preparation and its uses is lacking.

The toxic, vesicant and purgative properties of the oil obtained from the croton bean have been long recognized and according to the thesis of K. Bernhard[6] (1932) its pharmacological bibliography dates back to the sixteenth century. Probably the first attempt to isolate the vesicant principle of croton oil was due to Schlippe[7] (1850) who claimed to have obtained a resinous material having a formula of $C_{18}H_{28}O_4$, (or multiple thereof) which he called crotonol and to which he attributed the vesicant action of the oil. Schlippe's work however was not verified by later workers.

Buchheim[8] (1872) and later Kobert[9] (1887) and Hirschsohn[10](1890) obtained an acidic substance called

crotonoleic acid from the alcohol soluble part of croton oil by saponification with $Ba(OH)_2$. These workers considered this substance to be the vesicant and toxic principle of croton oil.

Stillmark[11] (1889) isolated a toxic albuminoid from croton seeds which he confounded with ricin and which Elfstrand[12](1898) subsequently showed to be a new material to which he gave the name crotine. Crotine has been studied more recently by Karrer[13] and co-workers (1925).

The crotonoleic acid of Kobert and Hirschsohn was found by Dunstan and Boole[14] (1903) to be a mixture of oily inactive acids and a resinous material which according to them possessed extraordinary vesicant properties. They proposed the name croton resin for this new substance to which they assigned the formula $C_{20}H_{30}O_3$. Their croton resin was obtained by treatment of that portion of croton oil which is soluble in alcohol with aqueous lead oxide and subsequent separation from the resulting lead salts of the neutral resinous material. The vesicant properties of the resin were shown to be lost by saponification, and it was demonstrated that the compound was not a glyceride.

R. Boehm[15](1913) has isolated from the methyl alcohol soluble fraction of croton oil a neutral resin which amounted to 2% (on the basis of optical activity a theoretical yield of 10% was calculated by Boehm) of the crude oil. His procedure was complicated and involved the use of lipolysis for the saponification of the fats, and the removal of the

fatty acids formed by means of treatment with barium hydroxide in alcoholic media. This process always involved a partial destruction of the resin which is quite sensitive to alkaline hydrolyzing agents. Finally a product insoluble in petroleum ether was obtained which was further purified by means of extraction with this solvent. Boehm's resin had a molecular weight and percentage composition which were satisfied by the formula $C_{36}H_{54}O_9$; it was saponifiable (destroyed toxic action), unsaturated, non-acidic, and possessed no free hydroxyl groups. Like the resin of Dunstan and Boole it was not a glyceride and it yielded a series of fatty acids upon hydrolysis. Boehm also made a study of its physiological action; at a concentration of $1:10^6$ this resin killed frog larvae in from three to four hours.

Recently Boehm and Flaschentrager[16] et al (1930), recognising that the resin of Boehm was a product already altered by the processes of its extraction, have succeeded in isolating from croton oil by a purely physical means, which they did not disclose, a yellow honeylike material (Giftiger Naturstoff) which they considered to be the pure toxic principle. This substance according to them forms about 3% of the oil and by controlled hydrolysis (method not described) these authors claim to have isolated a crystalline, non-toxic compound called phorbol. Phorbol upon acetylation was stated to acquire the

pharmacological properties of the unhydrolyzed resin. They concluded, therefore, that the toxic principle is an acyl derivative of phorbol.

Despite the claims of Boehm and Flaschentrager regarding the "Naturstoff" Cherbuliez, Maringer and Bernhard[6] (1932) believed it to be a non-homogeneous substance and they have shown the presence of an extremely active resin found in small quantity in croton oil and to a much larger extent in the seed. These authors have developed a physical means of obtaining croton resin starting either with the oil or the bean and they have followed the efficiency of the different steps of their extraction processes by testing experiments. The yield of croton resin obtained by them from the oil was only 0.05 to 0.1% whereas Boehm and Flaschentrager reported a yield of about 3% of the "Naturstoff".

Practically all of the published work of Cherbuliez et al. on croton resin has been confirmed in this investigation and the resin used has been obtained by a process essentially the same as that outlined by them.

II. SOURCE OF CROTON BEANS

Croton tiglium (Linné) is a species of the croton genus which belongs to the Euphorbiaceae family of plants. The seed or bean is the fruit of a tropical shrub which is common in the wild state as well as cultivated throughout Hindustan and some of the East Indian and Philippine Islands. It has also been introduced into Japan and other countries.

Croton beans are used as fish poison by the natives in some countries. No published accounts of its use in this connection were encountered by the author, but the following quotation taken from a communication from Mr. H. E. Heibert, Milbuk, South Mindanao, Philippine Islands describes its use for this purpose; " the natives use the scattered seeds between crops to kill fish by pounding the seeds and mixing the crushed seeds with some vegetable substance, after which the ingredients are thrown into the sea or stream. The fish turn 'belly up' in a few seconds after eating it".

The sample of croton beans (I.D.630) used by Spies[4] (1933), in which the extraordinary toxicity to goldfish of its acetone extract was first observed, was sent to the Insecticide Division by Dr. G. F. Jung. After it had been decided to continue the study of this substance it was of course necessary to obtain a larger supply of the croton seeds.

Inquiries to importers of crude drugs in this country however soon showed it to be impossible to obtain them here, as the demand in the United States previously had been solely for croton oil for medicinal purposes. This was obtained as such from foreign sources and not extracted or pressed from the seeds in this country.

In August (1932) a cablegram was dispached to Dr. Jung requesting him to send, if possible, a large supply of seeds. This inquiry was rewarded in October of the same year by the receipt of a shipment of 452 catties (the catty is a chinese and oriental measure of weight, it is equivalent to one and one-third pounds avoirdupois) of unshelled croton beans, contained in one package. Several other sources for the supply of croton beans were finally located and information concerning each lot is given in Table I. The I.D. No. (Insecticide Division number) is given as a convenient means of identification. If further information is desired it may be obtained from the Insecticide Division of the Bureau of Chemistry and Soils, Washington, D. C., by reference to that number.

TABLE I.

Information Concerning Shipments of Croton Beans

from Various Sources

Source	Description	I.D.No.	Price per lb.	% yield of resin	lbs. received
Dr. G. F. Jung, Nanking, China	unshelled seeds	830	0.63	-	8
Dr. G. F. Jung, Hankow, China	"	1273	0.036	0.54	607
Schimmel & Co. Akt.- Ges. Miltitz bei Leipzig(thru Fritzsche Bros., N.Y.)	shelled seeds	1381	1.88	0.60	20
Anandji Virgi & Co., P.O. Box 153,Bombay, India.	"	1444	0.33	0.04	100
Henry K. Heibert, Milbuk,Philippine Islands.	"	1530	1.00	-	5
J. L. Hopkins & Co., 125 William St., N.Y.	oil	-	3.50	-	5

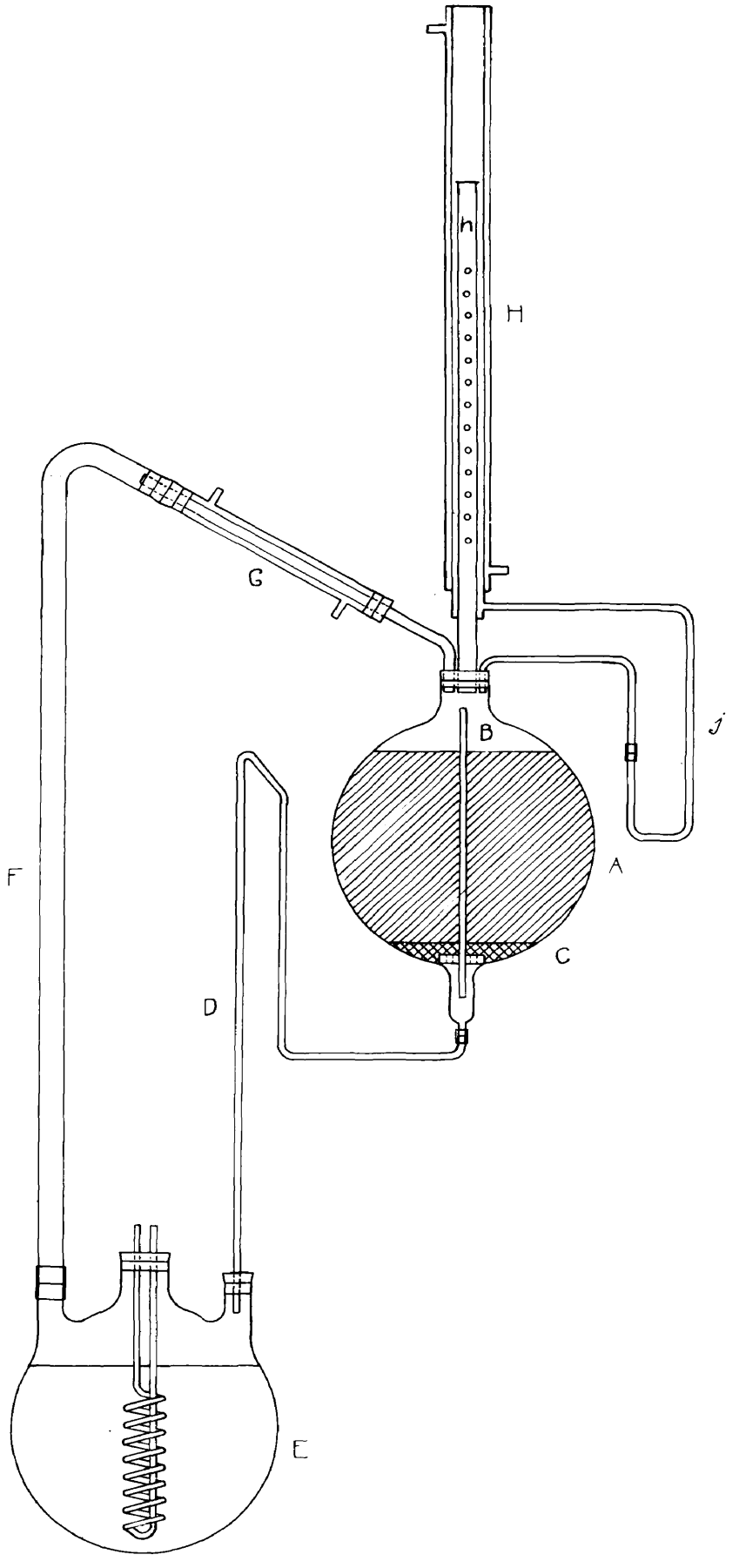
Probably the reason for the comparatively low cost (between 8 and 9 cents per pound including transportation) of the seeds obtained from Dr. Jung was the fact that they were purchased from various dealers in China who evidently had little or no demand for them. These seeds also were not of select quality, many of the beans were shriveled and dried. No doubt good quality seeds could be obtained (1932), in large quantities, for considerably less than one dollar per pound.

III. EXTRACTION OF CROTON RESIN

The croton resin used in this investigation was obtained by a process essentially the same as that employed by Cherbuliez et al., with, however, certain modifications which in the writer's opinion make the process more adaptable to a somewhat larger scale procedure. These authors have followed the efficiency of their extraction processes by carefully conducted testing experiments. They have also shown that there is little likelihood that any alteration in the composition of the resin has occurred during its extraction and purification, since all of the steps involved are in the main purely physical in nature. Check experiments on those steps where change in composition conceivably could have taken place showed that no such alteration had occurred.

In the isolation of a substance obtained as is croton resin, by extraction and evaporation processes where the final product is admittedly non-homogeneous, it is important that the method of preparation be clearly outlined, as the final composition must necessarily be dependent upon the process of preparation. The following description of the method used in isolating croton resin is given therefore in considerable detail.

The unshelled seeds were first carefully ground.


h
H
G
B
J
A
C
F
D
E

Cherbuliez et al. have shown that the outer shells do not contain any of the vesicant principle, nevertheless it is advantageous to make the extraction on the ground unshelled seeds as the shells tend to absorb much of the oil present in the bean. This forms a somewhat granular mass which allows more intimate contact with the extracting liquid. The shelled beans when ground form a heavy sticky mass which is very difficult to handle and which is not at all suitable for efficient extraction. When dealing with the latter it is well to mix about an equal volume of marc from former extractions with the unshelled beans at the time of grinding, this not only facilitates grinding and subsequent handling of the material but also forms a more readily extractable mass.

The extractions were made in a large-capacity Soxhlet designed by Dr. N. L. Drake[17]. The details of this apparatus are shown in Fig. 1 and since it is already described in the literature it will not be discussed further here. A photograph of the apparatus in operation is shown in Plate 1.

Five kilos of the ground unshelled beans were extracted with methyl alcohol for 24 hours. At the end of this time the solution of the extract was withdrawn from the boiler, a fresh portion of methyl alcohol was added and the extraction continued for 24 hours more. The liquors obtained in this manner from 30 kilos of croton beans were combined in five gallon glass bottles. The bottles containing the original

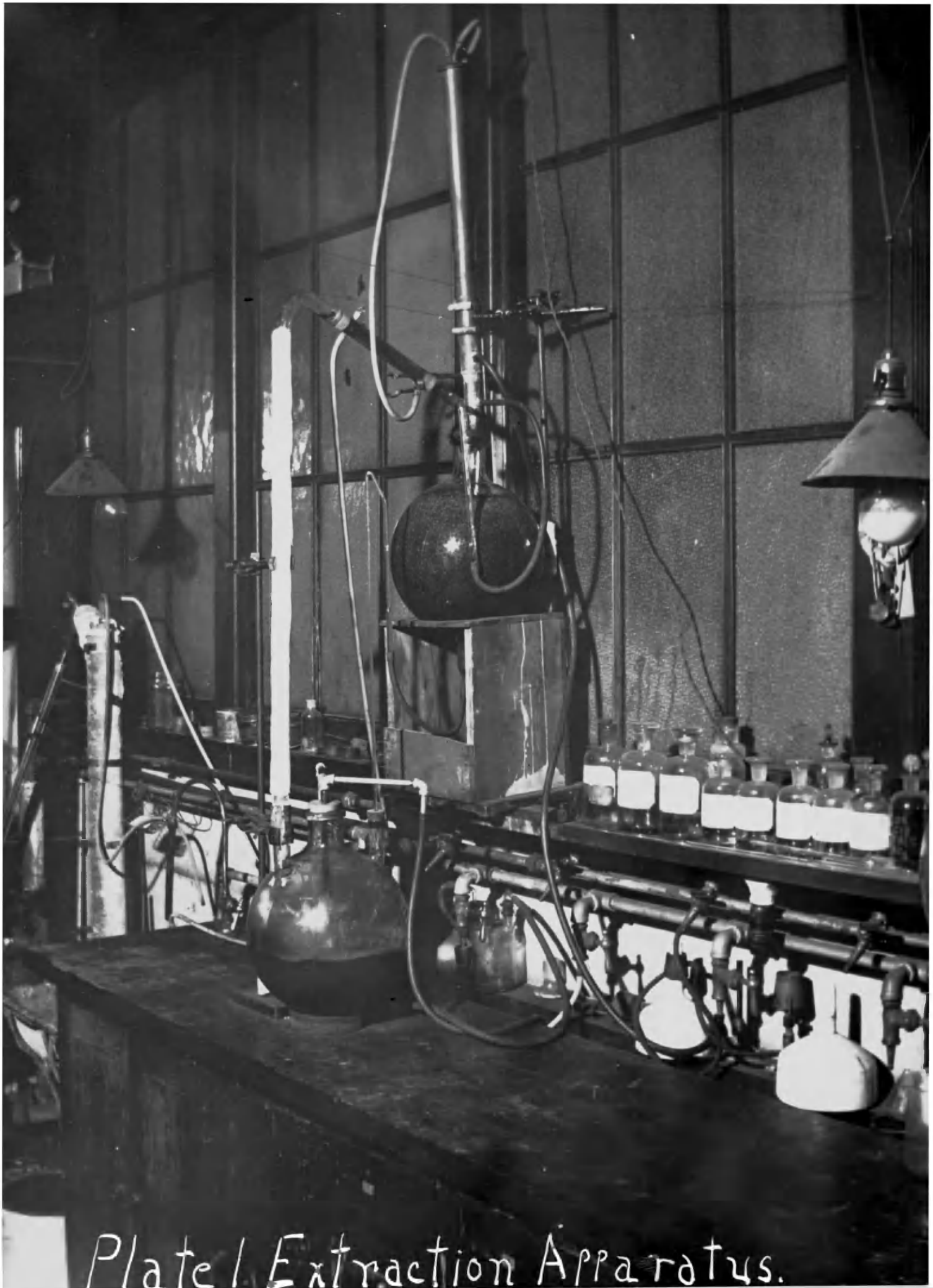


Plate I Extraction Apparatus.

extract were cooled over night in ice or by allowing to remain outside if the temperature were low enough. The contents of the bottles separated into two layers, the upper, a clear brown solution was siphoned off and placed directly in the solvent recovery apparatus (plate 2) for the removal of the methyl alcohol under diminished pressure. To the lower layer (containing oil, precipitated crotonoside[10], etc.) was added 2 liters of petroleum ether (30-70°) and the mixture was agitated to secure solution of the oil. This solution was next filtered through a large folded filter. The work was planned so this process could be completed over night as the filter drained slowly. The resin retained by the precipitate can be recovered by washing the solid on the filter with methyl alcohol, or at a later time when the crotonoside is purified. The filtrate separated into two layers, the upper containing petroleum ether and croton oil, and the lower the methyl alcoholic solution of resin, etc. The lower layer was siphoned off and added to the methyl alcohol solution already in the solvent recovery still, the petroleum ether layer was extracted with five 500 c.c. portions of 90% methyl alcohol. These methyl alcohol washings were also combined with the solutions in the solvent recovery still. After the removal of the methyl alcohol and water from these combined solutions 1 liter of pure methyl alcohol was added to the gummy brown

Plate 2. Solvent Recovery Still.

residue, while still warm, and the mixture was refluxed and shaken until no more of this matter seemed to dissolve. Upon cooling considerable dark gummy matter separated. The solution was decanted from this precipitate which was then boiled up with 600 c.c. of 90% methyl alcohol from which most of it again separated on cooling. This second extract was filtered and the filtrate was combined with the preceeding methyl alcohol extract. The insoluble acidic solid material was discarded. 100 c.c. of distilled water was now added to the methyl alcohol solution to give it a concentration of 90%. To this solution, contained in a large separatory funnel, was added 1200 c.c. of petroleum ether (30-70°) and the mixture was shaken well. This treatment usually caused the precipitation of more of the acidic gummy solid which prevented the ready separation of the petroleum ether and methyl alcohol layers. The liquid consequently was decanted from this solid and when entirely free from it the two layers separated rapidly. The precipitated solid was washed with 90% methyl alcohol to remove any resin retained by it. The petroleum ether layer was extracted with six 250 c.c. portions of 90% methyl alcohol, and then the combined methyl alcohol solutions were extracted with three 400 c.c. portions of petroleum ether. The combined petroleum ether extracts were in turn now extracted three times with 150 c.c. portions of 90% methyl alcohol. This involved process served to remove rather effectively most of the glycerides and free acids and

yielded finally a resin having the physical characteristics of the product described by Cherbuliez et al. Consequently, the second purifying process sometimes used by them, in cases where these impurities were not fully removed, was rendered unnecessary.

All of the methyl alcoholic extracts, obtained as just described, were combined and the methyl alcohol removed under diminished pressure. While the resulting aqueous suspension of resin, together with certain acidic impurities, was still warm, 500 c.c. of ether was added (despite some volatilization) and the mixture was shaken and refluxed for some time to secure solution of the resin. In cases where the aqueous suspension was allowed to cool before the ether was added a sticky mass was obtained which was difficult to disintegrate in order to dissolve the resin therefrom. The flask was then cooled and enough cold water was added to give mobility to the mixture. The contents of the flask was then poured into a suitable separatory funnel, 500 c.c. more ether was added and the whole shaken for 15 minutes and then allowed to stand over night to separate. Any solid matter which separated was removed, washed with ether, and the washings were combined with the main ether solution. The aqueous layer was washed with seven 500 c.c. portions of ether. This large quantity of ether was employed because a large volume of solution was desirable for the next step of the process. The ether extracts were combined,

washed with water and filtered.

It is important that all suspended solid particles should be removed at this point as their presence tends to stabilise the emulsion which readily forms in washing the ether solution of the resin with the aqueous alkali used in the next step of the process. This is undesirable because of the sensitivity of the resin to alkaline hydrolytic agents.

Finally the clear ether solution (4 to 4.5 liters) was washed with 200 c.c. portions of 0.2 N potassium hydroxide until no more acidic matter was removed. This point was indicated qualitatively by the lack of coloration of the alkaline layer, and could be verified by its failure to give a precipitate upon acidification. Usually about fifteen washings were necessary for the complete removal of these acidic impurities. In washing the ether solution of the resin with the dilute alkaline solution vigorous shaking should be avoided to prevent the emulsion formation already mentioned. The alkaline solution should instead be poured carefully down the side of the separatory funnel which is then agitated by a gentle rotatory motion. The small amount of emulsion which formed each time at the juncture of the two layers, despite these precautions, was removed and placed in a smaller funnel. If separation did not finally occur the emulsion was broken by acidifying with dilute hydrochloric acid. When free from acidic impurities the ether solution was finally washed with

water to remove all traces of alkali. It was then dried over night with anhydrous sodium sulfate. The solution was filtered and the ether was removed by evaporation on the steam bath. Constant stirring during the evaporation was necessary to prevent bumping or foaming due to super-heating.

Finally all but the last trace of ether was removed by placing the beaker containing the resin on a heated copper block in a desiccator and alternately evacuating and admitting air. Yield 100 gms. (0.5%).

In the manner just described a total of 110 kilos (255 lbs.) of croton beans were extracted and 670 gms. of resin was obtained therefrom.

All of the samples of resin were dissolved in ether, thoroughly stirred together and then the ether was evaporated as before. This gave a product of uniform composition for all of the experiments described later (unless otherwise stated). The resin was stored in the bottom of a desiccator out of contact with the air and away from the light. For removal of the resin from the desiccator it was found convenient to first warm it on the steam bath after which the semi-fluid material could easily be removed with a tablespoon.

IV. PHYSICAL AND CHEMICAL CHARACTERISTICS OF CROTON RESIN

Croton resin is an orange-yellow or light brown transparent resinous material. At ordinary temperatures it is fairly hard and brittle but as the temperature is raised it gradually softens and becomes quite fluid at 50-70°. It has a sharp penetrating odor, particularly when warmed, which after some time causes burning of the nose and upper part of the throat, this burning sensation persists for a considerable length of time. The resin is miscible in all proportions with all of the common organic solvents with the exception of petroleum ether in which, however, it is quite appreciably soluble, it is practically insoluble in water.

The resin showed no tendency to crystallize either by cooling or allowing the solvent to slowly evaporate from its solutions. Crystallization is no doubt prevented by the fact that the resin is an intimate mixture of several structurally similar chemical individuals.

In the preparation of samples of the resin or any of its derivatives for analysis it was of course quite important that all of the solvent should be removed since these samples were prepared by evaporation of and not crystallization from the solvent. Before all analyses the samples therefore were freed from final traces of solvent by heating in a platinum

boat in an abderhalden drier at a temperature of from 100-110°. Air was alternately evacuated from and admitted into the drying chamber until some time after no more bubbles formed in the resin when the pressure was reduced.

To test the efficiency of this method of solvent removal, the following experiment was performed. About 2 grams of resin was dissolved in 150 c.c. of pure chloroform and the solvent was then removed by distillation on a steam bath. The chloroform was distilled before use in order to insure removal of any non-volatile halogen containing residues. The sample of resin was then poured into the platinum boat and placed in the abderhalden drier which was heated to 110° by boiling toluene. Air was then alternately evacuated from and admitted into the drying chamber for two hours. At the end of this time halogen could just barely be detected in the resin by means of the Beilstein test. Finally when no more bubbles could be raised (at 22 mm. pressure) in the resin no appreciable Beilstein test could be obtained. This method therefore was followed for the removal of solvent before all of the analyses of non-crystalline compounds reported in this thesis.

Analysis of the original resin:

	Sample (mg)	H_2O (mg)	CO_2 (mg)	% H	% C
1.	3.637	2.934	9.209	8.60	68.55
2.	3.637	2.872	9.113	8.84	68.34
	Analyses of Cherbuliez et al.			8.98	69.2
				8.95	68.8

The molecular weight of croton resin was determined by the Rast[19(a)(b)] modification of the Barger method. The resin used for this determination was taken from the sample used for the toxicity tests, its preparation is described in Sec. VII (3). The following analysis of this sample shows that its composition was similar to the main supply of resin.

Analysis:

	Sample (mg)	H_2O (mg)	CO_2 (mg)	% H	% C
1.	3.546	2.788	8.914	8.79	68.56
2.	3.692	2.930	9.371	8.80	68.47

Following is the data obtained in the molecular weight determinations:

0.2431 gms. resin was dissolved in 3 c.c. of $CHCl_3$. Because of its red color azobenzene (Eastman) was used in the preparation of the standard solutions. The temperature of the capillary tubes was adjusted to within $\pm.5^0$ for the initial and final reading of the ocular micrometer.

The results obtained in the first determination are shown in Table II.

TABLE II.

Data Obtained in First Determination of the Molecular Weight of Croton Resin

Conc.of Azobenzene Solution.Normality	Initial reading of micrometer	Final reading of micrometer	Direction of change
0.10	4.75	9.13	+
0.12	5.89	7.00	+
0.14	5.17	4.70	-
0.16	7.33	4.04	-
0.18	6.29	2.37	-
0.20	5.49	1.45	opposite-side

Normality of resin solution = 0.13

Av. Mol. Wt. = 625.

The range of the standard solutions was then further limited and the results obtained are shown in Table III.

TABLE III.

Data Obtained in Second Determination of the Molecular Weight of Croton Resin

Conc. of Azobenzene Solution. Normality	Initial reading of micrometer	Final reading of micrometer	Direction of change
0.12	5.66	6.79	+
0.13	4.36	4.91	+
0.14	4.58	4.47	-
0.16	5.00	4.14	-

Normality of resin solution = 0.130

Av. Mol. Wt. = 600.

Average Mol. Wt. obtained by Cherbuliez et al. = 632.5.

The close agreement of the values obtained for the average molecular weight and the percentage composition shows the similarity in composition of the resin used in this investigation and that isolated by Cherbuliez et al. It is interesting to note that Boehm's resin, although already altered by the extraction process, also gave values very close to those reported here.

V. PREPARATION OF CROTADI DERIVATIVES

1. Unsaturation

Croton resin is an unsaturated substance as previous workers have shown. Its iodine number varies considerably for different samples, for Boehm's resin it was 77, while for the resin investigated by Cherbuliez et al. it was 48.4, 74.8, and 74.1 respectively for three different samples. The average value for the iodine number of the resin used in this investigation was 55.1 (Harms[20]).

Iodine number of original resin:

25.00 c.c. I_2 soln. ≙ 46.36 c.c. of 0.110 N $Na_2S_2O_3$ soln.

	Sample (gms.)	c.c. I_2 soln.	c.c. 0.110 N $Na_2S_2O_3$ soln.	I_2 No
1.	0.4660	35.00	47.37	52.5
2.	0.5027	35.00	45.60	53.8

It is of considerable interest to point out that Paul and Roth[21] have hydrogenated croton oil and later Boehm's resin using a colloidal palladium catalyst. They found the irritating properties of the oil to be proportional to the iodine number, these properties disappeared when the latter fell to zero. The iodine number of Boehm's resin fell from 77 to 12.5 upon hydrogenation and the resulting product was no longer toxic to frogs nor rabbits.

(a) Hydrogenation

It was found that only partial saturation (as shown by the fall of the iodine number) of the resin could be brought about by means of catalytic hydrogenation. Reduction using platinum black[22] as catalyst and 95% ethyl alcohol as solvent was tried in a preliminary experiment. Shaking was accomplished with the Adams machine and a pressure of about 60 lbs. per sq. in. and a temperature of 45-60° was used. By this treatment the iodine number (Gatterman and Wieland[23]) was lowered to 39.2.

Iodine number of hydrogenated resin:

5.00 c.c. I_2 $HgCl_2$ Soln. $\rightleftharpoons$ 41.00 c.c. 0.0190 N $Na_2S_2O_3$ soln.

Sample(gms.)	c.c.I_2 soln.	c.c.0.0190 N $Na_2S_2O_3$ soln.	I_2No.
1. .1512	15.00	99.46	30.2

Other catalysts were then tried in an effort to cause further lowering of the iodine number. Catalytic reduction using colloidal palladium[24] was next attempted. The catalyst was prepared in the following manner: 15 c.c. of 1% $PdCl_2$ solution, 3 c.c. of 5% gum arabic solution, and sufficient water (so that when the methyl alcoholic solution of resin was added about a 90% aqueous solvent would result)were mixed together and the $PdCl_2$ was reduced by shaking with hydrogen on the Adams machine. When the reduction of the catalyst was completed 10.5 gms. of resin in 100 c.c. of

methyl alcohol was added. The solution was shaken at room temperature and at a pressure of about 60 lbs. per square inch for about twenty minutes. At the end of this time the catalyst had precipitated, evidently coagulation was due to the specific nature of the resin solution.

The precipitated palladium was therefore filtered off and 3 gms. of nickel catalyst (Raney[25]) was added to the solution. A pressure of 50-60 lbs. per square inch and a temperature of 40-50° was employed. The solution was shaken on the Adams machine for at least one day and then the catalyst was filtered off. This treatment was repeated three times using a fresh portion of nickel each time. At the end of this procedure the resin was recovered and the iodine number was found to have an average value of 37.5.

Iodine number (Hanus) of resin hydrogenated with nickel catalyst.

25.00 c.c. I_2 soln. ⇄ 46.36 c.c. of 0.110 N $Na_2S_2O_3$ soln.

Sample (gms.)	c.c. I_2 soln.	c.c. 0.110 N $Na_2S_2O_3$ soln.	I_2 No.
1. 0.5084	25.00	32.30	38.8
2. 0.4400	25.00	34.93	36.2

This same sample of partially saturated resin (I_2 No.37.5) was then subjected to further hydrogenation using platinum black[22] as catalyst. Ethyl alcohol (95%) was

employed as the solvent and 0.49 gms. of PtO_2 was used. The solution and catalyst were shaken for five hours on the Adams machine under a pressure of 55-60 lbs. per square inch and a temperature of 48-50°. The catalyst was then filtered off and the iodine number was found to be practically the same as before the treatment.

Iodine number (Hanus) of resin hydrogenated with platinum black:

25.00 c.c. I_2 soln. $\rightleftharpoons$ 46.36 c.c. 0.110 N $Na_2S_2O_3$ soln.

Sample (gms.)	c.c. I_2 soln.	c.c. 0.110 N $Na_2S_2O_3$ soln.	I_2 No.
1. 0.6810	25.00	25.73	42.3

The partially hydrogenated resin still retained its resinant action and also at least most of its toxicity to goldfish, as will be shown later. In appearance the hydrogenated product was quite different from that of the original resin. It was no longer transparent but possessed a milky turbidity and was also somewhat harder than the starting material.

(b) Bromination

When treated with bromine in glacial acetic acid solution croton resin added bromine readily. Substitution also occurred as was shown by the evolution of hydrobromic acid.

Samples of brom resin were prepared for toxicity and vesicant studies by the following two methods:

1. About 1 gm. of resin was dissolved in 15 c.c. of cold glacial acetic acid. To the cold solution bromine was added drop by drop until an excess was present. The solution was then allowed to stand for one half hour. It was then poured slowly into a cold dilute $NaHSO_3$ solution. The bromide precipitated in white flocs, which were filtered off, then stirred up again with fresh $NaHSO_3$ solution and finally washed with water on the filter until the washings were neutral. The precipitate was then pressed on a porous plate and dried in a vacuum desiccator over P_2O_5. The dried material was amorphous and possessed a light yellow or orange coloration.

Bromine Analysis:

	Sample (mg.)	AgBr(mg.)	% Br
1.	3.483	2.920	35.67
2.	3.356	2.771	35.14
	Cherbulies et al.		31.1
			31.2

Carbon and Hydrogen Analysis:

	Sample (mg.)	H_2O(mg.)	CO_2(mg.)	% H	% C
1.	4.127	2.008	6.894	5.69	45.56
2.	3.627	1.800	6.050	5.55	45.50

The author wishes to express his appreciation to Mr. S. A. Shrader of the University of Maryland for the micro bromine determinations.

Differences in the method of preparation of the two samples of brom resin probably account for the rather wide variation in

the bromine content of the bromide of Cherbuliez et al. and the one reported in this thesis. Whereas Cherbuliez added bromine in 2% glacial acetic acid solution until it was no longer instantaneously discolored by the resin solution, the bromide whose preparation has just been described was allowed to stand in glacial acetic acid solution with an excess of bromine for one half hour. One would naturally expect a higher bromine content in the latter case.

2. Another 1 gm. sample of resin was dissolved in glacial acetic acid with gentle warming to hasten solution. The solution was then cooled and treated exactly as in the preceding case. More substitution seemed to take place, and the product was considerably darker in appearance.

The first sample of brom resin thus prepared still possessed a slight vesicant action (the second sample was not tested). They both possessed some toxicity to goldfish although to a greatly lessened extent than the original resin. Sample #1 was found to be slightly more toxic than #2.

Cherbuliez et al., however, reported their brom resin to be completely insipid. From this fact, together with the interesting findings of Paal and Roth on the hydrogenation of both croton oil and Beebe's resin, it would seem that the toxicity was dependent upon the presence of some unsaturated function existing within the molecule. Data will be presented in the section on the toxicity to goldfish studies showing that such does not represent the true situation.

2. Free Hydroxyl Groups

Croton resin contains free hydroxyl groups as was shown by active hydrogen determinations and by its reaction with acetylating and methylating agents. Beehm reported his resin to be free from hydroxyl groups, while Cherbuliez et al. reported the presence of 3.4% hydroxyl on the basis of the saponification numbers before and after acetylation. This value is probably not a true indication of the actual number of free hydroxyl groups present as the treatment used by them probably did not completely acetylate the resin. This contention will be proved by the experimental data presented in this section.

(a) Methylation

Aside from the desire to prepare new derivatives of croton resin, it was hoped that by protecting the free hydroxyls by means of ether formation that more tractable products would be obtained upon saponification. The resin was found to have an average original methoxyl content of 1.2%.

Methoxyl determination:

	Sample (mg)	c.c. 0.0193 N $Na_2S_2O_3$ soln.	% CH_3O
1.	79.20	9.30	1.13
2.	79.47	10.20	1.23
3.	82.30	6.45	1.19

Clark's[26] modification of the Viebock and Schwappach method was used for all of the methoxyl determinations reported herein.

The first attempt to methylate the resin involved the use of diazo methane[27] but was without success.

Methylation to the extent of 0.5% (corresponding to one hydroxyl) was obtained using the method of Haworth and Lapworth[28]. Following is a description of this method: about 2 gms. of resin was dissolved in benzene and enough of the latter distilled from the solution to insure dryness. 5 c.c. of acid-free $(CH_3)_2SO_4$ and 10 gms. of anhydrous K_2CO_3 were then added and the system was closed with a calcium chloride tube. The mixture was refluxed for 72 hours in such a manner that bumping insured thorough mixing. The K_2CO_3 was then filtered off, ether was added and the solution was washed with dilute ammonia to remove the excess $(CH_3)_2SO_4$. It was then washed with water until neutral and finally was dried over anhydrous Na_2SO_4. The ether and benzene were evaporated and the sample was prepared for analysis by the usual solvent removal procedure. The product was not discolored by this treatment. Using xylene as a solvent, however, considerable hydrolysis of the resin occurred when treated in this manner. The extent of this hydrolysis was shown by the characteristic deep discoloration of the product and the formation of acidic decomposition products.

Methoxyl determinations:

	Sample (mg)	c.c. 0.0186 N $Na_2S_2O_3$ soln.	% CH_3O
1.	18.46	12.15	6.32
2.	11.65	8.10	6.60

The gentlest and probably the most efficient means of methylating the resin was by the use of Purdie's reagents[29]. Following is a description of this method: 10 gms. of resin was dried by solution in benzene and subsequent removal of the solvent by distillation. 50 c.c. of pure dry CH_3I and 7 gms. of dry Ag_2O were then added, the system was closed with a calcium chloride tube and refluxed for 21 hours. A small flame was used to heat the flask so that vigorous boiling and consequent good agitation of the Ag_2O occurred. At the end of this time refluxing was stopped and 5 c.c. of the CH_3I solution was withdrawn in order to obtain a sample for analysis. This solution was boiled up with acetone to remove all traces of CH_3I, filtered through a hardened paper, and the solvent evaporated off. It was then redissolved in acetone, allowed to stand over night. (Centrifuging can be used in place of allowing the solution to stand over night in order to remove suspended Ag_2O which cannot be removed by filtration) and again filtered through a hardened paper. The solvent was evaporated and the sample dried as usual. The sample had an average methoxyl content of 10.5%.

Methoxyl determinations:

	Sample (mg)	c.c. 0.0196 N $Na_2S_2O_3$ soln.	% CH_3O
1.	27.78	30.40	10.41
2.	18.46	20.40	10.61

The CH_3I was then distilled from the remainder of the methylated product, and the water formed by the reaction was removed by distillation from its benzene solution. 50 c.c. of pure dry CH_3I and 7 gms. fresh Ag_2O was added and the resin refluxed as before for 20 hours. Finally a sample was prepared for analysis in a manner similar to that just described. It had an average methoxyl content of 11.7%.

Methoxyl determinations:

	Sample (mg)	c.c. 0.0196 N $Na_2S_2O_3$ soln.	% CH_3
1.	20.19	25.12	11.45
2.	27.46	34.00	11.85

This unquestionably represents the maximum amount of methylation attainable by this method inasmuch as a sample of methylated resin having a methoxyl content of 10.6% was refluxed with methyl iodide and silver oxide as just described for 258 hours. At the end of this period the methoxyl content had risen only to 11.3%.

Methoxyl determinations:

	Sample (mg)	c.c. 0.0201 N $Na_2S_2O_3$ soln.	% CH_3O
1.	13.73	14.80	11.32
2.	12.74	13.65	11.25

On the basis of 600 for the molecular weight of the resin and an average original methoxyl content of 1.2%, a methoxyl content of 0.4% and 11.6% would correspond to one and two hydroxyls respectively. The average value for the methoxyl content of the completely methylated resin was 11.7%, this is in excellent agreement with the value calculated for two hydroxyls. It is interesting to note that by treating the resin in dilute dry benzene solution with an excess of CH_3I and Ag_2O and refluxing for 92 hours a resin with a methoxyl content of 6.4% was obtained.

Methoxyl determinations:

	Sample (mg)	c.c. of 0.0303 N $Na_2S_2O_3$ soln.	% CH_3O
1.	14.22	8.80	6.49
2.	7.46	4.55	6.41

Thanks are due to Mr. S. A. Shrader for this methoxyl determination.

This result together with the fact that by methylation with Haworth and Lapworth's method a resin containing 6.5% methoxyl was obtained, makes it seem quite plausible that one

of the free hydroxyls is more acidic than the other.

The completely methylated resin possessed no vesicant action and was non-toxic to goldfish. In view of the mildness of the reaction used in its preparation it is exceedingly probable that the toxicity to goldfish and the vesicant action of croton resin are more intimately connected with the presence of its free hydroxyl groups than with the unsaturated function. The methylated resin in appearance gave no evidence of having suffered either hydrolysis or oxidation by the silver oxide. Indeed oxidation would be more likely in the bromination of the resin and yet the brom product still possessed both vesicant action and toxicity to goldfish, although to a much lesser degree than the original resin.

(b) Acetylation

The resin was acetylated by means of the two methods the description of which follow:

1.2 gms. of resin was dissolved in 25 c.c. of acetic anhydride, 2 gms. of anhydrous sodium acetate was added and the solution was refluxed for one hour. The excess acetic anhydride was then removed by distillation on the steam bath under diminished pressure. The residue was dissolved in ether and the solution was then washed with water until neutral. The solution was dried over anhydrous Na_2SO_4, filtered, the ether evaporated, and the residue dried in the usual manner.

The material was somewhat darker in appearance than the starting product. It was still vesicant and toxic to goldfish although to a lesser extent than the original resin. The average value of its saponification number was 358.

Saponification number:

	Sample(gms.)	c.c. 0.223N alc.KOH soln.	c.c.0.242 N HCl soln.	Sap. No.
1.	0.3463	40.00	31.40	355.3
2.	0.2875	40.00	30.2	360.9

The saponification values were all determined by refluxing the sample for 40 minutes with an excess of alcoholic KOH. Phenolphthalein was used as the indicator and the titration of the unused alkali was made in a volume of about 300 c.c. This large volume was necessary in order to distinguish the endpoint, as the saponification products of the resin and its derivatives possessed the usual dark discoloration.

2. 4 gms. of resin was dissolved in 10 c.c. of dry, pure, pyridine, the solution was cooled and 10 c.c. of distilled acetic anhydride was allowed to flow in slowly. The solution was allowed to stand at room temperature for 36 hours and was then poured slowly into cold distilled water. It was allowed to stand in contact with the water for one half hour in order to insure hydrolysis of the excess acetic anhydride. The aqueous suspension was then extracted with ether and the ether solution was washed with dilute HCl solution and then with water until neutral. It was dried over anhydrous

Na_2SO_4. the ether was evaporated, and the resin dried as usual. Its average saponification value was 336.

Saponification number:

	Sample (gms.)	c.c.0.220 N alc.KOH soln.	c.c.0.242 N HCl soln.	Sap. No.
1.	0.4040	40.00	27.70	341.6
2.	0.7693	40.00	19.10	331.1

Cherbuliez et al. obtained an acetylated product by this same method which had a saponification number of 341. They found this product to be about ten times more active (determined by tasting) than the resin acetylated by refluxing with acetic anhydride, and they concluded therefore that heating had destroyed some of the toxic properties of the resin. This conclusion is not supported by the data presented in the section devoted to the study of the toxicity of these derivatives to goldfish. It is true that the resin acetylated in the cold is more toxic to goldfish (conc. = 2 mg/l), but it also has a lower saponification number and hence more free hydroxyl groups than the resin acetylated with heat. The difference in the survival times of goldfish produced by the two samples is 14%. Gersdorff[30(a)] claims a 7% precision for the method, yet this may not be significant although it does support the theory that the toxicity of the resin is closely related to the presence of its free hydroxyls.

A sample of dimethyl resin (11.7% CH_3O) was also

acetylated by means of acetic anhydride and pyridine as has been described. Its average saponification value was 183.

Saponification numbers:

	Sample (gms.)	c.c.0.220 N alc.KOH soln.	c.c.0.342 N HCl soln.	Sap. No.
1.	0.3435	39.99	33.40	176.9
2.	0.3940	40.03	32.40	189.4

The average saponification number of the unacetylated dimethyl resin was found to be 181.

Saponification numbers:

	Sample(gms.)	c.c.0.220N alc.KOH soln.	c.c.0.342 N HCl soln.	Sap. No.
1.	0.3420	30.00	23.00	179.8
2.	0.3385	30.00	23.80	183.9

The average saponification number of the original unacetylated resin was found to be 244.

Saponification numbers:

	Sample (gms.)	c.c.0.220 N alc. KOH soln.	c.c.0.342 N HCl soln.	Sap. No.
1.	0.3803	40.00	31.10	254.5
2.	0.3617	40.00	33.50	233.2

The information obtained by these acetylation and methylation experiments is shown in condensed form in Table IV.

TABLE IV.

Methoxyl Content and Saponification Numbers of Derivatives of Croton Resin

Substance	Av. Saponification No.	Av. Methoxyl Content %
Original resin	244	1.2
Methylated resin	181	11.7
Acetylated Methylated resin	183	-
Acetylated resin #1	368	-
Acetylated resin #2	336	-

The increase in the saponification number of the acetylated resin over that of the original resin is not great enough to account for more than slightly over one hydroxyl group. An increase of approximately 87 in the saponification number is required for each acetylated hydroxyl. This shows that the acetylation did not extend as far as the methylation of the resin.

The fact that the saponification numbers of the dimethyl and acetylated dimethyl resins are the same indicates that no further esterification of any free unmethylated hydroxyl groups has taken place. Also the fact that this value is so much lower than that of the

original resin shows that at least one of the methylated hydroxyls possesses some appreciable acidity. This conclusion was also reached on the basis of methylation data which already has been discussed.

(c) Active Hydrogen Determinations

Active hydrogen determinations on both the methylated and unmethylated resin were carried out to determine the completeness of the methylation reaction. A modification of D. Flaschentrager's[31] method was used.

The pyridine used for the solvent was first dried by refluxing over calcium oxide and finally by distillation from Grignard reagent in pyridine to remove the last traces of moisture. It was then preserved in a glass-stoppered bottle, the stopper of which was well lubricated with vaseline to exclude all moisture. Another modification of the method consisted of sweeping out the apparatus with dry hydrogen gas after the sample and Grignard solution had been introduced. This procedure served to prevent the reaction of the Grignard reagent with the oxygen of the air, enclosed within the system, and consequent invalidation of the result.

Using these modifications good results were obtained on known substances; thus values of 1.07 and 1.05 active hydrogens were found for benzoic acid and chlorobenzoic acid respectively. The following formula given by Flaschentrager

was used to calculate the results obtained:

$\% H_{(a)} = \frac{0.004494Vo}{S}$. Vo is the volume of methane liberated (standard conditions), S is the weight of the sample.

Great care was used to insure dryness of the samples analysed. For the purpose of drying about two grams of each sample was dissolved in 150 c.c. of pure chloroform. The solvent was then distilled off and the last traces were removed by heating to 110° in the abderhalden in the usual manner. Finally the resin was allowed to remain for at least one hour at a pressure of about 20 mm. and a temperature of 110° in the presence of anhydrous calcium chloride.

Following is the data obtained by these determinations:

Original resin:

Blank = 0.64 c.c. at standard conditions (corrected for V.P of pyridine)

Sample gms.	P(mm)	T_K	V_S(cc)	% active* H	No. of OH groups
1. 0.0355	758	300	6.86	0.69	4.2
2. 0.0312	757	299	6.70	0.77	4.6
Dimethyl resin (11.3% CH_3O)					
1. 0.0449	757	299	4.13	0.31	1.9
2. 0.0336	757	299	4.46	0.28	1.7

* considering the mol.wt. 600, 1 active H corresponds to .166%.

These results show that the original resin contains approximately four free hydroxyl groups while the methylated resin contains two. These figures are borne out by the fact that the methoxyl content of the methylated resin corresponds to the presence of two free hydroxyl groups.

It can be concluded therefore that it is possible to methylate only two of the four free hydroxyl groups present in croton resin.

VI. PROOF OF THE NON-HOMOGENEITY OF CROTON RESIN

The lack of homogeneity of croton resin was recognised by Cherbuliez et al. and indeed one would scarcely expect to obtain a pure substance by the method used in its isolation. The experiment to be described was undertaken with the hope that a resolution of the components of the resin might be effected by which finally the pure toxic principle could be induced to crystallise. The experiment was based on the lack of miscibility of croton resin in petroleum ether and the fact that petroleum ether is very slightly soluble in 90% methyl alcohol.

50.0 gms. of croton resin was dissolved in 300 c.c. of 90% aqueous methyl alcohol (a slight flocculent precipitate did not dissolve) and the solution was placed in the body of a liquid extractor. 250 c.c. of petroleum ether (65-70°), which had previously been distilled, was then placed in the boiler of the extractor and the resin solution extracted for the periods of time recorded in the table. At the end of each period the quantity of resin extracted by the petroleum was determined by evaporation of the solvent and subsequent weighing. Sufficient fresh 90% methyl alcohol was added after each period to maintain a volume of 300 c.c.

for the resin solution. Samples of this material were prepared for the analyses and toxicity tests in the usual manner.

The results of this experiment are recorded in Table V.

TABLE V.

Results of the Extraction of a 90% Aqueous Methyl Alcoholic Solution of Croton Resin with Petroleum Ether

Hours Extracted.	Grams Extracted.	% of Residue Extracted.	Analyses[1] (Av. of 2 determinations) % H	Av.	% C	Av.	Conc. = .5 mg/ 1.27°C. survival time of 2 fish (min.)[2]
			9.19		69.98		
24	28.8	57.6	9.10	9.15	69.81	69.90	27
			8.55		68.08		
24	11.7	55.2	8.52	8.53	68.44	68.26	25
			8.39		67.43		
24	1.8	19.0	8.16	8.33	67.80	67.61	29
			8.15		67.02		
24	1.6	20.8	8.14	8.15	66.88	66.95	31
			8.33		67.05		
48	1.4	33.0	8.08	8.13	66.77	66.90	49
			8.14		66.76		
72	0.9	19.1	8.07	8.11	66.39	66.83	76
			8.27		66.70		
168	0.8	21.0	8.12	8.20	66.12	66.41	132

1. Average analysis of the original resin: % H 8.77; % C, 68.40.

2. Toxicity of original resin at conc. = 5 mg/ liter. 23 min. The 1.1 gm of resin which remained in the methyl alcohol did not affect the fish in six hours.

Although the appearance of the fractions changed

markedly from pale yellow to deep brown in color and from soft and sticky to hard and brittle in consistency from first to last respectively, nothing crystalline was obtained from any of them. It is interesting to note that 81% of the resin was extracted in the first 48 hours and that the toxic material concentrated in this portion. The percent of carbon and hydrogen in the fractions varied from 69.90 to 66.41 and 9.15 to 8.20 respectively; almost 1% in the case of hydrogen and about 3.5% in that of carbon. While this experiment was a failure in that no crystalline substances were obtained, it was of value in that it showed clearly that the resin is an intimate mixture of substances whose solubility ratios in petroleum ether and methyl alcohol are very similar. It also showed that the toxic fraction of the resin is the more readily extractable from its 90% methyl alcoholic solution with petroleum ether.

VII. TOXICITY STUDIES USING THE GOLDFISH

Geredorff[30] has made extensive use of the goldfish in the study of the toxicity of rotenone and its derivatives. When these tests are carried out in a uniform manner they serve as a very convenient means of comparison of the relative toxicity of various substances. The necessity for absolute uniformity of procedure cannot be too strongly emphasized if consistent results are to be secured. The factors influencing survival time, the method of determination of the death point, together with a discussion of the precision to be expected of the method is excellently given by Geredorff[30(a)]. It should be borne in mind that there is probably no direct correlation between the toxicity, of a given substance, to goldfish and to insects. It is, however, usually assumed that those substances which are toxic to insects will also be toxic to goldfish, although the reverse is less generally true. In any case the decision regarding the insecticidal value of a material must rest finally on tests made on insects.

The procedure followed in making all of the toxicity tests described herein was exactly similar to that used by Geredorff. Goldfish of the species carassius auratus were used, the fish were fairly uniform in size, the average weight of each was about two grams. The fish were removed from the aquaria on the afternoon of the day preceeding the tests

and were placed in either 3-liter wide-mouth candy jars or in 13-liter battery jars filled with water taken from the aquaria to avoid sudden temperature changes. One liter of water was allowed for each fish. The jars were placed in the constant temperature bath, in which the temperature was maintained at 27° ± .3° and allowed to come gradually to that temperature over night.

Gersdorff has shown that acetone at a concentration of 1:1000 has no apparent effect on goldfish. Consequently the samples of resin were added to the test jars in acetone solution in such quantity that its concentration at no time exceeded this value. By vigorous stirring at the time of the addition of the sample good dispersion of the precipitated toxic substances could be obtained. The death point was determined by noting as near as possible when all gill movements had ceased.

The number of fish used for the determination of the average survival time varied. At high concentrations, where the differences in survival time due to variations in individual resistance of the fish were small, four fish were usually used for each point. On that portion of the curve, where survival time was increasing rapidly with small decreases in concentration, a larger number of fish were used in order to minimize the effect of differences in individual resistance. At the lower concentrations fewer fish were again required since a comparatively large difference in survival time would

correspond to a small percent deviation from the mean.

1. Croton Oil

Crude croton oil was obtained in the following manner: 51 gms. of the ground beans (I.D. 330) was extracted in a Soxhlet with petroleum ether (max. B.P. 65°) for about 9 hours. The petroleum ether was then evaporated from the extract and the oil was dried at 105° to constant weight. Yield 12.9 gms. (25.3%). A further extraction with the same solvent for about 6 hours removed only .07 gm. of oil. The saponification number for croton oil is 200 - 215 according to the U. S. Pharmacopoeia IX, p. 305. The value for the saponification number of the croton oil isolated as just described was however 205.8.

Saponification number:

	Sample (gms.)	c.c. 1.049 N alc. NaOH soln.	c.c. 0.223 N HCl soln.	Sap.No.
1.	1.066	25.00	22.03	205.8

The toxicity to goldfish of this crude croton oil was determined in a manner similar to that just described. The results of this study are shown in Table VI. These results are shown graphically in Fig. 2, where average survival time is plotted as ordinates and concentration as abscissas.

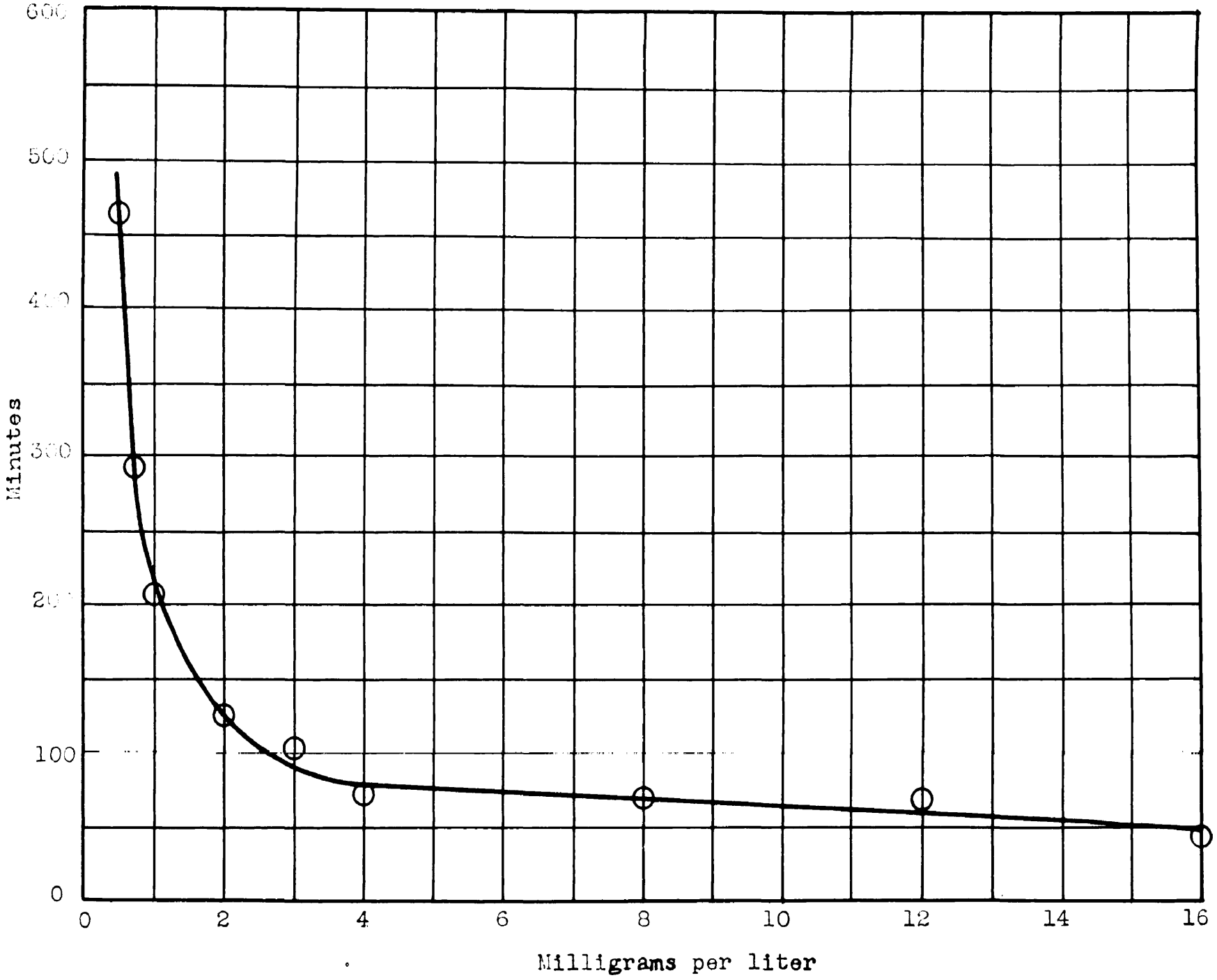


Fig. 2. - Curve showing toxicity of Croton Oil to Goldfish.

TABLE VI.

Toxicity of Croton Oil to Goldfish at 27° ± .3°

Conc. Mg/liter	No. fish used / test	Average survival time in minutes
16	4	43
12	8	66
8	12	71
4	8	71
3	8	102
2	8	126
1	12	209
0.75	4	296
0.50	4	432
0.25	4	2 fish 630 2 > 720 < 1440
0.10	4	2 > 720 < 1440 2 > 1440

In the determination of the average survival times no values were discarded and it was assumed that the number of fish removed before death had occurred would balance the number removed after death.

2. Alcohol Soluble Portion of Croton Oil

3374 gms. of the ground croton beans (I.D.830) were extracted by shaking with petroleum ether (60-70°) in the cold. This process was repeated three times and the petroleum ether was then removed from the combined extracts by evaporation on the steam bath. The final traces of solvent was removed by heating in a large claisen flask on the steam bath under reduced pressure while a slow current of air was drawn through the oil. Yield: 722 gms. (21.4%).

100 gms. of this oil was shaken in the cold with 150 c.c. of alcohol (95%), the alcoholic layer was withdrawn and the oil was then extracted with a further 100 c.c. portion of alcohol. The combined alcoholic extracts were filtered, centrifuged to remove suspended particles of oil, and finally the alcohol was evaporated off. Yield: 31 gms. (31%).

The toxicity to goldfish of this alcohol soluble portion of croton oil was carried out in the usual manner. The results of this study are shown in Table VII. Fig. 3 shows a comparison of the survival time - concentration curves of this material and of rotenone, the values for the latter were determined by Gersdorff[30(a)].

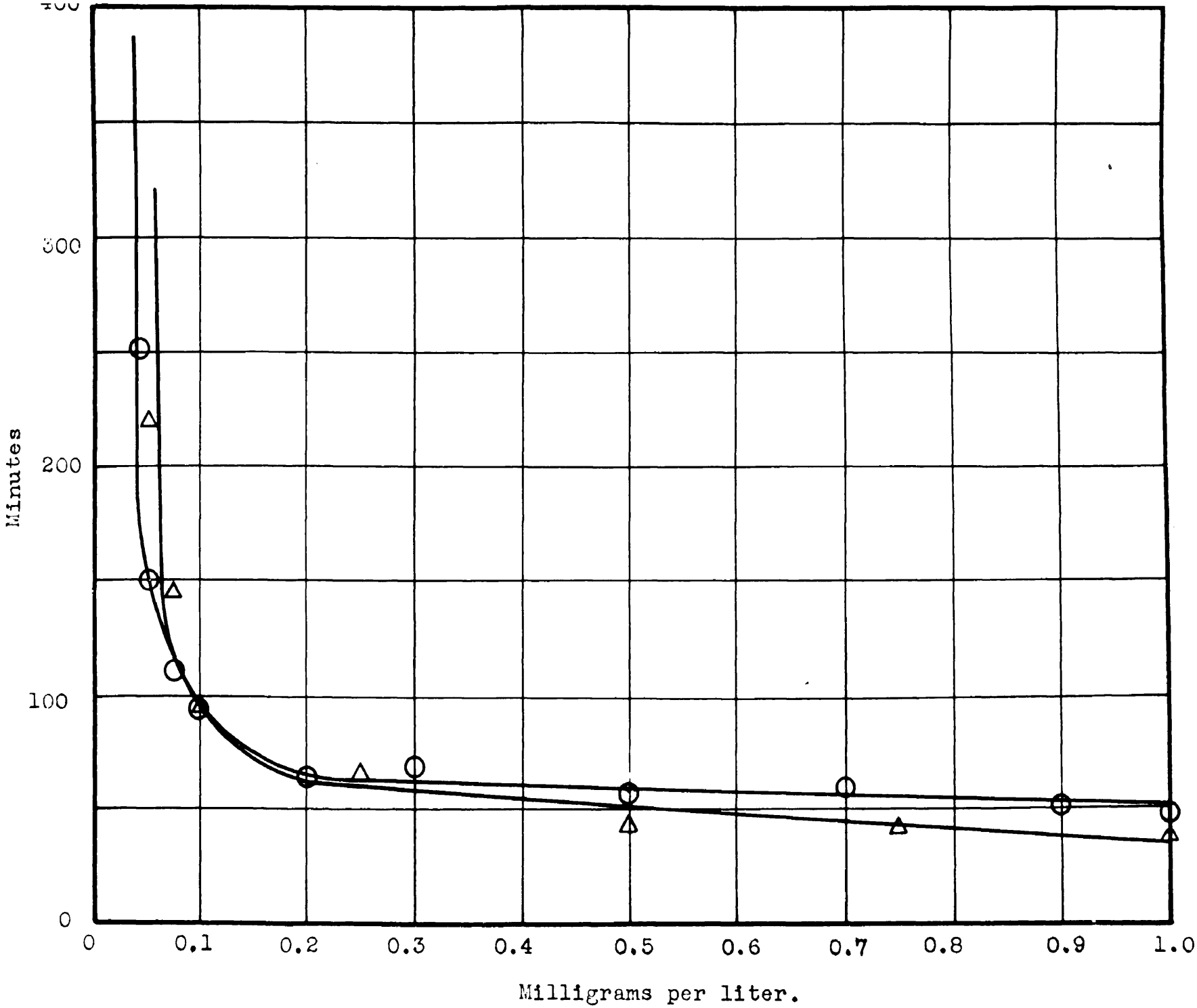


Fig. 3 - Curves showing toxicity of alcohol soluble fraction of Croton Oil and Rotenone. △ Alcohol soluble fraction of Croton Oil. ○ Rotenone.

TABLE VII.

Toxicity of Alcohol Soluble Portion of Croton Oil to Goldfish at 27° ±.3°

Concentration Mg/liter	No. fish used / test	Average survival time in minutes
12	6	24
8	5	20
4	6	27
3	4	26
2	4	32
1	4	33
0.75	9	42
0.50	8	43
0.25	7	66
0.10	8	97
0.075	5	146
0.050	6	220
0.025	6	4 dead < 700 2 dead < 1440
0.010	4	> 1440

It is interesting to note in Fig. 3 that the two curves are of the same general shape and are almost superimposable.

3. Croton Resin

The toxicity tests described here were made prior to the extraction of the large supply of resin (670 gms.) previously described in Sec. III, consequently a different sample was used for them. This sample was obtained, in a preliminary experiment, from the petroleum ether extracted mare (I.D. 830) and from some of the croton oil obtained therefrom by following essentially the procedure of Cherbuliez et al.

1000 gms. of this mare was shaken with 1000 c.c. of 90% aqueous methyl alcohol, it was allowed to stand 22 hours with this solvent and filtered warm. A fresh portion of methyl alcohol was then added and the extraction repeated. The methyl alcohol was removed from the combined extracts by distillation under diminished pressure. 200 gms. of croton oil was then added to the water-oil residue and the two layers were separated by centrifuging. The oil was extracted with eight 100 c.c. portions of methyl alcohol. These extracts were combined and the methyl alcohol removed as before. The residue was thoroughly extracted with petroleum ether to free the product from glycerides, free acids and other impurities. The aqueous suspension, containing the resin together with certain impurities, was then extracted thoroughly with ether. The ether solution,

was washed with three portions of 0.3 N KOH, then with water to remove all traces of alkali and finally was dried over anhydrous K_2SO_4. When the ether was evaporated 1.3 gm. of crude resin was obtained. For further purification the crude product was dissolved in 80% aqueous methyl alcohol, which was then extracted three times with petroleum ether. The combined petroleum ether extracts were then extracted with 90% aqueous methyl alcohol and this extract was combined with the 80% methyl alcoholic resin solution. This solution was filtered, the methyl alcohol evaporated and the aqueous suspension, which resulted, was extracted with five portions of ether. The ether solution was dried as just described, the last traces of solvent was finally removed in a vacuum. Yield: 0.61 gms.

The toxicity to goldfish of all of the by-products in the steps required to obtain the 1.3 gms. of crude resin was determined. By this means the efficiency of the extraction processes was found to be very good.

In appearance this sample of resin was similar to that described by Cherbulier et al. Its analysis(mixed however with another sample obtained in the same manner) checked that given by Cherbuliez et al, and also that of the large sample of resin whose isolation has already been described, thus demonstrating the similarity in composition of the two samples.

Analysis:

	Sample(mg)	H_2O (mg)	CO_2 (mg)	% H	% C
1.	3.546	2.735	8.914	8.70	68.56
2.	3.692	2.930	9.271	8.88	68.47
	Oberbulies et al:			8.95	69.2
				8.86	69.8

The toxicity to goldfish of this purified resin was determined in the manner previously described. The results of this study are shown in Table VIII.

TABLE VIII.

Toxicity of Croton Resin to Goldfish at 27° ±.3°.

Conc. mg. per liter	No.fish used per test	100 / Survival time	Av.survival time in min.
1	4		22
0.50	4		22
0.10	4	4.17	24
0.050	4	4.35	23
0.025	4	3.70	27
0.010	6	2.08	48
0.0090	7	1.89	53
0.0080	8	1.06	94
0.0075	8	0.51	197
0.0070	8	0.42	237
0.0060	8	0.60	167
0.0050	6	1 dead	1440.5 dead 1440
0.0025	10	10	1440
0.0010	6	6	1440

These data are shown graphically in Fig. 4 where survival time is plotted as ordinates and concentration as abscissas. A similar curve showing the toxicity of rotenone is also included. The data for this curve were taken from the work of Gersdorff.[30(a)] In the same figure is plotted also the velocity of fatality curve in which the reciprocals of the survival time (multiplied by 100 to avoid decimals) is plotted as ordinates against concentration as abscissas.

Powers[32] has found that the survival time curve is logarithmic in function. However, the middle portion of the curve (where velocity of fatality increases most rapidly with increase in concentration) approaches an equilateral hyperbola. Consequently the corresponding portion of the velocity of fatality curve, which is drawn with the reciprocals as ordinates, approaches a straight line. This straight line, called the theoretical velocity of fatality curve, when prolonged cuts the x-axis at a point designated by Powers as the theoretical threshold of toxicity "a".

On the basis of these considerations Powers suggested the following formula for use in determining comparative toxicities, T (toxicity) $= \sqrt{\frac{\tan \theta}{a}}$ where "a" is the value for the threshold of toxicity and θ is the angle formed by the theoretical velocity of fatality curve and the x-axis.

Using this formula Gersdorff obtained a value of T = 4 for rotenone at 27°. He calculated values of T = 0.008 and 0.16 for phenol and potassium cyanide respectively from

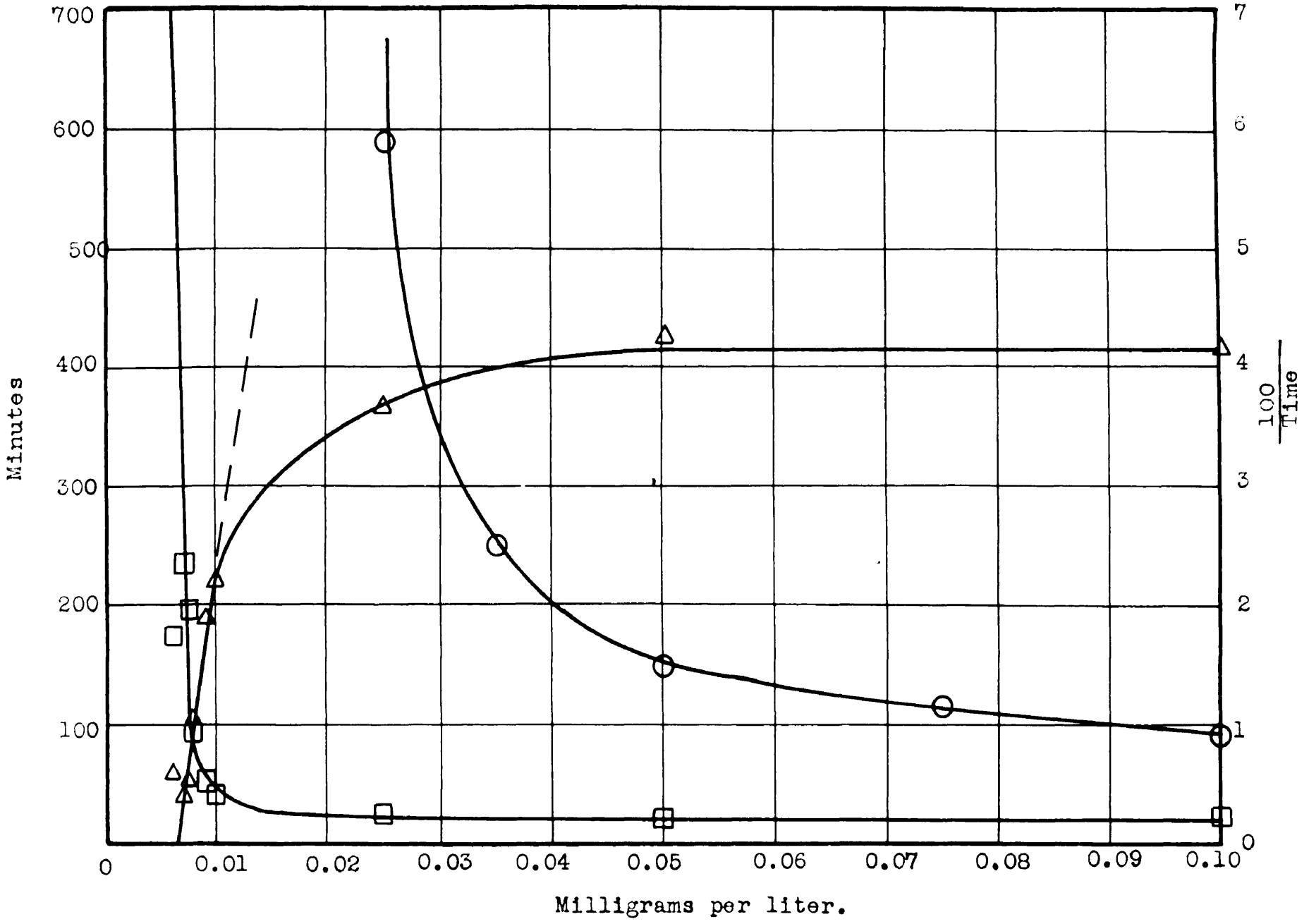


Fig. 4 - Curves showing toxicity of Croton Resin and Rotenone.
□ Croton Resin. ○ Rotenone. △ Velocity of fatality.

data given by Powers, and therefore concluded that rotenone was more toxic to goldfish than potassium cyanide and the latter was 200 times more toxic than phenol. These values are not entirely comparable since a temperature of 27° was used by Gersdorff and 21.5° by Powers.

From the velocity of fatality curve for croton resin the following values are obtained:

$$a = .0004$$

$$\theta = 81^{\circ}$$

$$\tan \theta = 6.31$$

$$Z = \sqrt{\frac{6.31}{.0004}} = \sqrt{980.0} = 31.3$$

Thus according to this method of comparison croton resin is 7.8 times more toxic than rotenone.

Gersdorff[30(d)] has pointed out that this method of comparison of the toxicities of various substances is not all that could be desired since the effect of the threshold value "a" was overemphasized while the third factor, the toxicity at high concentration as expressed by the horisontal asymtote, was not considered at all. Thus by the use of this formula he found the value of the threshold of toxicity "a" for rotenone hydrochloride to be 0.002 mg./l. and that this substance appears to be therefore, about ten times more toxic to goldfish than rotenone.

Table IX shows a comparison of the toxicities of

rotenone, rotenone hydrochloride and croton resin at corresponding concentrations. By inspection of the data it would seem obvious that the imperfections of the method, as pointed out by Gersdorff, are even more exaggerated and striking in the comparison of the toxicities of croton resin, rotenone, and rotenone hydrochloride for which the values of T are 31.3, 4 and 40(about) respectively.

TABLE IX.

Comparison of the Toxicity of Croton Resin, Rotenone, and Rotenone Hydrochloride to Goldfish at 27°.

Concentration mg./liter	Average Survival Time in Minutes		
	Croton Resin	Rotenone	Rotenone Hydrochloride
0.50	22	57	120
0.10	24	93	130
0.050	23	150	130
0.025	27	800	212
0.015	-	2400	300
0.010	40	-	-

4. Certain Derivatives of Croton Resin

The toxicity to goldfish of the derivatives of croton resin, whose preparation has been described in Sec. V, has been determined. The usual procedure was followed and for the sake of comparison all of the tests were made at a

concentration of two milligrams per liter. This is a rather high concentration but if a lower one had been used the survival time for some of these derivatives would have been inconveniently high. The average survival times were obtained by the use of four fish per test.

The results of these tests are tabulated in Table X.

TABLE X.

The Toxicity of Certain Derivatives of Croton Resin to Goldfish at 27° ± 0.3°

Substance	Average % CH_3O	Average Sap. No.	Average Iodine No.	Av. Survival time in min. (4 fish)
Original Resin	1.2	244	55.1	20
Hydrogenated "	-	-	39.2	18
Acetylated "(1)	-	350	-	35
Acetylated "(2)	-	336	-	30
Dimethyl-acetylated "	-	183	-	> 1740
Dimethyl "	11.7	181	-	> 1740
Brom "(3)	-	-	-	62
Brom "(4)	-	-	-	71

1 Sec. V, 2b, Prep. 1
2 " " 2b " 2
3 " " 1b " 1
4 " " 1b " 2

Hydrogenation seems to have had no effect on the survival time of the goldfish while acetylation and bromination decreased the toxicity to a considerable extent. The magnitude of this decrease is not so apparent from the figures in the table because of the comparatively high concentrations employed.

The dimethyl resin had little effect on the fish in 1740 minutes. Although all of the fish were alive and in no apparent distress, at the end of this period a slight irritation was noticed on the gills of some of them.

This table shows in striking fashion the important relation of the free hydroxyl groups of croton resin to its toxicity to goldfish.

VIII. VESICANT TESTS

From an insecticidal viewpoint croton resin has a serious disadvantage in its rubefacient properties. This property of the oil has been long recognised and use of it has been made in certain medical applications. No quantitative measure of the vesicant action of croton resin was recorded by either Boehm or Cherbuliez et al., the latter authors noted however that bromination rendered the resin completely inolpid. As has been previously mentioned Paal and Roth[30] showed that hydrogenation of croton oil caused the iodine number to fall to zero and the vesicant properties simultaneously to disappear. Hydrogenation of Boehm's resin caused the iodine number to fall from 77 to 12.5 and the product thereupon lost its toxicity to frogs and rabbits.

The vesicant tests herein described were all carried out in the following uniform manner: 3 drops from a pipette (calibrated 75 drops / c.c.) containing the test solution were placed on the skin and allowed to spread over an area of from one half to one square inch. Each drop was allowed to dry before the following one was applied. Alcohol or acetone was used as the solvent for the application of the resin. The treated area was left exposed to the air without washing for from 12 to 18 hours when observations were made to determine the results of the test. In cases of positive

reaction a redness and swollen feeling resulted in from 8 to 10 hours probably reaching a maximum within 24 hours. The appearance of the burns was red and slightly swollen with numerous small blisters or pustules. The redness and pustulation caused by slight burns usually disappeared within a few days but sometimes persisted several weeks in more severe cases, as was observed in some minor accidents incurred during the course of the extraction of the resin.

The results of the vesicant tests are shown in Table XI.

TABLE XI.

Vesicant Tests on Croton Resin and Certain of Its Derivatives

Substance	Conc. of test soln.	No. mg. per sq. in.[1]	Solvent used	No. of individuals	Result
Original Resin	1:1000	0.04	alcohol	1	moderate burn
Original Resin	1:100	0.4	"	1	pronounced burn
Hydrogenated" [2]	1:100	0.4	"	1	" "
Dimethyl "	1:100	0.4	acetone	2	no burn
Acetylated "[3]	1:100	0.4	"	2	pronounced burn
Brom Resin [4]	1-100	0.4	"	2	very slight burn

1. approximate area
2. Iodine number = 39.2 (Gattermann & Wieland[23])
3. Sec. V 2b Prep. 1
4. " " 1b " 1

The preparation of these derivatives has been described in Section V.

The dimethyl resin is the only derivative which had no vesicant action, although the bromination product possessed very little. A previous test had indicated that bromination completely destroyed the vesicant action but a repetition of the tests on two individuals both gave slight positive results. Plate 3 is a photograph of the burn produced by the original resin. The burn nearer the wrist was produced by 0.4 mg. while the one nearer the elbow was produced by 0.04 mg. of resin.

These data support the conclusions drawn from the study of the toxicity to goldfish of these derivatives of the resin; that is, the action of croton resin, both vesicant and toxic, is more intimately connected with the presence of its free hydroxyl groups than with the unsaturated functions. Here again the positive result obtained with the acetylated resin can be ascribed to lack of complete masking of these hydroxyls by this reaction.

Plate 3. Vesicant Action of Croton Resin.

IX. INSECT TESTS

1. Preliminary Tests on Acetone Extracts

Aside from the work of Dr. P. L. Campbell and Mr. W. N. Sullivan of the Division of Insect Toxicology and Physiology, Takoma Park, Maryland, on mosquito larvae, no toxicity studies have been made on insects using pure croton resin. Messrs. Davidson, Billings and Reynolds of the Food and Drug Administration at Silver Spring, Maryland, however have made quite extensive insecticidal tests using the acetone extracts of certain of the more promising plant materials (croton tiglium is included) as determined by Drake and Spies[3,4] in their study of the toxicity to goldfish of these extracts. Space forbids the presentation of the details of the tests made by Davidson et al. in this thesis but a condensed summary of their results has been prepared from Mr. Davidson's report. These data are tabulated in Table XII.

TABLE XII

CONDENSED SUMMARY OF INSECT TESTS[1]

(Acetone Extracts of Plants Used for these Tests)

Name of plant	I.D. No.	Av. Survival time of goldfish in min.[2]	Kind of Insect Used	Conc. of Extracts in water % [3]	Emulsifying Agent	Percent of Mortality	Remarks
Apuriznacia michelii	381	106	Aphis rumicis	5	none	-	Aphids driven from plant
Apuriznacia michelii	381	106	Grasshoppers	10	none	20	Some repellent action
Bejuco chilio[6]	843	90	Aphis rumicis	5	none	-	Plant injured
Covillia	382	46	Aphis rumicis	5	none	0.0	----
Cracca virginiana (56)	829	144	Aphis rumicis	1.25	1 - 800 Penetrol	100	----
Cracca virginiana (56)	829	144	Aphis rumicis	0.5	1 - 800 Penetrol	98.4	----
Cracca virginiana (55)	829	144	" gossypii	5	none	85.5 75.2	----
" vogelii	820	106	" gossypii	5	none	95	----
" "	820	106	" "	2	none	100	----
Croton tiglium	830	22	" "	5	none	100	----
" "	830	22	" "	1	none	99	----
" "	830	22	Myzus persicae	5	none	100	----

TABLE XII (CONT'D.)

Name of Plant	I.D. No.	Av. Survival time of goldfish in min.²	Kind of Insect Used	Conc. of Extracts in water % ³	Emulsifying Agent	Percent of Mortality	Remarks
Croton tiglium	830	22	White fly	5	none	90 94	----
" "	830	22	House fly	10	none	practically 0.0	----
" "	830	22	Red Spiders	5	0.25% Penetrol	92	----
" "	830	22	Mealy bugs	5	0.25% Penetrol	3.8	----
Derris root, 1.4% rotenone	402	--	Aphis gossypii	5	- - -	97 92	----
Derris root, 1.4% rotenone	402	--	Lygus pratensis	5	- - -	100	----
" " "	402	--	White fly	5	- - -	91	----
Diospyros maritima	825	67	Aphis rumicis	5	- - -	--	Plant injured Aphids driven off
Evonocarpus setigerus	789	33	" gossypii	5	- - -	18 49	----
Fohla tingui	366	72	" rumicis	5	- - -	0.0	----
Ichthyomethia piscipula	388	94	" "	5	- - -	2	----

TABLE XII (CONT'D.)

Name of Plant	I.D. No.	Av. Survival time of goldfish in min.[2]	Kind of Insect Used	Conc. of Extracts in water % [3]	Emulsifying Agent	Percent of Mortality	Remarks
Lonchocarpus velutinus*	785	148	Aphis gossypii	5	none	65	----
Manganille ecrum	390	42	" rumicis	5	none	0.0	----
Pogostemon cablin[4]	791	127	" "	5	- - -	0.0	----
Sinibuite *	844	57	" "	5	- - -	80	----
Spatholobus roxburghii[4]	873	105	" "	5	- - -	100	----
Symplocos tinctoria	716	70	" "	5	- - -	-	Plant injured
Urechites suberecta	519	48	" "	5	- - -	0.0	----

1. For full details see Mr. Davidson's (of Silver Spring) report which is attached to Spies' monthly report for March 1932.
2. Acetone extracts used as described in J. Econ. Ent. 25:(1)129-33(1932), Ibid 26 235 (1933)
3. Acetone extracts prepared the same as for the goldfish toxicity tests.
4. Contains about 1% rotenone. (J.A.C.S. 55 1737 (1933).

* Solutions not freshly prepared. Age probably as much as one year in some cases.

The acetone extracts used for these tests were freshly prepared in all cases where availability of material made this possible. They were prepared exactly as described by Drake and Spies[3] for their tests on goldfish; that is, at a concentration of 0.2 gm. plant material per c.c. of acetone.

The table is self-explanatory but should further detail be desired it can be obtained from Mr. Davidson's report, a copy of which is attached to Spies' monthly report for March 1932, on file in the offices of the Insecticide Division of the Bureau of Chemistry and Soils, Washington, D. C.

These results can only be considered as preliminary in nature but they are especially interesting in that a comparison with other plant extracts, some of which contain rotenone, is made. Not only is a sample of derris (1.4% rotenone) included but also two other plant materials, Spatholobus roxburghii[33] and Cracca virginiana[34], in both of which rotenone has since been discovered.

Using a 5% aqueous dilution of the acetone extract of croton tiglium, with no emulsifying agent, as spray solution against Aphis gossypii, Myzus persicae, and White fly, 100%, 100% and 90-94% mortality respectively was recorded. A 5% aqueous dilution with 0.25% penetrol emulsifying agent gave 93% mortality against red spiders, though this same

solution however had little effect on mealy bugs. On the basis of 1% croton resin in the seeds from which this extract was prepared, a 5% dilution would represent a concentration of 1:10000 of the pure resin in the spray mixture. This does not, however, preclude the possibility of other ingredients from also exerting an insecticidal action along with that of the resin. An important feature in the use of the acetone extract from croton tiglium is the lack of any reported plant injury due to its application.

2. Mosquito Larvae

Dr. F. L. Campbell et al. have studied the effect of croton resin upon mosquito larvae in the following manner: 50 mosquito larvae were added to 100 c.c. of water containing the resin suspension which had been previously dispersed in the water by the addition of its acetone solution. The appearance of the resulting solution was noted and observations were made at intervals to record the number of larvae killed. The results of this study are given in Tables XIII and XIV.

TABLE XIII.

Toxicity of Croton Resin to Mosquito Larvae

at 29.5° ± 0.1

Concentration	Appearance of Solution	No. down in hours indicated* 20 hrs.	28 hrs.	45 hrs.
1:100,000	light cloud	28	36	40
1:200,000	faint cloud	11	30	40
1:400,000	clear solution	10	20	42

* Acetone check in duplicate: none down in 45 hours.

TABLE XIV.

Toxicity of Croton Resin to Mosquito Larvae

at 29.5° ± 0.1

Concentration	Appearance of Solution	No. down in hours indicated* 9 hrs.	20 hrs.	45 hrs.
1:10,000	cloudy, clumps of resin on bottom	37	44	50
1:20,000	cloudy, small particles of resin on bottom	23	35	50
1:40,000	cloudiest of series, no particles visible	13	30	50
1:80,000	Light cloud	11	20	42
1:160,000	Faint cloud	6	11	34
1:320,000	Clear solution	7	14	42
1:640,000	Clear solution	3	26	30

* Acetone check: none down in 45 hours.

The results of these tests can best be discussed by quoting from Dr. Campbell's report: "Croton resin affects the larvae in the same way as the acetone extract of the plant. The action seems to be even slower than that of rotenone. The larvae remain suspended from the surface for long periods of time and are not easily disturbed. There is a curious tendency for the larvae to apparently shrink in length. This appearance may be due partly to the bending of the head and thorax. But these shrunken larvae live for many hours and are quite active when prodded. A considerable number of larvae when driven below the surface are fairly active but do not seem to be able quite to reach the surface of the liquid again".

"At 1:10,000, 1:20,000, 1:40,000 there is obviously a mechanical effect of the sticky resin. Groups of 3 or 4 larvae become attached to each other and struggle violently below the surface to get away from the sticky substance. It might be assumed that this mechanical action plays a part even at lower concentrations where such action is not apparent".

"In conclusion, it appears that croton resin is very effective against mosquito larvae over a long period of exposure".

The author wishes to express at this time, to Dr. Campbell and Messrs. Davidson, Sullivan, Billings and Reynolds, his appreciation for their efforts in carrying out the

entomological tests just described.

In conclusion it appears that croton resin holds considerable promise as an insecticidal material. Preliminary experiments have shown it to possess appreciable toxicity to a variety of insects, and it seems to have no injurious effect upon plant foliage. The author feels that the serious disadvantage to its use as an insecticidal material (its powerful vesicant action) can probably be obviated by its application as a dust. This dust could be prepared by its deposition upon some inert clay material such as kaolin or other suitable substance. For its commercial application it would not be necessary to carry out the involved processes required to obtain the "pure" resin, but rather the alcoholic extract of the bean could be used as it contains all of the resin and only a small fraction of the inert oil. Should the resin fail to find any practical applications as an insecticidal agent its exceedingly powerful toxic action still offers an inviting field for a theoretical study of the little known relationship of toxicity and chemical constitution.

X. THE SAPONIFICATION PRODUCTS OF CROTON RESIN

Dunstan and Boole observed that boiling their resin with potassium hydroxide solution decomposed it, destroying its vesicant properties and yielding several acids, some of which were volatile. They, however, claimed that their evidence pointed to a lactone or anhydride structure.

Boehm stated that by allowing his resin to stand 12 hours with 30% aqueous potassium hydroxide a dark brown, completely water soluble product was obtained. When this solution was acidified a brown resinous mass separated, whereby an intense odor of fatty acids was observed with the odor of isobutyric acid predominating. A yield of 36% of this acid mixture was recorded by him. He also observed besides the fatty acids an amorphous solid, resinous material which he called resin alcohol. This substance was non-toxic and insoluble in petroleum ether though partially soluble in hot water. It was alkali soluble and an amorphous benzoyl derivative was obtained from it. The aqueous solution gave a red-violet color with ferric chloride solution. Formic and acetic acids were identified qualitatively from the saponification products by Boehm. He also isolated isobutyric and tiglic acids and suggested the probable presence of other higher fatty acids. From his observations Boehm concluded that croton resin is not homogeneous but is probably a

mixture of different esters of the so-called resin alcohol and fatty acids. These esters are not separable because of their similar solubility properties.

Cherbuliez et al. did not study the saponification products of their resin although they probably were aware of its nature.

In this research the saponification products of croton resin have been studied and all of the petroleum ether soluble fatty acids have been identified. Upon treatment of the resin with alkaline hydrolysing agents the characteristic dark brown coloration of its solution was observed. This coloration seemed to be just as intense whether the saponification was carried out in an inert atmosphere or in the presence of the oxygen of the air.

Following is a description of a typical saponification experiment together with the yield of products obtained therefrom: 30.4 gms. of croton resin was refluxed for one and one-half hours on the steam bath with 135 c.c. of 1.6 N alcoholic KOH in an atmosphere of nitrogen gas. The alcohol was then removed under diminished pressure in a current of nitrogen. The dark colored residue was dissolved in 300 c.c. of distilled water (all dissolved) and the alkaline solution was extracted with two 80 c.c. portions of ether (I). This ether solution was dried over anhydrous Na_2SO_4. When dry it was filtered and the ether evaporated. Yield 0.3 gi.

The aqueous alkaline solution was then made acid to congo with dilute H_2SO_4 whereupon a layer of dark fatty-acid-smelling material separated. 100 c.c. of petroleum ether (30-60°) was added and the mixture thoroughly extracted in a separatory funnel. A large lump of dark brown material remained insoluble in either the petroleum ether or aqueous layers. This substance was separated mechanically and washed several times with petroleum ether and finally with water. It was then dissolved in methyl alcohol, the solution dried over anhydrous Na_2SO_4, filtered, and the alcohol was evaporated to constant weight on the steam bath in a current of nitrogen. The product when cold was a hard brittle dark-brown amorphous mass (II). Yield 11.6 gms.

The acidified aqueous solution was thoroughly extracted with petroleum ether and all of the petroleum ether solutions so obtained were combined with the washings of the resin alcohol and the main petroleum ether solution of fatty acids. These combined solutions were washed once with water, filtered and dried over anhydrous Na_2SO_4. The solution was then filtered and the solvent evaporated on a steam bath under reduced pressure in a current of nitrogen until a drop of fatty-acid-smelling material distilled over. The product was a brown liquid (III). Yield: 9.6 gms.

The clear, golden yellow, acidified, aqueous solution gave a beautiful purple or deep red-violet color with $FeCl_3$

solution. This color was very persistant. The solution was made slightly alkaline to litmus and the water removed by distillation under reduced pressure in a current of nitrogen. As the solution became more concentrated a little dark gummy matter separated on the sides of the flask. This was removed twice during the course of the evaporation. The golden yellow residue consisted of the water soluble saponification products together with the inorganic salts produced by the neutralization of the KOH. To remove these salts the residue was refluxed with absolute alcohol, cooled and filtered. The insoluble inorganic residue was washed with two 25 c.c. portions of absolute alcohol. Finally the alcohol was removed from the combined solutions by distillation on the steam bath under reduced pressure in a current of nitrogen. The light-brown amorphous residue (IV) so obtained was readily soluble in the alcohol and water but insoluble in ether. It was not possible to extract this material from its acidified aqueous solution by means of ether. The procedure just described was resorted to because it was found that evaporation of the solution even when neutral or just acid to litmus caused considerable darkening and the resulting product would no longer give the color test with ferric chloride. This darkening and evident decomposition occurred when HCl was used to acidify the solution and was therefore not due to the charring action of the H_2SO_4.

The results of this saponification are shown in Table XV. The numbers assigned to each fraction will be used as a means of designation in future reference to the corresponding product.

TABLE XV.

Data Concerning the Saponification Products of Croton Resin

Substance	No.	Yield Gms.	% of Starting Product
Ether extract of alkaline solution	I	0.6	1.6
Pet. ether insoluble material	II	11.6	33
Pet. ether soluble acids	III	9.0	32
Water soluble material	IV	8.7*	29

* Yield by difference

Product #I gave a negative Liebermann Buchard[35] color test for sterols. The residue (IV) left by the evaporation of the water gave a negative test for glycerol[36] as shown by its failure to yield acrolein when heated with $KHSO_4$.

2. Lead-Salt-Ether Separation of the Liquid (unsaturated) and Solid (saturated) Fatty Acids

There are several methods available for the separation of the liquid (unsaturated) from the solid(saturated) fatty acids in a mixture containing both. All of these methods are however subject to certain limitations which, especially in cases where the lower members of the saturated series are present, decrease the quantitative value of the results obtained by their use. The lead-salt-ether method is one of the oldest employed for this purpose. It is based on the fact that the lead salts of the saturated fatty acids are insoluble in ether while those of the liquid fatty acids are soluble. However, the solubility of the lead salts of the saturated series increases as the hydrocarbon residue decreases, and also the presence of the unsaturated lead salt in solution tends to increase the solubility of even the higher members of the saturated series. It should also be borne in mind that only the liquid unsaturated fatty acids yield ether soluble lead salts.

The procedure described by Jamieson[37] was used to obtain the results shown in Table XVI. Since this procedure is lengthy and is described in great detail by Jamieson it does not warrant repetition here.

TABLE XVI

Lead-Salt-Ether Separation of Fatty Acids (III) from Croton Resin

Gms. of Mixed Fatty Acids III	Milli equivalents of KOH to neutralize	Gms. saturated acids	Gms. unsaturated acids
3.3	17.1	0.94	1.53
3.4	17.5	0.98	1.74

Work, which will be described subsequently, has shown the presence of a large amount of the lower saturated fatty acids in this mixture, consequently these results given have little quantitative significance. It is interesting to point out however, that about 46% unsaturated fatty acids (by volume) was shown by distillation of the methyl esters (to be reported later) while an average of 49% was obtained by the method just described.

The lead salts obtained from the mixture (III) were completely soluble in ether at room temperature (30-33° summer). This is significant in that it shows the absence of any appreciable quantity of the higher saturated fatty acids. This conclusion was substantiated by the data obtained by the distillation of the methyl esters of this fatty acid mixture to be described later.

3. Identification of Oleic and Linolic Acids

(a) Oxidation of the Fatty Acid Mixture (XII).

Oxidation with cold alkaline potassium permanganate by the method of Lapworth and Mottram[38] was used to show the presence of oleic and linolic acids. Following is a description of the method used: 5.1 gms. of the fatty acid mixture (III) was dissolved in 500 c.c. of water containing 5 gms. of NaOH. The solution so obtained was then poured into a battery jar containing 4 liters of ice water. The solution was kept cold in an ice bath and stirred vigorously with a mechanical stirrer while 400 c.c. of a cold 1% $KMnO_4$ solution was added. The stirring was continued for 5 minutes after the addition of the $KMnO_4$ solution. The liquid was then decolorized and the oxides of manganese were dissolved with SO_2, after which 150 c.c. of concentrated HCl was added. While still cold (to keep the low melting saturated acids solid) the white flocculent precipitate was filtered with suction through a large Buchner funnel, 2.41 gms. of crude solid was obtained. This crude product was then extracted with about 100-150 c.c. of petroleum ether to remove the saturated acids and any unoxidized unsaturated acids. 0.73 gms. of white solid material remained insoluble while 1.7 gms. was recovered

upon evaporation of the petroleum ether.

A method based on the solubility of dihydroxy-stearic acid in ether and the insolubility of tetra hydroxystearic acid in the same solvent was used for the resolution of the 0.78 gms. of petroleum ether insoluble acids. The crude dried mixture of di and tetra hydroxy acids was shaken with 300 c.c. of ether and then filtered or decanted from the insoluble residue. Upon concentration of the ether solution so obtained some beautiful white crystals separated which when dried melted at 98° and remelted at 112°.

Because of the danger of lactone formation of this compound it was dried after each recrystallization in a vacuum dessicator at room temperature. For the same reason the melting points were taken with the bath already at a temperature near the melting point of the compound.

Two further recrystallizations from ether gave a product which shrunk together at 117° and melted sharply at 123.5°. The dihydroxy stearic acid was then recrystallized three more times from ethyl acetate with subsequent drying in the usual manner. The melting points after each recrystallization were as follows: (1) shrink 117-118°, melted sharply 123-124°; (2) shrink -, melted sharply 124-124.5°; (3) shrink 119-120°, melted sharply at 123.5-124 respectively.

Analysis:

	Sample (mg)	H_2O(mg)	CO_2(mg)	% H	% C
1.	3.194	3.391	8.023	11.98	68.50
2.	3.904	4.079	9.770	11.69	68.25
	Calc. for $C_{18}H_{36}O_4$			11.44	68.29

A great number of isomeric dihydroxystearic acids are possible and they have been reported with melting points ranging from as low as 69.5° to as high as 141-43°. An excellent discussion of these acids is given by Lewkowitsch[39] and judging solely from a melting point basis the acid just described might correspond to one of the following. Vongerichten and Kohler[40] reported a dihydroxystearic acid, which melted at 122°, obtained from petroselinic acid while LeSueur[41] reported another obtained by the permanganate oxidation of 2-3 oleic acid which crystallised from ethyl acetate in slender needles and melted at 126°. Drum[42] resolved the supposedly pure dihydroxystearic acid (M.P. 66-68°) obtained by the action of concentrated sulfuric acid on ricinoleic into several isomerides one of which also melted at 126°.

The ether insoluble residue was recrystallised from water and dried in a vacuum desiccator. It softened at 145° and melted clear at 155-6°. The same precautions regarding the drying and determination of the melting points were observed in the case of this acid as were employed with

dihydroxystearic acid just described. This recrystallized product was boiled up with 50 parts of benzene, the solution was cooled and filtered (see Lewkowitsch 6th Ed. 1 203). The insoluble residue was then recrystallised from alcohol, filtered and washed with ether on the filter. After drying it shrunk together at 155-6° and melted clear at 166-7°. This treatment was repeated and the product again shrunk at 155-6° and melted clear at 167-8°.

Rollett[43] reported having obtained 40.7% of a tetrahydroxystearic acid upon the oxidation of pure linolic acid which melted at 158°. After recrystallising this acid six times from alcohol the melting point was 160-169° but by boiling the substance with fifty parts of benzene (whereby a small amount of foreign material was removed) and afterwards recrystallizing from alcohol, sativic acid was obtained which melted at 171-173°. It is evident that this treatment has failed to effect a similar separation in this case.

The tetrahydroxy acid was recrystallised from water, filtered, washed with a little cold alcohol on the filter and then dried as usual. The product softened at 156-7° and melted clear at 167-8°. A further recrystallization from alcohol with subsequent drying failed to raise the melting point.

Analysis:

	Sample (mg)	H_2O (mg)	CO_2 (mg)	% H	% C
1.	3.375	3.213	7.677	10.65	62.03
2.	3.867	3.668	8.809	10.66	61.80
	Calc. for $C_{18}H_{36}O_6$			10.42	62.02

Softening at 156-7° and melting at 167-8° was very characteristic of this acid. Even extraction with benzene as recommended by Hollett followed by alternate recrystallization from water and alcohol failed to cause any change in this characteristic softening point or any elevation in the final melting point of the sativic acid.

(b) Bromination of the Liquid Fatty Acids

The absence of unsaturated acids containing three or more double bonds in the fatty acid mixture (III) was shown by application of the bromination test[44]. Following is a description of this test: 1 gm. of the fatty acid mixture (III) was dissolved in 40 c.c. of ether containing 2 c.c. of glacial acetic acid. To the solution, cooled to 0-5°, bromine was then added drop by drop until the red color persisted. The solution was kept in ice for three hours and at the end of that time no precipitate had formed. This indicated that no acids capable of yielding hexa or octabromides were present.

5.3 gms. of unsaturated fatty acids, obtained by

the lead-salt-ether separation, was dissolved in 80 c.c. of ether containing 4 c.c. of glacial acetic acid. The solution was cooled to 0-5° and bromine was added drop by drop until the permanent red color indicated the presence of an excess of this reagent. The solution was allowed to stand for three hours at this temperature (no precipitate formed) and was then washed with dilute $NaHSO_3$ solution to remove the excess bromine. It was then washed with saturated NaCl solution until neutral to litmus. When water was used for washing this solution stable emulsions tended to form which prevented the separation of the two layers. The ether solution of bromides was dried over anhydrous Na_2SO_4, filtered and the ether was finally evaporated. The residue was dissolved in 20 c.c. of petroleum ether and filtered to remove a small quantity of insoluble tarry material. The solution was then concentrated to about 10 c.c. and allowed to cool. The crystalline tetrabromide separated slowly due to the presence of the oleic dibromide and some saturated fatty acids. Finally the crude brown tetrabromide was filtered off. It was then dissolved in 50 c.c. of petroleum ether (50-70°), and the solution was refluxed with bone black, filtered while hot and then concentrated to about 10 c.c. Beautiful white crystals formed upon cooling which were filtered off and dried in a vacuum desiccator. Melting point 114-114.5°. Two further recrystallisations from the same solvent failed to

raise the melting point which was finally determined as 113.5-114.5°. The petroleum ether solutions were filtered through hardened papers before each crystallization in order to make certain the removal of all charcoal.

Analysis:

	Sample (mg)	AgBr(mg)	% Br_2
1.	6.070	7.611	53.36
	Calc. for $C_{18}H_{32}O_2Br_4$		55.29

	Sample (mg)	H_2O (mg)	CO_2(mg)	% H	% C
1.	2.932	1.462	3.681	5.59	36.11
2.	3.670	1.749	4.664	5.33	36.30
	Calc. for $C_{18}H_{32}O_2Br_4$			5.38	36.00

Thanks are due to Mr. R. P. Jacobsen of the University of Maryland for this micro bromine determination and to Mr. S. A. Shrader, also of the University, for the micro combustions.

Literature values for the melting point of the tetrabromide of linolic acid of 114-115°, 114°, and 113-114° are given by Hazura, Lewkowitsch[45] and Farnsteiner respectively.

From 3.64 gms. of the unsaturated fatty acids, as separated by the lead-salt-ether method, 4.66 gms. of crude brominated product was obtained from which 1.55 gms. of crude linolic tetrabromide separated upon crystallization from

petroleum ether. Matthes and Boltze[46] reported the formation of a so-called liquid tetrabromide which is soluble in petroleum ether. They succeeded in crystallising this substance by evaporation of the petroleum ether and solution of the residue in methyl alcohol from which they obtained the tetrabromide as silky scales melting at 54-55°. Subsequent recrystallisations failed to raise the melting point above 57-58°.

To ascertain therefore the presence or absence of this second linolic tetrabromide the residue obtained by evaporation of the petroleum ether (after the removal of the insoluble tetrabromide) was dissolved in methyl alcohol and allowed to stand for 2.5 days in the ice box. No further crystals separated from the solution during this period.

Attempts were made to prepare stearolic acid from the petroleum ether soluble oleic dibromide by heating it with alcoholic potassium hydroxide under pressure but these experiments were not successful.

4. Separation of the Saturated Fatty Acids by Distillation of their Methyl Esters

Satisfactory separation of a mixture containing several fatty acids can only be accomplished by means of fractional distillation of either the acids themselves or

their esters. For this purpose distillation of the methyl or ethyl esters is much to be preferred to that of the free acids. Not only do the methyl esters boil at lower temperatures than the corresponding acids, but in most cases there is a greater difference in the boiling points of the individuals of the series. There is also less tendency for decomposition in the fractionation of the esters due not only to their inherently more stable nature, but of course also to the lower distillation temperatures which may be employed. Unfortunately the boiling points of the esters of the unsaturated acids, oleic, linolic, linolenic, together with that of stearic acid lie so close together that separation of them by this means is impossible. The presence of oleic and linolic acids, however, has been shown by chemical means, therefore it was only necessary, in this case, to effect a separation of those esters boiling below 200° at 15 mm., and to show the presence or absence of stearic acid in the undistilled residue.

A preliminary experiment on the distillation of the methyl esters obtained from the fatty acid mixture (III) showed the presence of fractions boiling all the way from 97° at 760 mm. to 225° at 15 mm. Consequently quite a complex mixture of saturated acids was anticipated.

(a) Preparation of the Methyl Esters of the Fatty Acid Mixture (III)

33.7 gms. of the fatty acid mixture (III)was dissolved in 337 c.c. of absolute methyl alcohol and the solution saturated with dry HCl gas. The reaction vessel was kept cooled to room temperature during the 3.5 hours required for saturation and was then allowed to stand for an additional hour. The contents of the flask were then poured into 3 volumes (1035 c.c.) of cold water and the separated esters were removed by thorough extraction with ether. The ether solution was washed with 0.25 N KOH until all free acid was removed and then with water until neutral. The solution was then dried over anhydrous Na_2SO_4, filtered and the ether distilled off. The last traces of solvent were removed by heating on the steam bath in a current of nitrogen. Yield: 30.6 gms. The ester mixture was placed in a sealed tube and kept in the dark prior to the distillation.

(b) Distillation

The author was fortunate to have available an excellent still in which to carry out the fractionation of the ester mixture. This still was similar to that described by Podbielniak[55] and with the exception of the vacuum-jacketed column and receiver change block was constructed by

Mr. W. G. Rose of the University of Maryland. The author wishes to express his appreciation to Mr. Rose for its use and also for the time spent unsparingly by him in preparation for the actual distillation.

Following is the data regarding the distillation:

Volume of methyl esters ..... 34 c.c.

Size of distilling flask ..... 125 c.c.

Pressure at start of distillation.... 15.1 mm.

Pressure at end of distillation..... 13.3 mm.

Time required for distillation..... 6 hrs.

Size of column used ..... 8 mm (I.D.)

Data concerning the collection of the fractions together with distillation temperatures, as determined by means of a triple junction copper-advance thermocouple at the top of the column, are given in Table XVII.

TABLE XVII

Data Concerning Distillation of Methyl Esters

Fraction No.	Total Volume of distillate c.c.	M.V. at top of column	Temperature
	0.2	3.93	34.7
	0.6	3.93	34.7
I	1.0	3.93	34.7
	1.5	3.93	34.7
	1.6	-	-
Cut I	1.7	7.6	65.5
	1.9	8.1	69.5
II	2.2	8.43	72.3
	2.8	8.50	73.5
	2.9	9.58	81.5
Cut 2	3.1	12.06	101.0
	3.2	12.54	104.5
	4.2	12.63	107.0
	5.2	12.55	104.0
	5.8	12.65	105.0
III	6.2	12.65	105.0
	7.2	12.47	104.0
	8.2	12.44	104.0
	9.2	12.72	106.0
	9.3	14.12	117.0
	9.5	15.19	125.5
Cut 3	9.9	15.09	124.5
	10.1	15.65	129.0
	11.6	16.02	132.0
IV	12.1	16.20	133.5
	13.0	16.43	135.0
	13.1	18.40	150.0
Cut 4	13.7	19.10	155.5
	13.9	19.00	154.5
	14.0	19.40	157.5
	14.2	19.40	157.5
V	15.0	19.30	157.0
	16.1	20.10	163.5
	16.5	20.10	163.5
	16.8	21.40	171.0
Cut 5	17.2	23.10	183.0
	17.3	23.02	182.0
	18.2	23.60	186.0
VI	18.7	23.60	186.0
	18.8	24.00	189.0
	19.0	24.70	194.0
	19.8	-	-

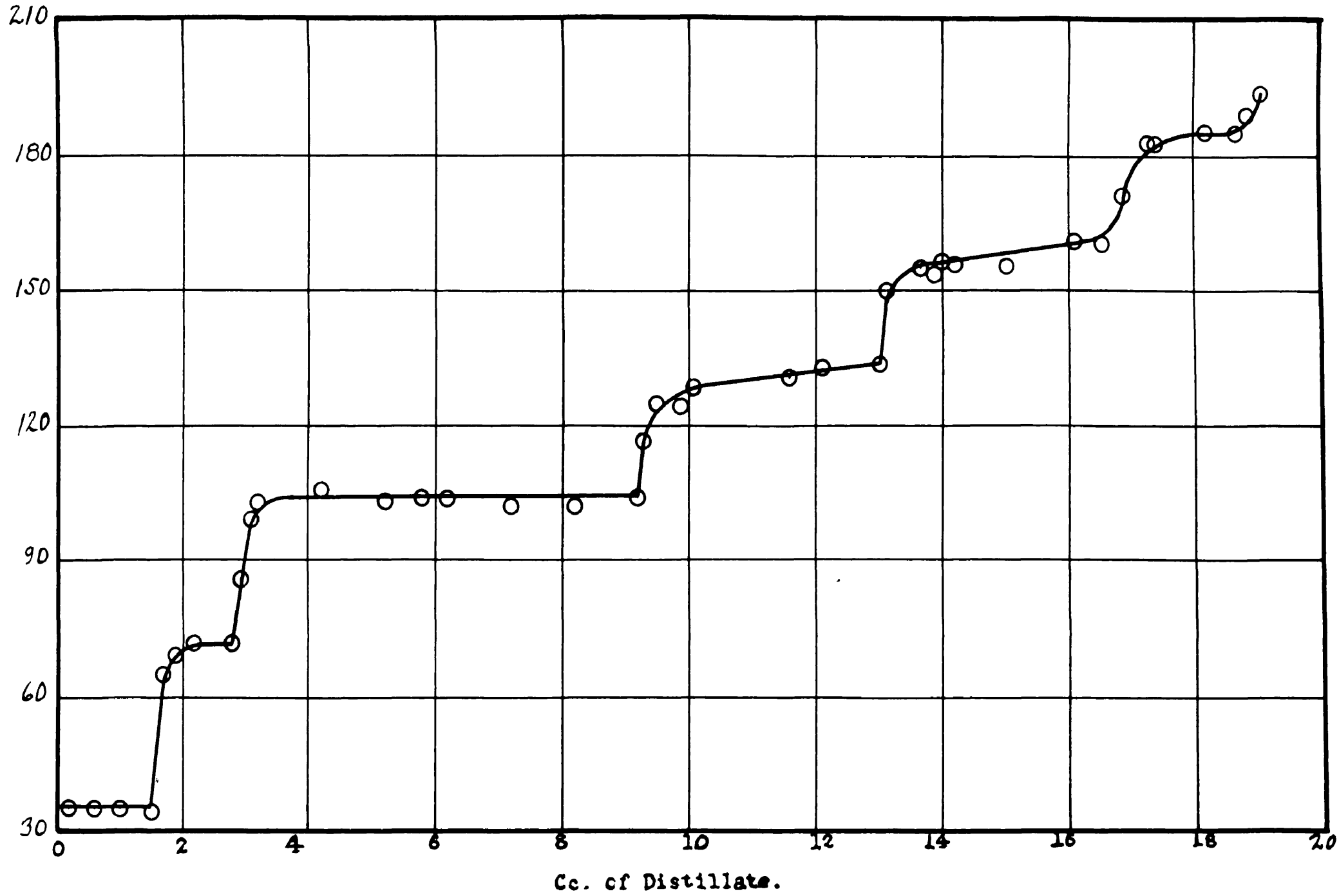


Fig. 5. - Distillation Curve of Methyl Esters of Fatty Acids from Croton Resin.

Six distinct fractions were obtained and the separations were very sharp through the first three, while in the last three the breaks although very pronounced were not so sharp. The rate of distillation at no time exceeded 0.1 c.c. per minute and of course was much slower than this in the cuts between fractions. The strip heater in the column was turned on after the third fraction was collected in order to prevent flooding of the column and the necessity of superheating the distilling pot. The temperatures recorded at the top of the column are in some cases as much as 20° lower than the literature values for the corresponding esters at 10 mm. pressure. This is no doubt due to the slow rate of distillation and to variations in the methods of determination of boiling points employed by different experiments.

The results of the distillation are shown in condensed form in Table XVIII and are shown graphically in Fig. 8, where temperatures are plotted as ordinates against c.c. distillate as abscissas.

TABLE XVIII

Data Concerning Distillation of the Methyl Esters

Fraction No.	Approximate B.P. at 15 mm.	Volume c.c.	% of total volume
I	95°	1.6	4.7
II	73°	0.9	2.6
III	105°	6.0	17.7
IV	133°	2.9	8.5
V	157°	2.6	7.8
Cut 5	160°	0.8	2.4
VI	166	1.5	4.4

6. Identification of Tiglic, Caprylic, Capric, Lauric, Myristic and Palmitic Acids

The density and refractive index were taken on each fraction including the 0.8 c.c. of material called "Cut 5". This "Cut 5" was found to be a purer sample of methyl palmitate than fraction #VI which had considerable yellow color and was evidently contaminated with esters of the higher boiling unsaturated acids. These data are tabulated in Table XIX.

(a) Density Determinations

Standardization of pycnometer:

Wt. of pycnometer + H_2O^{20} (upper mark)	2.6309 gms.
Wt. of pycnometer	1.8549 "
	0.8360 "
Wt. of pycnometer + H_2O^{20} (lower mark)	2.5214 "
Wt. of pycnometer	1.8549 "
	0.6665 "

1 ml. H_2O weighs 0.9982 gms. at $20^{0}47$

$\frac{0.8360}{0.9982}$ = 0.8375 ml. = vol. at upper mark.

$\frac{0.6665}{0.9982}$ = 0.6677 ml. = vol. at lower mark.

TABLE XIX

Table Showing Density and Refractive Index of Fractionated Methyl Esters

Fraction No.	Wt. of Pycnometer Full Gms.	Wt. of Pycnometer Empty Gms.	d_{20}^{20}	n_D^{20}
I	2.6490	1.8549	0.9402	1.4361
II*	2.4522	1.8549	0.8946	1.4253
III	2.5857	1.8549	0.9262	1.4269
IV	2.5891	1.8549	0.9267	1.4330
V	2.5947	1.8549	0.9714	1.4387
"Cut 5"	2.5922	1.8549	0.9304	1.4512
VI	2.5971	1.8549	0.9362	1.4361

* Pycnometer filled to lower mark.

(b) Molecular Weights

Molecular weight determinations were made on each fraction by means of the micro method of Charggaff[48]. This method consists of saponification of 10 to 30 mg. of sample by means of 0.1 N sodium n-propylate in n-propyl alcohol and then titration of the excess base by means of 0.05 N sulfuric acid using phenolphthalein as indicator. Since no n-propyl alcohol was available sodium isobutylate in isobutyl alcohol was substituted as the saponifying agent and 25 to 50 mg. samples were used for each determination.

Fraction I: Methyl tiglate

Using the micro method, just described, for the determination of the molecular weight of this fraction values of 141.7 and 135.9 were obtained. These values are fairly close to that required for methyl caproate (130) and the p-toluidide obtained from it melted within 1.5° of that reported for caproic acid. In addition to this combustion of the p-toluide gave a value for carbon which checked the theory for the toluidide of caproic acid but the hydrogen was 1% too low. It was found, however, that the combustion values checked those required for the toluidide of tiglic acid, the melting point of which is not given in the literature.

In view of these facts it was thought advisable to saponify the remaining portion of Fraction I and attempt to isolate the free acid produced, which if tiglic acid could be readily characterized by its melting point and analysis.

0.3427 gms. of Fraction I was refluxed for one hour with 39.9 c.c. of 0.239 N alcoholic KOH. 27.3 c.c. of 0.242 N HCl was required to titrate the excess alkali using phenolphthalein as indicator. From these data a molecular weight of 116.9 is obtained for this substance. The molecular weight calculated for methyl tiglate is 114.

The saponification solution was acidified to congo and the alcohol was then distilled off under reduced pressure. The remaining aqueous solution was then thoroughly extracted with ether. The ether solution was dried over anhydrous Na_2SO_4, filtered and the ether evaporated. The residue crystallised readily. It was recrystallised from petroleum ether but the product, however, was colored slightly yellow and melted at 59-61°. It was purified by sublimation at a pressure of about 20 mm. and a temperature of 90°. The sublimed product was white and beautifully crystalline, it melted at 64° (ordinary thermometer). Upon resublimation the product melted at 65.5°. The melting point of tiglic acid is given as 64.5 by Kopp[49]. (See Beilstein 3rd Ed. 1 513).

Analysis:

	Sample (mg.)	H_2O (mg.)	CO_2 (mg.)	% H	% C
1.	3.772	2.662	8.267	7.90	59.76
	Calc. for $C_9H_8O_2$			8.00	60.00

In the following molecular weight determinations 0.06804 N H_2SO_4 was used in all of the titrations. The samples were refluxed with the sodium isobutylate for one half hour in each case and 3 drops of 1% alcoholic phenolphthalein solution was used as the indicator. The standardization of the sodium isobutylate solution was carried out in a manner exactly similar to the actual saponifications, and its concentration was checked by frequent restandardizations.

Fraction II: Methyl caprylate

5.00 c.c. alkali ⇄ 6.64 c.c. 0.06804 N acid.

	Sample (mg)	c.c. alkali	c.c. acid reqd. for excess	Mol. Wt. found
1.	38.23	5.00	3.31	155.9
2.	38.95	5.00	2.97	156.8
	Calc. for $C_9H_{18}O_2$			158

Fraction III: Methyl caproate

5.00 c.c. alkali ⇄ 6.63 c.c. 0.06804 N acid.

	Sample (mg.)	c.c.alkali	c.c.acid reqd. for excess	Mol.Wt. found
1.	43.27	5.00	3.05	186.3
2.	43.49	5.00	3.17	185.1
	Calc. for $C_{11}H_{22}O_2$			186

Fraction IV: Methyl laurate

10.00 c.c. alkali ⇄ 11.11 c.c. 0.06804 N H_2SO_4

	Sample (mg.)	c.c.alkali	c.c.acid reqd. for excess	Mol.Wt. found
1.	47.40	10.00	7.75	205.1
2.	36.72	10.00	8.60	214.7
	5.00 c.c. alkali ⇄ 6.63 c.c. 0.06804 N H_2SO_4			
1.	47.98	5.00	3.37	215.2
	Calc. for $C_{13}H_{26}O_2$			214

Fraction V: Methyl myristate

10.00 c.c. alkali ⇄ 11.11 c.c. 0.06804 N H_2SO_4

	Sample (mg.)	c.c. alkali	c.c.acid reqd. for excess	Mol.Wt. found
1.	44.30	10.00	8.33	234.4
2.	43.86	10.00	8.42	239.7
	Calc. for $C_{15}H_{30}O_2$			242

"Cut 5": Methyl palmitate

5.00 alkali ⇄ 6.63 c.c. 0.06604 N H_2SO_4

	Sample (mg.)	c.c. alkali	c.c.acid reqd. for excess	Mol. Wt. found
1.	40.40	5.00	4.80	278.3
	Calc. for $C_{17}H_{34}O_2$			270

(c) Preparation of p-toluidides

The preapration of a suitable derivative of the acid corresponding to each fraction might have entailed some difficulty (particularly in the case of Fraction No. II where only 0.8 c.c. of ester was available) but for the excellent method recently published by Koelsch and Tenenbaum[50]. This method involves the formation of the p-toluidide of an acid directly from the corresponding methyl or ethyl ester in yields reported as high as 66%. The p-toluidide is soluble in ether while the by-products of the reaction are soluble in water (or dilute HCl). This therefore yields a product already in a high state of purity.

The procedure used in the preparation of each derivative was the same, consequently it will be described in detail only in the case of Fraction I.

Fraction I

0.6 gm. of dry Mg was covered with absolute ether and

about 3 gms. of pure dry ethyl bromide was added. The reaction was started with a crystal of iodine. When the formation of the ethyl magnesium bromide was complete 2.68 gms. of p-toluidine was slowly added. To this mixture was then added 0.7 gm. of Fraction I in absolute ether. After the mixture had been refluxed for five minutes it was cooled and acidified with dilute HCl. The acid solution was extracted with ether and the ether layer was then washed with dilute HCl solution and finally with water. The solution was dried over anhydrous Na_2SO_4, filtered, and the ether evaporated. Yield 0.13 gm. of beautiful needle crystals. Softened 69-70°, melted 70-70.5°.

66% aqueous methyl alcohol was used for recrystallising this substance and some difficulty was encountered in this case in the removal of a trace of yellow-brown impurity. When the solution was cooled in ice-HCl the p-toluidide crystallized in clusters of needles or rosettes. The product was filtered off and dried in the Abderhalden at 61°. It melted at 70-71.5°. A third recrystallisation from 66% methyl alcohol followed by drying for 4-5 hours at 61° in the Abderhalden yielded a product which again melted at 70-71.5°. The melting point of this derivative is not reported in the literature.

Analysis:

	Sample (mg.)	H_2O (mg.)	CO_2 (mg.)	% H	% C
1.	3.485	2.518	9.716	8.09	76.05
2.	4.432	3.258	12.316	8.13	76.16
	Calc. for $C_{12}H_{15}OH$			7.94	76.19

Fraction II

Materials used:

0.46 gms. ester
0.29 " Mg
>1.31 " ethyl bromide
1.28 " p-toluidine

After recrystallisation of the crude product from 80% alcohol 0.32 gms. of material was obtained. When dried at 61° in the vacuum Abderhalden it melted at 67-68°. A further recrystallisation from aqueous alcohol and subsequent drying gave a product which melted at 68° - 68.8°. It was then recrystallised from aqueous methyl alcohol and dried as usual. Melting point 67.5 - 68.8°. Robertson[51] reported the melting point of the p-toluidide of caprylic acid as 70°.

Analysis:

	Sample (mg.)	H_2O (mg.)	CO_2 (mg.)	% H	% C
1.	4.318	4.029	12.731	9.98	76.96
	Calc. for $C_{19}H_{23}NO$			9.04	77.19

Fraction III.

Materials used:

1.0 gm. ester

0.52 gm. Mg

2.35 gm. ethyl bromide

2.31 gm. p-toluidine

After recrystallization of the crude product from 80% alcohol 0.92 gms. of material was obtained which when dried at 61° in the vacuum Abderhalden melted at 76-76.6°. A further recrystallization in the same manner gave a product which melted at 77-78°. It was finally recrystallized from pure methyl alcohol and dried as usual. Melting point 76.5° - 77.2°. Heberteon reported the melting point of the p-toluidide of capric acid as 78°.

Analysis:

	Sample (mg.)	H_2O (mg.)	CO_2(mg.)	% H	% C
1.	3.879	3.650	11.099	10.53	78.04
	Calc. $C_{17}H_{27}NO$			10.42	78.10

Fraction IV.

Materials used:

1.0 gm. ester

0.46 gm. Mg

2.03 gm. ethyl bromide

2.03 gm. p-toluidine

After recrystallization of the crude product from 80% alcohol 1.0 gm. of material was obtained which when dried at 61° in the vacuum Abderhalden melted at 82.5 - 83°. A further recrystallization in the same manner gave a product which melted at 83-83.8°. It was finally recrystallised from pure methyl alcohol and dried as usual. Melting point 82.5 - 83.8°. Robertson reported the melting point of the p-toluidide of lauric acid as 87°.

Analysis:

	Sample (mg.)	H_2O (mg.)	CO_2 (mg.)	% H	% C
1.	3.659	3.570	10.611	10.92	79.09
	Calc. for $C_{19}H_{31}ON$			10.90	78.81

Fraction V.

Materials used:

1.0 gm. ester
0.39 gm. Mg
1.79 gm. ethyl bromide
1.75 gm. p-toluidine

After recrystallization of the crude product from 80% alcohol 1.07 gms. of material was obtained which when dried at 61° in the vacuum Abderhalden melted at 89-90°. A further recrystallization in the same manner but with subsequent drying at 78° gave a product which still melted

at 89-90°. It was finally recrystallized from pure methyl alcohol and dried at 78°. Melting point 89-90.2°. Robertson reported the melting point of the p-toluidide of myristic acid as 93°.

Analysis:

	Sample (mg.)	H_2O (mg.)	CO_2(mg)	% H	% C
1.	3.826	3.826	11.096	11.19	79.10
	Calc. for $C_{21}H_{35}ON$			11.12	79.42

"Cut 5"

Materials used:

0.98 gms. ester.(all of cut 5 + some of Fraction VI)
0.36 " Mg
1.64 " ethyl bromide
1.61 " p-toluidine

After recrystallisation of the crude product from 80% alcohol 0.54 gms. of material was obtained which when dried at 61° in the vacuum Abderhalden melted at 89-90°. It was then recrystallised several times from 80% alcohol and dried at 78° after each. The melting points were as follows: 94-95.2°; 95-95.9°; 95-95.9° respectively. It was finally recrystallised from pure methyl alcohol and dried as usual. Melting point 95-96°. Robertson reported the melting point of the p-toluidide of palmitic acid as 98°.

Analysis:

	Sample (mg.)	H_2O(mg.)	CO_2(mg)	% H	% C
1.	4.164	4.226	12.130	11.36	79.43
	Calc. for $C_{23}H_{39}OH$			11.38	79.03

The sample of ester represented by "Cut 5" was evidently contaminated with other esters as no less than five recrystallizations were required to reach constant melting point.

A search of the literature revealed wide discrepancies in the values of the melting points recorded for the p-toluidides of the various acids. Comparison was made with those reported by J. Robertson[81] inasmuch as his determinations were made on compounds of tested purity using a standardized thermometer. His melting point determinations were made by the capillary tube method using a castor oil bath.

In the work just described each derivative was recrystallized to constant melting point. The melting points were determined by the usual capillary tube method using however an air bath heated by means of concentrated sulfuric acid. An Anschutz 0.2° thermometer which had been calibrated by the U. S. Bureau of Standards was used throughout. The purity of each derivative was shown by analysis. It is to be observed that the melting points of the p-toluidides reported by Robertson are in every case

a little higher than those obtained in this research. In view of the considerations just mentioned this variation can probably only be due to differences in the technique employed in the two cases. This conclusion is further supported by the fact that the melting points reported herein are consistently lower than those of Robertson.

Table XX shows in condensed form the refractive index, density, molecular weight and the melting point of the corresponding toluidide of each fraction. There is also included for the sake of comparison Robertson's value for the melting point of the p-toluidides of the corresponding acids.

TABLE XX.

Table Showing Density, Refractive Index, Molecular Weight and Melting Point of the p-toluidide of Fractionated Methyl Esters

Fraction No.	n_D^{20}	D_{20}^{20}	Mol.Wt. found	Mol.Wt. calc.	M.P. of p-toluidide	M.P. of p-toluidide literature[61]
I	1.4351	0.9492	116.9 155.9	114	70-71.5°	-
II	1.4253	0.8946	155.8 186.3	158	67.3-68.8°	70°
III	1.4269	0.8782	185.1 214.7	186	76.5-77.2°	78°
IV	1.4338	0.8767	215.2 234.4	214	82.5-83.2°	87°
V	1.4387	0.8714	239.7	242	89-90.2°	93°
"Cut 5"	1.4512	0.8804	278.3	270	95-96°	98°
VI	1.4581	0.8862	-	-	-	-

The experiments on the fatty acid mixture (III) just described have not provided for the detection and isolation of stearic or any of the other higher molecular weight saturated fatty acids. Therefore, to ascertain the presence or absence of these substances the following experiment was performed. 23 cc. of methyl esters, obtained by esterification of some of the fatty acid mixture (III) with CH_3I and Ag_2O, were subjected to distillation at 15 mm. pressure in the Podbelniak column. The first 14.7 c.c. was distilled over and the high boiling residue (which should contain the methyl

esters of oleic, linolic and any of the saturated series above palmitic) was saponified with alcoholic KOH. A lead-salt-ether separation was run on the resulting potassium salts. The ether solution of lead soap was cooled for three hours in an ice bath and then filtered. Only a small quantity of insoluble lead soap was thereby obtained from which 0.3 gm. of free acid was isolated. This free acid was practically completely soluble in methyl alcohol and since the higher saturated fatty acids are insoluble in this solvent their absence in the mixture is indicated.

8. The Resin Alcohol

Little information was gained regarding the untractable material (II) called resin alcohol by Boehm. Its properties seem to be similar to those attributed to it by him.

The resin alcohol (II) is a dark brown amorphous material, insoluble in petroleum ether, ether, benzene and water, but readily soluble in methyl and ethyl alcohol. Its dilute alcoholic solution gives a reddish color with $FeCl_3$ solution. It is soluble in aqueous alkali and upon boiling with acetic anhydride (+ a trace of H_2SO_4) an amorphous neutral ether soluble derivative is formed. It also reacts vigorously with CH_3I and Ag_2O in acetone solution. Part of the product formed by this reaction is an ether

soluble liquid possessing a highly characteristic pleasant odor. A considerable quantity of a light yellow ether insoluble solid material is also formed. This substance is soluble in acetone but only slightly soluble in methyl alcohol. These facts indicate that the resin alcohol is a polyhydroxy substance at least some of the hydroxyls of which are phenolic or enolic in character.

The resin alcohol reduces permanganate solution and oxidation by boiling with 45% HNO_3 yielded some crystalline material which though not positively identified, was probably oxalic acid.

7. The Water-Soluble Saponification Products

The isolation of this material has been described under the saponification of croton resin Sec. X, Part 1. When dry it is an amorphous light brown powder which readily takes up water from the atmosphere to become pasty in consistency. It is soluble in water, methyl and ethyl alcohol but is insoluble in the other common organic solvents.

Following is a tabulation of some of the properties of this material:

1. Its aqueous solution gives a beautiful purple or red-violet color with $FeCl_3$. This color is very persistent.

2. In dilute ammonia solution it gives no precipitate with $(NH_4)_2MoO_4$, hence it is probably not a glucoside which had not been hydrolysed by the alcoholic KOH.

3. Bromine precipitates it from its aqueous solution yielding an ether soluble amorphous substance.

4. It yields an amorphous acetyl derivative with acetyl chloride.

5. Its clear aqueous solution when just acid to litmus possesses a greenish-yellow flouresence. When just alkaline to litmus it has an orange-green flouresence.

6. It gives a negative test for resorcinol with formic acid[52].

7. Upon ignition it burns like a sugar yet when compared with a known carbohydrate it is doubtful if it gives a positive Molisch Test[53]

8. Reduces Fehling's solution readily.

9. Its aqueous solution gives no precipitate with saturated picric acid solution.

10. It is interesting to note that upon saponification of the dimethyl resin, the acidified aqueous solution (after the removal of the fatty acids and the resin alcohol) no longer gives the characteristic purple color with $FeCl_3$. This is a peculiar fact inasmuch as an alcoholic solution of the original resin itself gives no color with $FeCl_3$.

Consequently it would be expected that the OH group responsible for this color test would be generated from the resin upon its saponification and could not be masked by methylation of the original substance. It is possible that methylation of other hydroxyls in the same molecule has rendered the product water insoluble and the material may have been removed along with the fatty acids or resin alcohol.

In conclusion it may be stated that this material is probably a polyhydroxy enolic or phenolic substance, it may also possess carboxylic functions. Since it is amorphous and there is no guarantee of its homogeneity it is possible that the $FeCl_3$ color test is due to a small quantity of some foreign substance and not to the major portion of the material.

XI. DISCUSSION OF RESULTS

The apparatus employed for physical measurements in this research was all standardized before use (exception refractometer). The weights were compared by the method of T. W. Richards[54] and although the 10 mg. weight used for comparison was not standardized, its accuracy to within reasonable limits had been previously tested scores of times by its use in micro chemical work on compounds of known composition.

Melting points were taken by the capillary tube method using an air bath heated by means of concentrated sulfuric acid. The thermometers used for the melting points were of the Anschutz 0.2° type. They were standardized by the U. S. Bureau of Standards and the melting points were taken with the mercury thread enclosed in the heated air bath or protruding so little that stem corrections were negligible.

In addition to the ordinary calibration of apparatus the validity of all determinations was further checked by the use of compounds of known composition and purity.

Boehm concluded from his work that croton resin is an intimate mixture of esters formed from the resin alcohol and fatty acids, probably tiglic acid predominating. He

noticed that croton oil freed from resin no longer contained tiglic acid. His resin lost its toxicity to frogs and rabbits upon hydrogenation in which the iodine number fell from 77 to 12.5. Boehm and Flaschentrager claim to have isolated from croton oil the pure toxic principle which they suggested was possibly an ester formed from the crystalline phorbol and tiglic acid. They were able to obtain this crystalline non-toxic phorbol by the controlled hydrolysis of their resin, which according to them regained its toxicity upon acetylation.

It is apparent from the large number of fatty acids isolated from croton resin and the fact that its composite molecular weight is only 600 that all of these acids could not be combined with a single polyhydroxy compound. From the facts presented in this thesis one is forced to conclude, as did Boehm, that croton resin is an intimate mixture of esters formed by the esterification of probably both the resin alcohol and the water soluble saponification products with fatty acids. Of these acids capric occurs in the largest proportion, while tiglic is found in relatively smaller quantity.

From the data presented it is apparent that the toxicity of croton resin is not dependent solely on the presence of unsaturated functions as Boehm's data would

indicate, neither is it a matter of simple esterification of an hydroxy compound as Boehm and Flaschentrager have recently suggested, but rather it is more intimately related to the presence of the two free hydroxyl groups. Thus, hydrogenation of the resin did not seem to diminish its toxicity and vesicant action at all, and even the brominated product possessed some toxicity and vesicant action though to a greatly lessened extent. However, simple masking of the two hydroxyls by the gentle reaction of dry silver oxide and methyl iodide gave a product which possessed no toxic effect on goldfish in 1740 minutes at a concentration of two milligrams per liter and which was completely without vesicant action.

Because of the important relationship of these OH groups to the toxicity of the resin an excellent means is afforded for the isolation of the individual compound, or compounds in the complex resin mixture, which is responsible for the remarkable action of croton resin. The original resin is practically free from methoxyl content, hence these OH groups can be methylated, the product saponified and the important constituent of the resin could thereby be detected by its methoxyl content. In this manner it could be determined whether the resin alcohol or the water soluble saponification products were the alcoholic part of the toxic

resin ester.

Certain considerations have arisen since the writing of the main body of the thesis which have caused the author to question the findings of the active hydrogen determinations. Consequently these results should be accepted as subject to verification.

XII. SUMMARY

1. Samples of croton beans from four different sources have been obtained. A yield of 0.5% resin has been obtained from one sample of unshelled beans and 0.6% and 0.9% from shelled beans from two different sources.

2. The method of Cherbuliez et.al.for the isolation of croton resin has been modified. The efficiency of the method has been followed by means of determination of the toxicity to goldfish of the by-products of the extraction processes.

3. Practically all of the results of Cherbuliez et al have been confirmed.

4. The resin has been hydrogenated, brominated, methylated and acetylated. The toxicity to goldfish and the vesicant action of these derivatives has been studied.

5. Croton resin with a methoxyl content of 11.7% has been found to be non-toxic to goldfish and to possess no vesicant action.

6. Croton resin has been shown to possess approximately four free hydroxyl groups. (See Sec. XI.)

7. The non-homogeneity of croton resin has been shown.

8. In the extraction of a 90% aqueous methyl alcoholic solution of resin with petroleum ether the toxic principle

concentrates in the petroleum ether extract.

9. Complete curves showing the toxicity to goldfish of croton oil, the alcohol soluble portion of croton oil and croton resin have been determined.

10. Using the formula of Powers it has been shown that croton resin is 7.8 times more toxic to goldfish than rotenone.

11. As little as 0.04 mg. of croton resin per square inch of skin causes definite vesicant action.

12. Preliminary studies of the toxicity of the acetone extracts of croton beans to a variety of insects has been made by Davidson et. al. Using a spray solution calculated to contain 1:10,000 parts of resin mortalities of 100%, 100% and 92% were obtained against Aphis gossypii, Myzus persicae and white fly respectively.

13. The toxicity of croton resin to mosquito larvae has been studied by Campbell et al.

14. Tiglii, caprylic, capric, lauric, myristic, palmitic, oleic and linolic acids have been isolated from the saponification products of the resin.

15. Some information has been obtained regarding the nature of the other saponification products of croton resin.

BIBLIOGRAPHY

1.(a) LaForge, Haller, and Smith - Chemical Reviews, 12 No. 2 181 (1933).
(b) Roark - J. Ind. Eng. Chem. 25 639 (1933).

2. Meyers et al - J. Ind. Eng. Chem. 25 624 (1933).

3. Drake and Spies - J. Econ. Entomol. 25 No. 1 129 (1932).

4. Spies - Ibid. 26 No. 1 235 (1933).

5. Mr. Davidson's report is attached to Spies' monthly report for March, 1932, which is on file in the offices of the Insecticide Division, Bureau of Chemistry and Soils, Washington, D. C. A condensed summary of this report is given in Sec. IX of this thesis.

6. Cherbuliez, Ehninger and Bernhard - Helv. Chim. Acta. 15 638 (1932)

7. Schlippe - Annal. d. Chem. u. Pharm. 108 1 (1858).

8. Buckheim - Chemisches Central-Blatt (3) 4 676 (1873).

9. Kobert - Chemisches Central-Blatt 58 575 (1887). Inaug. Diss. Dorpat Chem. Ztg. 11 416.

10. Hirschsohn - Chemisches Central-Blatt 61 214 (1890). Arb. a.d. Pharm. Inst. Dorpat 4 R Phm. 1 173.

11. Stillmark - Chemisches Central-Blatt 60 978 (1889). Arb. pharmakol Inst. Dorpat. P.C.H. 30 680 (31/10).

12. Elfstrand - Habilitationsschrift, Upsala (1909) or (1907).

13. Karrer, Weber, and Sloeten - Helv. Chim. Acta. 8 384 (1925).

14. Dunstan and Boole - Proc. Royal Soc. (London) 80 238 (1908).

15. Boehm - Arch. fur exptl Path. Pharmakol. 79 138 (1915).

16.(a) Boehm and Flaschentrager - Arch. fur exptl Path. Pharmakol. 157 115 (1930).
(b) Flaschentrager - Z. Angew. Ch. 43 1011 (1930).

17. Drake and Spies - Anal. Ed. J. Ind. Eng. Chem. 5 284 (1933).

18. Cherbuliez and Bernhard - Helv. Chim. Acta. 15 464 (1932).

19.(a) Kast - Ber. 54 1979 (1921).
(b) Spies - J. Am. Chem. Soc. 55 250 (1933).

20 - Official Methods of Analysis of the A. O. A. C. p. 344 (1919).

21. Paal and Roth - Ber. 42 1644 (1909).

22. Adams, Voorhees and Shriner - Organic Syntheses Collective Vol. 1 462.

23. Gattermann and Wieland - Laboratory Methods of Organic Chemistry p. 142 (1932).

24. Ibid - 367 (1932).

25. Covert and Adkins - J. Am. Chem. Soc. 54 4116 (1932).

26. Clark - J. Assoc. Off. Agri. Chem. 15 136 (1932).

27. Houben - Die Methoden der Organischen Chemie. 3 124 (1932).

28. Haworth and Lapworth - J. Chem. Soc. 123 2986 (1923).

29. Purdie and Pitkeathly - J. Chem. Soc. 75 157 (1899).

30.(a) Bergdorff - J. Am. Chem. Soc. 52 3440 (1930).
(b) " " " " " 53 5051 (1930).
(c) " " " " " 53 1997 (1931).
(d) " " " " " 55 1147 (1933).
(e) " " " " " 55 2941 (1933).
(f) " " " " " 56 979 (1934).

31. Pregand - Quantitative Analytische Mikromethoden der Organischen Chemie p. 200 (1931).

32. Powers - Ill. Biol. Mono. 4 No. 2 (1917).

33. Jones - J. Am. Chem. Soc. 55 1737 (1933).

34. Clark - Science 77 311 (1933).

35. Lewkowitsch - Chemical Technology and Analysis of Oils, Fats and Waxes 6th Ed. 1 276 (1921).

36. Ibid - 1 253.

37. Jamieson - J. Assoc. Off. Agric. Chemists 11 303 (1928).

38. Lapworth and Mottram - J. Chem. Soc. 127 1628 (1925).

39. Lewkowitsch - Chemical Technology and Analysis of Oils, Fats and Waxes. 6th Ed. 1 233-5 (1921).

40. Vongerichten and Kohler - Ber. 42 1638 (1909).

41. LeSueur - J. Chem. Soc. 85 1712 (1904).

42. Orun - Ber. 42 3759 (1909).

43. Rollett - Zeits. f. Phys. Chem. 68 420 (1909).

44. Lewkowitsch - Chemical Technology and Analysis of Oils, Fats and Waxes. 6th Ed. 1 276 (1921).

45. Ibid - 1 202 (1921).

46. Matthes and Boltze - Arch. d. Pharm. 250 235 (1912).

47. International Critical Tables 5 25 (1933).

48. Chargaff - Z. Physiol. Chem. 192 221 (1931).

49. Kopp - Ann. 195 84 (1879).

50. Koolsch and Tenenbaum - J. Am. Chem. Soc. 55 3049 (1933).

51. Robertson - J. Chem. Soc. 115 1211 (1919).

52. Mulliken - Identification of Pure Organic Compounds. 1 24 (1904) Test 114.

53. Williams - Introduction to Biochemistry 446 (1931) (D. Vanlostrand Co., Inc. 250 Fourth Ave., N. Y.).

54. Richards - J. Am. Chem. Soc. 22 144 (1900).

55. Podbielniak - Anal. Ed. J. Ind. Eng. Chem. 5 135, (1933).

College Park, Maryland,

April 27, 1934.

[Reprint from the Journal of the American Chemical Society, **57**, 774 (1935).]

d-Ribose from the Croton Bean

By Joseph R. Spies with Nathan L. Drake

[Reprint from the Journal of the American Chemical Society, 57, 774 (1935).]

d-Ribose from the Croton Bean

By Joseph R. Spies with Nathan L. Drake

Cherbuliez and Bernhard[1] have recently isolated from the croton bean, *Croton tiglium* (L), a new glycoside, which they identified as 2-hydroxy-6-amino-purine-*d*-riboside, and which they proposed to call a "crotonoside." They did not, however, succeed in crystallizing the sugar residue obtained by hydrolysis.

The accumulation of a quantity of this material has afforded us an opportunity to examine it. We have succeeded in crystallizing the sugar residue and have conclusively established its identity as *d*-ribose, thus confirming the findings of these workers.

Experimental

Isolation of the Crotonoside.—The glycoside was extracted from the ground unshelled beans with methanol and isolated as described by Cherbuliez and Bernhard.[1] From 125 kg. of beans was obtained 345.0 g. of crude product which contained about 25% of pure crotonoside (0.07%). When recrystallized to constant melting point and dried in the Abderhalden at 110°, it decomposed at 245–247°. (All melting points were taken with standardized Anschütz thermometers.)

Anal. Calcd. for $C_{10}H_{13}O_5N_5$: C, 42.37; H, 4.59; N, 24.73. Found: C, 42.01; H, 4.47; N, 24.56.[2]

Crystallization of *d*-Ribose.—The crotonoside was hydrolyzed and the ribose isolated as described by Cherbuliez and Bernhard.[1] The sirup obtained was dried in a vacuum over phosphorus pentoxide, and when nucleated with an authentic specimen of *d*-ribose it slowly crystallized.[3] Recrystallization three times from absolute ethanol and once from dry isopropanol gave a product that melted constantly at 83–87° with previous softening at 80°. The melt, however, was cloudy; $[\alpha]^{20-25}D$ −17.5° (c = 5.00 g./100 ml.); (Levene and Jacobs, 85°, $[\alpha]^{20}D$

(1) Cherbuliez and Bernhard, *Helv. Chim. Acta*, **15**, 464 (1932).

(2) The authors are indebted to Mr. H. M. Duvall of the University of Maryland for the micro Kjeldahl determination.

(3) The writers are indebted to Mr. F. P. Phelps, of the Bureau of Standards of the U. S. Dept. of Commerce, for this material.

$-19.2°$)[4] (Phelps, Isbell and Pigman, 87°, $[\alpha]^{1}D$ $-23.7°$.)[5]

Tetraacetate.—The tetraacetate (tetraacetylribose) was prepared as described by Levene and Tipson,[6] who give 110° as the melting point and $[\alpha]^{24}D$ $-52.0°$ and $[\alpha]^{25}D$ $-54.3°$ in chloroform. The present writers' product was recrystallized to constant melting point from ethanol; m. p. 108–109°; in chloroform $[\alpha]^{24}D$ $-54.1°$ (*c*, 6.52 g./100 ml.).

Anal. Calcd. for $C_{13}H_{18}O_9$: C, 49.06; H, 5.66. Found: C, 49.33; H, 5.88.

Phenylosazone.—The phenylosazone was recrystallized to constant melting point from 40% ethanol; m. p. 165–165.5° (Levene and LaForge, 166°).[7]

***p*-Bromophenylhydrazone.**—The *p*-bromophenylhydrazone was prepared as described by Cherbuliez and Bernhard.[1] It was recrystallized to constant melting point from an absolute ethanol–ether solution; m. p. 164° (Ekenstein and Blanksma,[8] 164°) (Levene and Jacobs,[9] 170°).

(4) Levene and Jacobs, *Ber.*, **42**, 2469, 2474 (1909).
(5) Phelps, Isbell and Pigman, THIS JOURNAL, **56**, 747 (1934).
(6) Levene and Tipson, *J. Biol. Chem.*, **92**, 109 (1931).
(7) Levene and LaForge, *ibid.*, **20**, 429 (1915).
(8) Ekenstein and Blanksma, *Chem. Weekblad*, **10**, 664 (1913).
(9) Levene and Jacobs, *Ber.*, **42**, 2703 (1909).

BUREAU OF ENTOMOLOGY
AND PLANT QUARANTINE
U. S. DEPARTMENT OF AGRICULTURE
COLLEGE PARK, MD.
RECEIVED FEBRUARY 14, 1935

[Reprint from the Journal of the American Chemical Society, **57**, 180 (1935).]

Croton Resin. I. Toxicity Studies Using Goldfish

By Joseph R. Spies

[Reprint from the Journal of the American Chemical Society, 57, 180 (1935).]

[CONTRIBUTION FROM THE UNIVERSITY OF MARYLAND STATION OF THE INSECTICIDE DIVISION, BUREAU OF CHEMISTRY AND SOILS]

Croton Resin. I. Toxicity Studies Using Goldfish[1]

BY JOSEPH R. SPIES

The oil of the croton bean[2] has been the subject of numerous investigations during the last century. Its purgative action as well as its vesicant and toxic properties were noted by earlier investigators, and various attempts have been made to isolate the active principle. Recently Cherbuliez[3] and his co-workers have isolated an extremely active, non-homogeneous, resin from both the oil and beans which is undoubtedly responsible for their vesicant and toxic properties.[4]

In a search for new natural insecticides, in which a number of plant materials were examined,[5] it became apparent that the croton bean[6] contains a substance which surpasses rotenone in its toxicity to goldfish. The process of Cherbuliez was modified to obtain the resin for this study, and it was shown by tests on goldfish that no appreciable quantity of toxic material was lost in the process.

In the hope that some resolution of components

(1) From a thesis submitted by Joseph R. Spies to the Graduate School of the University of Maryland in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(2) *Croton tiglium* (Linné) is a species of the *Croton* genus of the *Euphorbiaceae* family. The seed or bean is sometimes used as a fish poison.

(3) Cherbuliez, Ehninger and Bernhard, *Helv. Chim. Acta*, **15**, 658 (1932).

(4) Boehm and Flaschenträger, *Arch. Path. Pharmakol.*, **157**, 115 (1930), claim to have isolated the pure toxic principle from croton oil by purely physical means which they did not describe.

(5) (a) Drake and Spies, *J. Econ. Entomol.*, **25**, 129 (1932); (b) Spies, *ibid.*, **26**, 285 (1933).

(6) Dr. G. P. Jung of the Bureau of Entomology of China kindly furnished the major supply of beans for this study. According to Jung a "croton emulsion" made from the beans is used as an insecticide in China; other sources from which the beans were obtained: (a) Schimmel & Co., A. G., Miltitz bei Leipzig (through Fritzsche Bros., N. Y.), shelled beans, yield of resin 0.6%; (b) Anandji Virgi & Co., Box 153, Bombay, India, shelled beans, yield of resin 0.94%; (c) H. C. Neibert, Milbuk, P. I. *Cf.* Jung, *Lingnan Sci., J.*, [3] **13**, 557 (1934).

leading to crystalline material might be effected, a solution of 50 g. of resin in 300 ml. of 90% methanol was extracted continuously with redistilled petroleum ether (b. p. 55–70°). The color of successive fractions deepened materially; the first extracted material was light yellow and the resin soft and sticky, that obtained toward the end of the experiment was brown and yielded a hard and brittle resin. About 81% of the resin, in which portion the more toxic substances were concentrated, was extracted during the first forty-eight hours. The carbon and hydrogen content of the fractions decreased from 69.9 to 66.4% and 9.2 to 8.2% from first to last, respectively, showing that a constituent which contains more oxygen than the most toxic substances concentrated in the methanol. The extraction was continued for one hundred and sixty-eight hours and the residue which remained in the methanol was not toxic to goldfish. No crystalline products were obtained from any extract.

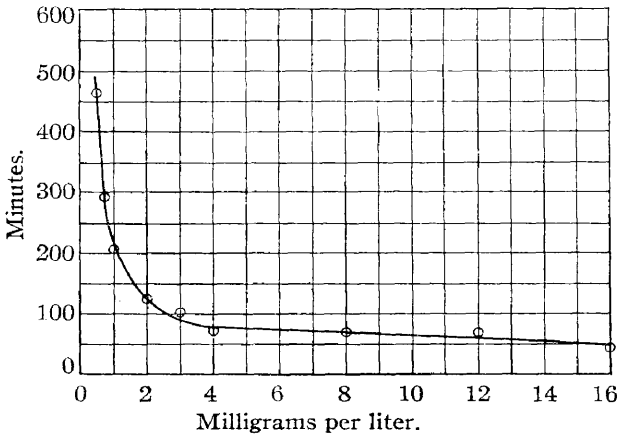


Fig. 1.—Curve showing toxicity of croton oil to goldfish.

The method of Gersdorff[7] using goldfish[8] (*Carassius auratus*) was employed to study the relative toxicity of croton oil, the alcohol-soluble portion of the oil, and croton resin, which constitute, respectively, about 25, 8 and 0.5% of the unshelled croton bean. The results obtained are illustrated graphically in Figs. 1, 2 and 3, where average survival time is plotted as ordinates against concentration as abscissas. The survival time–concentration curve for rotenone published by Gersdorff[7] has been included in Figs. 2 and 3 for the sake of comparison. Since the tests were not made on the same lot of fish, exact comparison is not possible, but it is evident that croton resin is appreciably more toxic than rotenone.

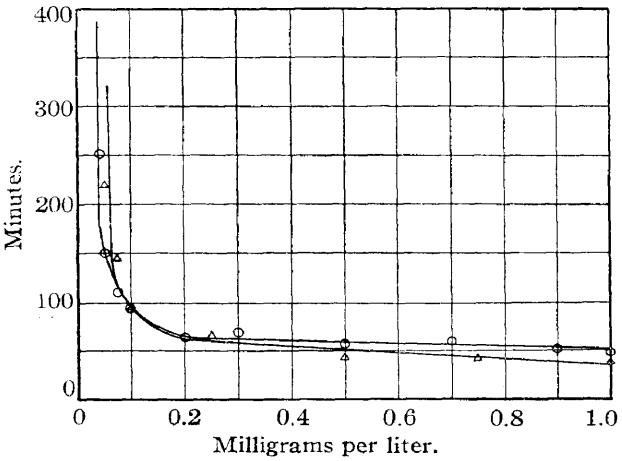


Fig. 2.—Curves showing toxicity of alcohol-soluble fraction of croton oil and rotenone: △, alcohol soluble fraction of croton oil; ○, rotenone.

Experimental

Isolation of Croton Resin.—The extraction with methanol of the ground unshelled beans was carried out on 5-kg. lots in a large capacity Soxhlet.[9] The resin was isolated from the extract by a modification of the process of Cherbuliez.[3] The details of this procedure can be obtained from the author's dissertation. The resin was stored in a desiccator out of contact with air and protected from light.

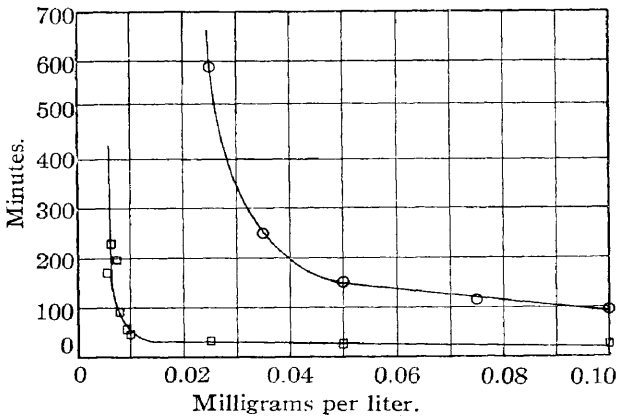


Fig. 3.—Curves showing toxicity of croton resin and rotenone: □, croton resin; ⊙, rotenone.

For removal of solvent from the resin before analysis or toxicity studies, the following procedure was adopted. The sample was placed in a platinum boat in an Abderhalden drier heated by boiling toluene. Air was alternately removed and admitted until no more bubbles formed in the resin when the pressure was reduced (water pump). To test the efficiency of this method the following experiment was performed. About 2 g. of resin was dissolved in 150 ml. of pure distilled chloroform and most of the solvent was removed on the steam-bath. The sample was

(7) Gersdorff, THIS JOURNAL, **52**, 3440 (1930).

(8) The average weight of each fish was about 2 g. Four fish were used to determine the average survival times on the straight portions of the curves while eight to ten fish were used on the curved portions.

(9) Drake and Spies, *Ind. Eng. Chem., Anal. Ed.*, **5**, 284 (1933).

then treated as just described. After two hours halogen could just barely be detected in the sample and finally when no more bubbles could be raised in the resin no appreciable Beilstein test could be obtained.

The physical characteristics of the resin are similar to those described by Cherbuliez. It showed no tendency to crystallize. The resin is very sparingly soluble in water, slightly soluble in petroleum ether and miscible in all proportions with alcohol, benzene, ethyl acetate and chloroform. It contains no nitrogen. *Anal.* Found: C, 68.6, 68.3; H, 8.69, 8.84; mol. wt., 600;[10] Hanus iodine no. 52.5, 53.6; OCH_3, 1.13, 1.23, 1.19;[11] sap. no. 254.5, 233.2.

Preparation of Croton Oil.—Fifty-one grams of ground croton beans was extracted in a Soxhlet with petroleum ether (max. b. p. 85°) for about nine hours. The petroleum ether was evaporated from the extract and the oil dried to constant weight at 105°; yield 12.9 g. (25.3%). A further extraction with the same solvent for about six hours removed only 0.07 g. of oil of sap. no. 265.8.

(10) (a) Rast, *Ber.*, **54**, 1979 (1921); (b) Spies, THIS JOURNAL, **55**, 250 (1933).

(11) Clark, *J. Assoc. Off. Agr. Chem.*, **15**, 136 (1932).

Preparation of Alcohol-Soluble Portion of Croton Oil.—One hundred grams of croton oil (obtained by petroleum ether extraction) was shaken in the cold with 150 ml. of alcohol (95%). The alcoholic layer was withdrawn and the oil was then extracted with a further 100-ml. portion. The combined alcoholic extracts were filtered, centrifuged to remove suspended particles of oil and finally the alcohol was evaporated; yield 31 g. (31%).

Summary

1. Croton resin has been separated into fractions which have different compositions and possess different toxicities to goldfish.

2. Complete survival time–concentration curves, using goldfish as the test organism, have been determined for croton oil, the alcohol-soluble portion of croton oil and croton resin.

3. Croton resin has been shown to be more toxic to goldfish than rotenone.

COLLEGE PARK, MD. RECEIVED OCTOBER 26, 1934

[Reprint from the Journal of the American Chemical Society, 57, 182 (1935).]

Croton Resin. II. The Toxic and Vesicant Action of Certain of its Derivatives

By Joseph R. Spies

[Reprint from the Journal of the American Chemical Society, 57, 182 (1935).]

[Contribution from the University of Maryland Station of the Insecticide Division, Bureau of Chemistry and Soils]

Croton Resin. II. The Toxic and Vesicant Action of Certain of its Derivatives[1]

By Joseph R. Spies

The work of previous investigators has indicated that the croton bean contains a physiologically active principle which owes its toxic and vesicant properties to a condition of unsaturation. A more extensive study, using goldfish as the test organism, has demonstrated, however, that the physiological action of the active constituent is more intimately related to the presence of free hydroxyl groups.

Croton oil was first hydrogenated by Paal and Roth,[2] who used a palladium catalyst and found the irritating property of the oil to be proportional to the iodine number. This property disappeared entire y when saturation was complete. These authors also hydrogenated Boehm's[3] resin, causing a drop in its iodine number from 77 to 12.5. The product was no longer toxic to frogs or rabbits. Catalytic hydrogenation of our croton resin,[4] with both nickel and platinum, caused a reduction of the iodine number from 53 to 38 but no apparent decrease in toxic or vesicant action at the concentration used for the tests. The product, a harder resin, had lost its transparency and assumed a turbid or milky appearance.

Cherbuliez *et al.*[5] brominated the resin and found the product to be without physiological activity, as shown by tasting. Bromination of our resin produced a marked decrease in toxic and vesicant properties but did not destroy them completely.

The important relation of the free hydroxyl groups to the physiological activity of croton resin was not observed by earlier investigators. Boehm[3] reported the absence of free hydroxyl groups in his resin, while Cherbuliez *et al.*[5] found that their resin contained approximately 3.4% hydroxyl on the basis of the saponification number before and after acetylation. The latter authors noted a decrease in activity of the acetylated product but did not attribute this to esterification of the hydroxyl groups. Acetylation of our resin, which, however, resulted in only partial esterification of the hydroxyl groups shown

(1) From a thesis submitted by Joseph R. Spies to the Graduate School of the University of Maryland in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(2) Paal and Roth, *Ber.*, **42**, 1544 (1909).

(3) Boehm, *Arch. Path. Pharmakol.*, **79**, 138 (1915). This resin was obtained by a process which altered its nature. Despite this fact its composition, physical characteristics and toxic action are similar to the resin used in this study. At a concentration of $1:10^8$ Boehm's material killed tadpoles in three to four hours.

(4) Isolated as described in the first article of this series. This Journal, **57**, 180 (1935).

(5) Cherbuliez, Ehninger and Bernhard, *Helv. Chim. Acta*, **15**, 658 (1932).

to be present by methylation by Purdie's reagents, produced some decrease in physiological action. Complete methylation to the extent of 11.7% methoxyl, however, yielded a resin which is without either toxic or vesicant action. The average methoxyl content of the original resin is 1.2%. Detailed results of these studies are collected in Tables I and II.

TABLE I

THE TOXICITY OF CERTAIN DERIVATIVES OF CROTON RESIN TO GOLDFISH

Temperature 27 ± 0.3°; concn. 2.0 mg./liter[a]

Substance		OCH_3, %	Sap. no.	Iodine no.	Survival time, min.[b]
Original resin		1.2	244	53.1	20
Hydrogenated resin		..	...	39.2	18
Acetylated resin	(1)[d]	..	358	..	35
Acetylated resin	(2)	..	336	..	30
Methylated resin		11.7	181	..	no deaths
Methylated acetylated resin	(3)	..	183	..	in 1740[c]
Bromo resin	(1)	..	...	..	62
Bromo resin	(2)	..	...	..	71

[a] This relatively high concentration was used to reduce the survival times to convenient limits. [b] Average of four fishes. See this series, article I, *loc. cit.* [c] A few fishes showed slight irritation of the gills. [d] These numbers refer to the number of the preparation in the experimental section.

TABLE II

VESICANT ACTION OF CROTON RESIN AND CERTAIN OF ITS DERIVATIVES

Substance		Concn. of test soln.	Mg./ sq. in. of skin[a]	Solvent used	Individuals tested	Results
Original resin		1:1000	0.04	Alcohol	1	Moderate burn
Original resin		1:100	.4	Alcohol	1	Serious burn
Hydrogenated resin	[b]	1:100	.4	Alcohol	1	Serious burn
Methylated resin		1:100	.4	Acetone	2	No burn
Acetylated resin	(1)[c]	1:100	.4	Acetone	2	Moderate burn
Bromo resin	(1)	1:100	.4	Acetone	2	Very slight burn

[a] Approximate. [b] Iodine number 39.2. [c] These numbers refer to the number of the preparation in the experimental section.

Croton resin forms no water-soluble salt when its ethereal solution is agitated with cold dilute aqueous potassium hydroxide and its alcoholic solution gives no color with ferric chloride. It is probable nevertheless that the free hydroxyl groups are phenolic or enolic in nature but are masked by substituents which repress their normal reactions. This conclusion is based on the fact that the average saponification number of the methylated resin is 181, while that of the original resin is 244, indicating that part of the alkali was neutralized by the free hydroxyls after saponification. The saponification number would otherwise be lowered only in the ratio of the molecular weights of the original and methylated resin, *i. e.*, in the ratio 600:628.

Experimental

Hydrogenation.—In a preliminary experiment 16.4 g. of resin was dissolved in 200 ml. of alcohol and the solution was shaken for five hours with hydrogen and 0.2 g. platinum oxide[6] at 50–60 lbs. per sq. in. This process was repeated twice using fresh portions of catalyst at 45–55° for one and three hours, respectively. The hydrogenated product, which was still toxic[7] and vesicant, was harder than the starting resin and possessed a turbid or milky appearance, iodine number 39.2.[8]

To 18.5 g. of resin in 110 ml. of 90% methanol, 3 g. of nickel catalyst (Raney)[9] was added. This solution was shaken for twenty-four hours with hydrogen at 50–60 lb. per sq. in. at 40–50° and the process was repeated three times with fresh portions of nickel. The resin was recovered and dried as usual, iodine no. (Hanus) 38.8, 36.2. In an attempt to lower further the iodine number this sample was subjected to further hydrogenation in alcoholic solution to which 0.5 g of platinum oxide was added. The solution was shaken for five hours with hydrogen at 55–60 lb. at 48–58°. The product, however, showed no decrease in iodine number.

Bromination. 1.—To 1 g. of resin dissolved in 15 ml. of *cold* glacial acetic acid bromine was added drop by drop until an excess was present. Liberation of hydrobromic acid showed that some substitution occurred. After half an hour the solution was poured into dilute sodium bisulfite. The bromide, which precipitated in white flocs, was filtered off and again stirred up with fresh bisulfite solution, filtered, and washed with water on the filter until the washings were neutral. The precipitate was pressed on a porous plate and dried in a vacuum desiccator over phosphorus pentoxide. The material was amorphous and possessed a light yellow or orange color.

Anal. Found: Br, 35.67, 35.14;[10] C, 45.56, 45.50; H, 5.69, 5.55.

2.—One gram of resin was dissolved, with *gentle warming* to hasten solution, in glacial acetic acid, the solution was cooled and treated with bromine as before. More substitution seemed to occur and the product was much darker in appearance. This material was less toxic than the preceding preparation.

Acetylation. 1.—One gram of resin was dissolved in 25 ml. of acetic anhydride, 2 g. of anhydrous sodium acetate was added and the solution was refluxed for one hour, after which the excess acetic anhydride was removed by distillation under diminished pressure on the steam-bath. The residue was dissolved in ether and the solution washed with water until neutral. The solution was dried over anhydrous sodium sulfate, and the resin recovered by

(6) Adams, Voorhees and Shriner, "Organic Syntheses," Coll., Vol. I, 1932, p. 452.

(7) Samples of non-crystalline derivatives were prepared for analysis and toxicity tests as described in the first article of this series, THIS JOURNAL, **57**, 180 (1935).

(8) Gattermann and Wieland, "Laboratory Methods of Organic Chemistry," 1932, p. 142.

(9) Covert and Adkins, THIS JOURNAL **54**, 4116 (1932).

(10) The author is indebted to Mr. S. A. Shrader of the University of Maryland for the micro bromine analyses.

evaporation of the ether. The product was darker in appearance than the starting material, sap. no. 355.3, 360.9.[11]

2.—Four grams of resin was dissolved in 10 ml. of dry pyridine, the solution was cooled and 10 ml. of distilled acetic anhydride was added slowly. After thirty-six hours the solution was poured into water and allowed to stand for one-half hour to ensure hydrolysis of the excess acetic anhydride. The aqueous suspension was extracted with ether and the ether solution washed with dilute hydrochloric acid and then with water until neutral. It was dried over anhydrous sodium sulfate and the resin recovered as before, sap. no. 341.6, 331.1.

3.—Six grams of methylated resin (OCH_3 11.7%) was acetylated with acetic anhydride in pyridine as described under 2, sap. no. 176.9, 189.4.

Methylation.—Ten grams of resin was dried by solution in benzene followed by distillation of the solvent. Fifty ml. of methyl iodide and 7 g. of dry silver oxide were added and the flask was connected to a reflux condenser closed with a calcium chloride tube. The mixture was refluxed for twenty-one hours over a small flame so that vigorous boiling and consequent thorough agitation of the silver oxide occurred. Five ml. of the solution was withdrawn to obtain a sample for analysis. This solution was boiled up twice with acetone to remove all trace of methyl iodide and the resin was recovered and dried as before. *Anal.* OCH_3, 10.41, 10.61.[12]

The methyl iodide was distilled from the remainder of the methylated product, which was then dried with benzene as before. The methylation process was repeated with twenty hours of refluxing. *Anal.* OCH_3, 11.45, 11.85.

This is believed to represent the maximum amount of methylation attainable by this method because, when another sample with a methoxyl content of 10.6% was refluxed as before for two hundred and fifty-eight hours, the methoxyl content increased only to 11.3% The product gave no evidence of having been decomposed as a result of this prolonged treatment.

Vesicant Tests.—The tests for vesicant action were carried out in the following manner: 3 drops from a pipet (calibrated 75 drops/ml.) containing the test solution were placed on the skin and allowed to spread over an area of about 6.5 sq. cm. Each drop was allowed to dry before the following one was applied. The treated area was left exposed to the air and in cases of positive reaction redness, swelling and vesication resulted in from eight to ten hours, probably reaching a maximum within twenty-four hours. The pustulation caused by slight burns disappeared within a few days but sometimes persisted for several weeks in more severe cases, as was observed in some minor accidents that occurred during the course of extraction of the resin.

Summary

A study of the toxicity to goldfish and the vesicant action of croton resin and certain of its derivatives has shown that the free hydroxyl groups, probably enolic or phenolic, are more intimately related to maximum physiological activity than is the condition of unsaturation.

COLLEGE PARK, MD. RECEIVED OCTOBER 26, 1934

(11) Titration of the unused alkali was carried out in a volume of about 500 ml.; this large volume was necessary in order to distinguish the end-point, as the saponification products produced a characteristic dark discoloration which tended to obscure the end-point.

(12) Clark, *J. Assoc. Off. Agr. Chem.*, **15**, 136 (1932).

[Reprint from the Journal of the American Chemical Society, 57, 184 (1935).]

Croton Resin. III. The Combined Acids

By Nathan L. Drake and Joseph R. Spies

[Reprint from the Journal of the American Chemical Society, 57, 184 (1935).]

[CONTRIBUTION FROM THE UNIVERSITY OF MARYLAND STATION OF THE INSECTICIDE DIVISION, BUREAU OF CHEMISTRY AND SOILS]

Croton Resin. III. The Combined Acids[1]

BY NATHAN L. DRAKE AND JOSEPH R. SPIES

Saponification of the complex mixture known as "croton resin" has been the subject of several investigations. Dunstan and Boole[2] noted the loss of vesicant action and the formation of acids resulting from treatment of the resin with boiling potassium hydroxide. Later Boehm[3] studied the action of 30% aqueous potassium hydroxide at room temperature on a "croton resin," and found that a dark brown solution resulted. Acidification produced a mixture of acids in a yield of 35%, among which formic and acetic acids were identified qualitatively, and isobutyric and tiglic acids by actual isolation.[4] Cherbuliez *et al.*[5] also recognized that the resin contained esters, but did not study the products of their hydrolysis.

The present paper is a report of a study of the saponification products of the resin, and in particular of the acids liberated. The presence of tiglic, caprylic, capric, lauric, myristic, palmitic, oleic and linoleic acids has been demonstrated conclusively, as well as the absence of any ap-

(1) From the Ph.D. dissertation of Joseph R. Spies. Presented at the 88th meeting of the American Chemical Society held at Cleveland, Ohio, September, 1934. Parts I and II, THIS JOURNAL, **57**, 180, 182 (1935).

(2) Dunstan and Boole, *Proc. Roy. Soc.* (London), **58**, 238 (1895).

(3) Boehm, *Arch. Path. Pharmakol.*, **79**, 138 (1915).

(4) It should be noted that the resin used by Boehm in his early work was not prepared in the same manner as the material employed here. Our "croton resin" was prepared by the method described in the first article of this series, Spies, THIS JOURNAL, **57**, 180 (1935).

(5) Cherbuliez, Ehninger and Bernhard, *Helv. Chim. Acta*, **15**, 658 (1932).

preciable quantity of unsaturated acids containing three or more double bonds. Furthermore, absence of any large quantity of stearic or other higher saturated acid has been shown by means of the lead salt–ether method applied to the residue after removal of the more volatile fractions from the mixture of methyl esters of all the acids.

Experimental

Saponification of Croton Resin.—30.4 g. of resin was heated under reflux for one and one-half hours with 125 ml. of 1.6 *N* alcoholic potassium hydroxide in an atmosphere of nitrogen.[6] After removal of the alcohol (nitrogen atmosphere) under diminished pressure, a dark-colored residue remained which dissolved completely in 200 ml. of distilled water. Evaporation of a dried ether extract of this alkaline solution yielded 0.5 g. of brown resinous material. This substance gave no Liebermann–Burchard test for sterols.

The alkaline solution was made acid to congo red with dilute hydrochloric acid, causing the separation of a dark-colored material with a pronounced fatty acid odor. Thorough extraction of the acidified mixture with petroleum ether (b. p. 50–60°) removed a part of the substances thrown out by the acid, but left a large lump of dark-brown gum. The latter was separated mechanically, washed several times with petroleum ether and finally with water. It was then dissolved in methyl alcohol, the solution dried over sodium sulfate and filtered, and the alcohol evaporated, leaving a residue which was heated under nitrogen on the steam-bath to constant weight (11.6 g.).

The combined petroleum ether extracts were washed once with water, dried, separated from drying agent and evaporated until all solvent was removed. There remained 9.6 g. of a brown liquid mixture of acids.

The aqueous solution remaining from the petroleum ether extraction process was a golden yellow. It gave a very persistent deep purple color with ferric chloride solution. The solution was made slightly alkaline to litmus and the water removed by distillation under reduced pressure in a current of nitrogen. As the solution became more concentrated a small quantity of dark gummy matter separated. Such material was removed on two occasions during the evaporation. When all the water had been removed, there remained a golden-yellow residue consisting of the water-soluble saponification products and some potassium chloride. To remove the major part of the inorganic salts the residue was treated with absolute alcohol, filtered and the alcoholic solution evaporated to dryness under reduced pressure in a current of nitrogen. The light-brown amorphous residue so obtained was readily soluble in water or alcohol but not in ether (8.7 g.). Warming an acid (HCl) aqueous solution of this substance caused some change in the material which gives the color test with ferric chloride, for attempts to isolate the organic material from its alkali salt by acidification (litmus) and evaporation of the acid solution resulted in a product which was much darker in color and would no longer give the ferric chloride color test. This water-soluble hydrolysis product gave a negative test for glycerol. Further work on the identification of this material is in progress.

Lead Salt–Ether Separation of the Liquid and Solid Acids.—The method described by Jamieson[7] was used. The results are given in the summary:

Mixed acids, g.	3.3	3.4
KOH to neut., milli-equiv.	17.1	17.5
Solid acids, g.	0.94	0.92
Liquid acids, g.	1.53	1.74

The presence of a considerable quantity of saturated acids of low molecular weight gives these figures little quantitative significance. The lead salts obtained, however, were completely soluble in ether at room temperature (30–33°), thus indicating the absence of any appreciable quantity of the higher saturated fatty acids.

The Bromides of the Unsaturated Acids.—To 5.3 g. of unsaturated fatty acids, obtained by a lead salt–ether separation and dissolved in 80 ml. of ether containing 4 ml. of glacial acetic acid, bromine was added at 0–5°, drop by drop, until an excess was present. The solution was allowed to stand for three hours at this temperature. Lack of any precipitate indicated the absence of acids containing three or more double bonds. The ethereal solution was washed with dilute sodium bisulfite to remove any excess bromine and then with saturated sodium chloride solution (to prevent emulsification) until neutral to litmus. The dried ethereal solution was evaporated, the residue taken up in 20 ml. of petroleum ether (b. p. 50–70°), and filtered to remove a small quantity of insoluble tarry material. Concentration to about 10 ml. and cooling caused separation of the tetrabromide of linoleic acid. Owing to the presence of the dibromide of oleic acid and some saturated fatty acids, separation of the tetrabromide was slow. After careful purification by recrystallization from petroleum ether, the tetrabromide melted from 113.5–114.5°.[8] In another experiment 3.64 g. of mixed acids yielded 4.66 g. of bromides, from which 1.55 g. of crude tetrabromide was obtained.

Anal. Calcd. for $C_{18}H_{32}O_2Br_4$: C, 36.00; H, 5.38; Br, 53.29. Found: C, 36.11, 36.30; H, 5.59, 5.33; Br, 53.36.[9]

The absence of Matthes and Boltze's[10] so-called liquid tetrabromide of linoleic acid, which is soluble in petroleum ether, was demonstrated in the experiment described above by dissolving the residue after removal of the petroleum ether in a small quantity of methanol and allowing this solution to stand for two and one-half days in the ice box. No crystals were formed.

Permanganate Oxidation of the Unsaturated Acids.—The method of Lapworth and Mottram[11] was used to show the presence of oleic acid and to confirm the presence of linoleic acid; 5.1 g. of the fatty acid mixture was used in the experiment, and 2.4 g. of crude polyhydroxystearic

(6) In alkaline solution a characteristic brown color is produced which appears to be equally intense whether air or nitrogen forms the atmosphere above the solution.

(7) Jamieson, *J. Assoc. Off. Agr. Chem.*, **11**, 303 (1928).

(8) All melting points were taken with standardized Anschütz thermometers.

(9) We wish to thank R. P. Jacobsen for the bromine determination, and S. A. Shrader for the carbon and hydrogen analysis.

(10) Matthes and Boltze, *Arch. Pharm.*, **250**, 225 (1912).

(11) Lapworth and Mottram, *J. Chem. Soc.*, **127**, 1629 (1925).

acids was obtained. This material was treated with 100–150 ml. of petroleum ether (50–70°) to remove the saturated acids and any unoxidized acids; 0.8 g. of product remained undissolved, and 1.6 g. dissolved. The dried mixture of di- and tetrahydroxystearic acids (0.80 g.) was shaken with 200 ml. of ether, and the solution was decanted from the residue. Concentration of the ethereal solution resulted in the separation of crystals which melted at 98°. Two further recrystallizations from ether yielded a product which shrunk at 117° and melted sharply at 123.5°. Three more recrystallizations from ethyl acetate resulted in a product which shrunk at 119–120° and melted sharply at 123.5–124°.[12] Analysis showed this substance to be a dihydroxystearic acid.

rated with dry hydrogen chloride at room temperature. After saturation the mixture was allowed to stand for one hour and then poured into 1685 ml. (5 vol.) of cold water. The esters were removed by ether extraction and isolated in the usual way, yield, 30.6 g.

Fractional Distillation of the Methyl Esters.—An apparatus similar to that described by Podbielniak[14] was used. The rate of distillation at no time exceeded 0.1 ml./min., and was much less than this between pure fractions; Table I gives the details. The temperatures recorded at the top of the fractionating column were considerably lower than the recorded boiling points at 15 mm. for the corresponding esters. The very slow rate of distillation is responsible.

TABLE I

DISTILLATION DATA AND CHARACTERISTICS OF THE ESTERS

Vol. of esters, 34.1 ml. Pressure (start), 15.1 mm. Pressure (finish), 13.1 mm. Time, 6 hrs. Total distillate, 19.8 ml.

Fraction	Vol., ml.	n_D^{20}	d^{20} g./cc.	Mol. wt. of ester from saponification equiv. Found		Calcd.	M. p. of *p*-toluidide of acid, °C. Found	Recorded[a]	Acid in *p*-toluidide
I	1.6	1.4351	0.9482	116.9		114	70–71.5	..	Tiglic
Ia[b]	0.3	....	...	...		...	.....	..	.....
II	0.9	1.4253	.8946	155.9	155.8	158	67.5–68.8	70	Caprylic
IIa	0.4	....	...	...		...	.....	..	.....
III	6.0	1.4269	.8762	186.3	185.1	186	76.5–77.2	78	Capric
IIIa	0.9	....	...	...		...	.....	..	.....
IV	2.9	1.4338	.8767	214.7	215.2	214	82.5–83.2	87	Lauric
IVa	0.9	....	...	...		...	.....	..	.....
V	2.6	1.4387	.8741	234.4	239.7	242	89–90.2	93	Myristic
Va	0.8	1.4512	.8804	278.3		270	95–96	98	Palmitic
VI[c]	1.5	1.4581	.8862	...		...	.....	..	.....

[a] The melting points recorded in the literature vary widely. The values given are those of Robertson, *J. Chem. Soc.*, **115**, 1211 (1919). [b] The fractions labeled a are mixtures between purer fractions. [c] The distillation was not carried farther because the boiling points of the methyl esters of stearic, oleic and linoleic acids are so close that separation is impossible.

Anal. Calcd. for $C_{18}H_{36}O_4$: C, 68.29; H, 11.44. Found: C, 68.50, 68.25; H, 11.88, 11.69.

The ether-insoluble residue from the experiment described above was recrystallized from water and dried. It softened at 143° and melted at 155–156°. After a lengthy purification by crystallization[13] from alcohol, water and again from alcohol, the melting point of the acid was raised to 167–168° with previous softening at 156–157°. Further crystallization from alcohol failed to raise the melting point. Analysis demonstrated this compound to be a tetrahydroxystearic acid.

Anal. Calcd. for $C_{18}H_{36}O_6$: C, 62.02; H, 10.42. Found: C, 62.03, 61.80; H, 10.65, 10.56.

Preparation of the Methyl Esters of the Mixed Acids.—33.7 g. of the mixed acids above described was dissolved in 337 ml. of absolute methanol, and the solution was satu-

Isolation of Tiglic Acid.—0.3 g. of Fraction 1 was saponified with alcoholic potassium hydroxide. After acidification the organic acid was extracted with ether and isolated by evaporation of the ether. The crude tiglic acid was crystallized from petroleum ether and finally sublimed at 20 mm. The purified product melted at 63.5°.[15]

Anal. Calcd. for $C_5H_8O_2$: C, 60.00; H, 8.00. Found: C, 59.76; H, 7.90.

Preparation of the *p*-Toluidides.—*p*-Toluidides were prepared directly from the methyl esters by the method of Koelsch and Tenenbaum.[16] All the products were crystallized from aqueous alcohol and finally from methanol to constant melting point before analysis. Table II lists the analyses of the various *p*-toluidides; the related acids are tiglic, caprylic, capric, lauric, myristic and palmitic, respectively.

Search for Other Saturated Fatty Acids.—Stearic acid or any higher homolog would have escaped detection in the experiments so far described; 23 ml. of mixed methyl esters were therefore subjected to fractional distillation in the Podbielniak apparatus, and the high boiling residue, which should contain any methyl stearate present origi-

(12) Because of the danger of lactone formation, this compound was dried in a vacuum at room temperature. Melting point capillaries were introduced into the bath only when the latter was near the melting point of the substance. A large number of isomeric dihydroxystearic acids are possible; *cf.* Lewkowitsch, "Chemical Technology and Analysis of Oils, Fats and Waxes," 6th ed., Vol. I, pp. 233–235.

(13) Rollett, *Z. physiol. Chem.*, **62**, 420 (1909), has discussed the purification of the tetrahydroxystearic acid resulting from linoleic acid on oxidation. His method was used in this instance.

(14) Podbielniak, *Ind. Eng. Chem., Anal. Ed.*, **5**, 135 (1933).

(15) The value 64.5° is given in Beilstein, 3d ed., Vol. I, p. 513.

(16) Koelsch and Tenenbaum, THIS JOURNAL, **55**, 3049 (1933).

TABLE II

THE ANALYSIS OF THE *p*-TOLUIDIDES

No.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
I	$C_{12}H_{15}ON$	76.19	76.03	7.94	8.09
			76.16		8.13
II	$C_{15}H_{23}ON$	77.19	76.86	9.94	9.98
III	$C_{17}H_{27}ON$	78.10	78.04	10.42	10.53
IV	$C_{19}H_{31}ON$	78.81	79.09	10.80	10.92
V	$C_{21}H_{35}ON$	79.42	79.10	11.12	11.19
VI	$C_{23}H_{39}ON$	79.93	79.45	11.38	11.36

nally, was saponified with alcoholic potassium hydroxide. The lead salt–ether method was then employed to separate any stearic acid from the liquid unsaturated acids. Only a small quantity of insoluble lead salt was obtained, from which 0.5 g. of free acid was isolated. This free acid was practically completely soluble in *cold* methanol; the higher saturated fatty acids are insoluble in methanol. It is unlikely, therefore, that any considerable quantity of stearic acid or higher homolog is combined in the original resin.

The Saponification Equivalents of the Methyl Esters.—The method of Chargaff[17] was used. Since no *n*-propyl alcohol was available, isobutyl alcohol was used instead; 25 to 50 mg. samples were used for each determination. The results are given in Table I.

Summary

1. The saponification of croton resin has been studied.

2. The petroleum ether–soluble fatty acids have been shown to comprise approximately 32% of the saponification products.

3. Tiglic, caprylic, capric, lauric, myristic, palmitic, oleic and linoleic acids have been shown to be present in the mixed acids obtained by saponification.

(17) Chargaff, *Z. physiol. Chem.*, **199**, 221 (1931).

COLLEGE PARK, MD. RECEIVED OCTOBER 26, 1934