A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS

THE IDENTIFICATION OF THE ACTIVE FACTOR IN THE LOW-MELTING FRACTION OBTAINED FROM CANE JUICE

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INTRODUCTION

Wulzen and Bahrs (3, 27, 28) during investigations of the nutritional requirements of planarian worms found that guinea pigs developed degenerative changes when the animals were fed grain diets lacking in green feeds, but including the necessary vitamins. Animals, raised on this diet, developed a stiffness in the wrist joints over a period of 4-8 weeks. The stiffness increased during the syndrome until it was no longer possible to bend the wrist joint.

Autopsy showed that the stiffening of the wrist joints was not the only effect of the deficient diet. The muscles were severely atrophied, with closely packed, fine, white lines of calcium phosphate deposits running parallel to the muscle fibers. Frequently, lumps of calcium phosphate were deposited under the skin, in the joint regions, between the ribs, and in many body organs, i.e., heart and aorta.

From physiological studies it would appear that the antistiffness factor has a regulatory effect upon the phosphorus metabolism. One of the most prominent

changes found was a sharp decrease in the easily hydrolyzable phosphorus fraction in the liver and kidney during the deficiency (24). This fraction responded immediately to the administration of the factor to the affected animals, in that the values returned to normal after a short-time treatment. Similar changes were observed in the concentration of the acid soluble phosphorus in the muscle (25).

CHAPTER II

THE STIFFNESS TEST

The activity tests of all of the fractions used and separated in this work were done by Dr. R. Wulzen. The assay for the antistiffness factor was performed on animals which had been deficient for at least a month. The test was carried out in the following way:

The foreleg of the guinea pig on the opposite side from the experimenter was extended posteriorly, close to the body wall of the animal, by pressing the thumb on the olecranon process and at the same time supporting the proximal and distal portions of the leg with the fingers. The leg should be as straight as possible. The disengaged hand of the operator was then used to superextend the foot gently by pressing upward on its medial aspect. The foot of a normal animal would bend easily until it formed a right angle with the leg. The nutritionally deficient animals were very sensitive towards the treatment and manifested pain at once when the foot was forced beyond the point of easy bending. This stiffness disappeared if active fractions were administered to the animals. The results are recorded in terms of arbitrary figures. A normal joint is designated as 4, a completely rigid joint as 1. Intermediate conditions are indicated

by such symbols as: 1+, 2-, 2, 2+, 3-, 3, 3+, 4-, 4. The superscript as in 4^{p}, indicates that although normal mobility has been regained, the joint is still painful under manipulation. The fractions were dissolved in cottonseed oil. In order to express activities in a quantitative way, one unit was arbitrarily defined as: a solution of an active fraction in cottonseed oil which, when 1 cc. is administered daily for five consecutive days to a sick animal, cures the animal.

The determination of the easily hydrolyzable phosphorus in the liver has occasionally been used as a method of assay (26).

It was found earlier (9) that the degenerative changes due to the lack of the antistiffness factor in the diet could be alleviated by feeding the affected animals one gram of raw cream per day, for five days. However, if the cream were heated under oxygen, the curative factor was destroyed. Cream, which was heated in the presence of nitrogen, showed no loss of activity. Gouley (9) claimed methyl vinyl ketone as the active factor present in raw cream. van Wagtendonk and Wulzen (23) were unable to confirm the presence of methyl vinyl ketone in raw cream. Synthetic methyl vinyl ketone in dosages of 5 mg. per day for six consecutive days cured the guinea pigs, but it also had at the same time, very toxic effects.

CHAPTER III

PREVIOUS METHODS OF ISOLATION

R. W. Gouley (9) was the first to attempt the isolation of the antistiffness factor from raw cream. The procedure was as follows: The raw cream was churned, and the butter therefrom, was saponified by refluxing with 30% alcoholic KOH for three and one-half hours under nitrogen. The mixture was cooled and neutralized with dilute sulfuric acid. The solution was then cooled for twenty-four hours at 0°C. The solid fatty acids were filtered off, and washed with cold water. The solid fatty acid fraction was steam distilled for eight and one-half hours under nitrogen. A small amount of highly volatile and very active material was collected from the steam distillation in a trap cooled by a dry ice-acetone mixture. However, not sufficient quantities were collected to characterize the material. The oily layer which had steam distilled was qualitatively tested for alcohols, aldehydes, and ketones.

The tests for aldehydes and alcohols were negative, but a slight amount of bromine was absorbed, and a slight pink coloration appeared on long standing with Schiff's reagent. A crystalline product was obtained on reaction with semicarbazide hydrochloride. This

crystalline material had the same melting point as that reported by Shriner and Fuson (22) for methyl vinyl ketone.

The procedure used by van Wagtendonk and Wulzen (23) to isolate the antistiffness factor from raw cream was similar in some respects to the above isolation. Their procedure is summarized below:

The raw cream was churned and the resulting butter washed twice with water, and pressed free of wash-liquid. The butter was added to a boiling 20% solution of alcoholic KOH and refluxed for four hours under an atmosphere of nitrogen. The mixture was cooled and acidified with 5% sulfuric acid. The fatty acids were separated and washed with water until free of sulfuric acid. The fatty acids were then steam distilled for twelve hours in an atmosphere of nitrogen, and the steam-distillate was extracted with peroxide-free ether. The ether extract was treated with 5% KOH until the water layer remained clear on acidifying. The ether layer was washed with water until alkali-free, and dried over anhydrous sodium sulfate. The ether was removed by distillation under nitrogen, leaving a yellow oil. The oil was dissolved in a solution of glacial acetic acid and absolute ethanol (9:1), and refluxed with trimethyl acethydrazide ammonium chloride for seven hours (7). The mixture was neutralized

to a pH between 6.5 and 7.0 with 1.0 N sodium hydroxide. The resulting solution was continuously extracted with ether for 36 hours, at which time the extraction was stopped and the ether layer removed. Since this layer did not contain any active compound, it was discarded. The aqueous layer was acidified with 1.0 N sulfuric acid, and again extracted with ether for 24 hours. The ether layer was removed and the ether distilled off under nitrogen, again leaving a yellow oil. The mercuric iodide complex of the trimethyl acethydrazide ammonium chloride derivative was prepared according to Hughes (10). The molecular weight in camphor was determined to be 820, which would give the active factor a molecular weight of 200. The mercuric iodide complex was dissolved in 1.0 N sulfuric acid, and treated with hydrogen sulfide. The solution was freed of hydrogen sulfide, and extracted with ether. The ether layer was dried and the ether removed in vacuo, leaving a pale yellow oil, which was active in a dosage of 0.1 gamma. The antistiffness factor was thus believed to be a compound with a molecular weight of about 200, and to contain a carbonyl group.

All fractions were tested for activity by Dr. R. Wulzen according to the procedure described in Chapter II.

CHAPTER IV

THE ISOLATION FROM CANE JUICE

Since only very small amounts of the antistiffness factor were obtained from raw cream, other sources of raw material were tested for the active factor. van Wagtendonk and Wulzen (26) found that crude cane molasses and crude unheated cane juice were considerably more active, the former ten times, and the latter 100-1000 times as active as the raw cream.

This discovery led to the isolation of the anti-stiffness factor from crude cane molasses and crude unheated cane juice on a semi-pilot plant scale.

The crude cane juice was extracted at room temperature with peroxide-free ethyl ether in a semi-continuous extraction apparatus. To lessen emulsification, the cane juice was passed through a column of ethyl ether. The column was filled with wooden blocks, 2x2x2 cm., which had been soaked in the cane juice. The cane juice was passed through the ether column six times, and the emulsion was centrifuged to recover the ether extract. The operation was repeated three times with fresh ether. The entire ether extract was washed with water, dried over anhydrous sodium sulfate and concentrated under nitrogen.

The resulting wax was dissolved in petroleum ether,

extracted four or five times with 90% methanol. The petroleum ether solution was dried over anhydrous sodium sulfate and concentrated under nitrogen, yielding a green wax. The green wax was dissolved in a 90% petroleum ether-benzene mixture, and adsorptive magnesium oxide (Adsorptive powdered magnesia #2641, California Chemical Company, Newark, California) was added. The mixture was thoroughly shaken and centrifuged. The pale yellow supernatant was concentrated under nitrogen.

The wax was then sublimed in a simple pot still. The condensing surface was cooled with a dry ice acetone mixture. The normal operating pressure was 0.1 micron. By varying the bath temperature of the still, some fractionation was possible. At a bath temperature of 70° C., a yellow inactive oil condensed. When the temperature of the bath was raised to 140-170° C., the active material condensed along with some yellow oil.

The wax that had sublimed at 140-170° was dissolved in benzene and nine volumes of 95% ethanol were added. The solution was cooled overnight in a refrigerator at 5° C., the precipitate filtered, washed with cold 95% ethanol, and dried over phosphorus pentoxide in a vacuum desiccator. This process removed the major part of the oil. The material was then resublimed in a molecular still. The condensate, which was only slightly

colored, was recrystallized from purified petroleum ether[1]. After ten to fourteen crystallizations, the material crystallized in pure white leaflets, with a melting point from 81.5-82.0° C.

The compound showed curative powers when fed to affected animals in a dosage of 0.01 gamma per day, for five days, Table I.

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1 Two liters of Skellysolve H were shaken five times with 100 cc. of fuming sulfuric acid, washed acid-free with distilled water. Dried over anhydrous sodium sulfate, and distilled over sodium. The fraction boiling from 62-78° C. was collected and used for the recrystallization procedure.

CHAPTER V

EXPERIMENTAL

A uniform product had apparently been isolated in the manner previously described. However, carbon-hydrogen determinations of different batches of the product did not correlate. When larger amounts of the material became available, it was apparent that the isolated substance represented a mixture. Therefore, several attempts were made to separate the components by chromatographic adsorption, after two or three recrystallizations from petroleum ether of the condensate from the molecular still.

a. Adsorption of the crude material on magnesia and alumina.

The column was prepared by pouring small portions of a mixture of celite (Johns-Mansville) and magnesium oxide (Adsorptive powdered magnesia, #2641, California Chemical Company, Newark, California) (1:5) into a 20 mm. tube until the column was 700 mm. deep. A slight vacuum was drawn on the tube and each portion was tamped lightly after its addition to the tube.

The column was wetted with petroleum ether, and a solution of 0.843 g. of the crude material in 100 ml. of petroleum ether was passed through the column. The

chromatogram was developed with a petroleum ether-benzene mixture (9:1), and eluted with a petroleum ether-benzene mixture (7:3). Since the material was colorless, and since no part of the column fluoresced when illuminated with ultraviolet light, fractions of 100 ml. were collected. Each fraction was concentrated, the residue transferred to a small vial, and dried in vacuo. The first six fractions consisted of small amounts of yellow oil. The next three fractions contained a few crystals as well as some yellow oil. The 10th, 11th, and 12th fractions were white solids, melting from 72-78° C. The rest of the thirty fractions had varying amounts of white solid material, melting from 80-140° C.*

A column, 17x700 mm., of activated alumina (Activated alumina, grade- F-20, mesh-minus 80, Aluminum Ore Company, East St. Louis, Illinois) was prepared in the same manner as the magnesia column. A solution of 0.341 g. of the material in 50 ml. of petroleum ether was passed through the column. The chromatogram was developed with a petroleum ether-benzene mixture (2:1), and eluted with an ethyl ether-benzene mixture (1:1). Thirty fractions of 100 ml. were collected. However, the separation was not any better than with the magnesia column.

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* All melting points reported in this work are uncorrected.

b. Solubility tests on the crude material.

The crude distillate was tested for solubility in solvents other than petroleum ether.

TABLE II

Solvent	Cold	Hot
Cyclohexane	Slightly soluble	Very soluble
Ethanol, 95 per cent	Very slightly soluble	Slightly soluble
Ethanol, absolute	Slightly soluble	Very soluble
Ethanol, 95 per cent benzene (3:1)	Very soluble	Soluble
1,4-Dioxane	Very soluble	-------
Isopropyl ether	Very soluble	-------
Ethyl acetate	Slightly soluble	Soluble
Dipentene	Insoluble	Soluble
Acetone	Soluble	Very soluble

Ninety-five per cent ethanol seemed to offer the greatest possibilities as a solvent. Recrystallization of the crude alcohol precipitate from 95 per cent ethanol is shown in Plate I. From 24.930 g. of crude material, 1.680 g. melted from 81.5-82.0° C. This material was active in dosages of one gamma. Of the material isolated from the mother liquor, 1.131 g. melted from 90-225° C., 8.606 g. melted from 90-130° C., 3.197 g. melted from

120-150° C., 2.874 g. melted from 110-130° C. This material was also active in dosages of one gamma. However, it was considered advantageous to attempt identification of the fraction melting from 81.5-82.0° C., since it was the most pure of the fractions on hand.

c. Carbon-hydrogen analysis of the low-melting fraction.

The carbon-hydrogen determinations, performed by Dr. A. J. Haagen-Smit of California Institute of Technology, gave the following results:

Found:		C 81.55	H 14.33
		81.48	14.53
		82.35	14.55
		82.44	13.85
		82.28	13.70
		82.77	13.69
		82.95	14.15
		82.82	14.16
Average:		82.33	14.11
Calculated:	$C_{27}H_{56}O$	C 81.74	H 14.23
	$C_{28}H_{58}O$	81.85	14.25
	$C_{29}H_{60}O$	81.98	14.25
	$C_{30}H_{62}O$	82.10	14.25
	$C_{31}H_{64}O$	82.21	14.25

d. Molecular weight determination.

The determination of the molecular weight was carried out in exaltone since it was found that some reaction took place if camphor were used as the solvent.
0.311 mg. compound in 3.032 mg. exaltone $\Delta = 4.4$ MW 497
0.348 mg. compound in 2.181 mg. exaltone $\Delta = 6.8$ MW 500

e. Hydrogenation of the compound and its acetate.

Although it did not seem likely from the carbon-hydrogen analyses that the compound was unsaturated, a microhydrogenation was run according to the method of Johns and Serfele (11). The apparatus used for this experiment was a van Slyke Deaminization Apparatus (Eimer and Amend, New York, N. Y.), which was modified in this laboratory to conform with the apparatus used by Johns and Serfele. The volume of the apparatus was determined by filling with mercury and weighing the mercury. Platinum oxide was used as the catalyst. It was prepared by the method of Adams, Voorhees, and Shriner (1). The hydrogenation of the acetate of the compound is included here. The preparation will be described later.

Three mg. of platimum oxide were placed in the flask and 5 ml. of glacial acetic acid were added. The boat containing the sample was suspended above the solution. Hydrogen from a tank was bubbled through a solution of sodium stannite to remove traces of oxygen, and

then through a tower filled with glacial acetic acid to saturate the hydrogen with the solvent. The apparatus was swept out with hydrogen for five minutes and the buret was filled. The volume of hydrogen in the buret, the temperature, and the atmospheric pressure were read. The apparatus was shaken for ten minutes to hydrogenate the catalyst. The volume, temperature, and pressure were read. The apparatus was then shaken for five minutes, and the volume, temperature, and pressure were read again. The boat containing the sample was dropped into the solution and the apparatus was shaken for 30 minutes. The volume, temperature, and pressure were read. These readings were taken again after a 10-minute period of shaking.

TABLE III

	mg. catalyst	ml. H_2 taken up	mg. sample	ml. H_2 taken up	Time of hydrog.
Low-melting fraction	2.707	1.078	6.784	0.020	30 min.
	2.660	1.050	7.617	0.010	2 hrs.
Acetate	2.842	1.125	9.859	0.000	30 min.

Dr. A. J. Haagen-Smit and Dr. H. L. Hunter confirmed our results on the hydrogenation of the acetate. They reported no uptake of hydrogen in four hours by a sample of

the acetate. From this evidence, it was concluded that the compound was saturated.

f. Preparation and analysis of the acetate.

The acetate of the compound was prepared as follows:

387 mg. of the compound were refluxed with 10 ml. of acetic anhydride for one-half hour. The mixture was cooled and filtered. The residue was recrystallized four times from 95 per cent ethanol. Yield: 300 mg. of waxy, colorless leaflets. M.P. 64.5-65.0° C.

Found:		C 79.75	H 13.22
Calculated:	$C_{29}H_{59}O_2$: ($C_{27}H_{56}O$) :	C 79.18	H 13.54
	$C_{30}H_{61}O_2$: ($C_{28}H_{58}O$) :	79.38	13.56
	$C_{31}H_{63}O_2$: ($C_{29}H_{60}O$) :	79.57	13.58
	$C_{32}H_{65}O_2$: ($C_{30}H_{62}O$) :	79.75	13.60
	$C_{33}H_{67}O_2$: ($C_{31}H_{64}O$) :	79.92	13.62

g. Preparation, chromatographic analysis, and analysis of the 3,5-dinitrophenyl urethane.

The 3,5-dinitrophenyl urethane was prepared according to the method of Smith and Sprung (21) as follows:

500 mg. of the compound were refluxed with 500 mg. of 3,5-dinitrobenzazide[1] in 10 ml. of dry toluene for one hour. The solution was cooled and the toluene removed in vacuo at room temperature. The residue was dissolved in 95 per cent ethanol and recrystallized four times from this solvent. Yield: 475 mg. of pale yellow powder. M.P. 103.5-104.0° C.

250 mg. of the urethane were dissolved in 250 ml. of benzene and adsorbed on a column of magnesia (adsorptive powdered magnesia, #2641, California Chemical Co., Newark, California) and celite (Johns-Mansville) (1:1). The column, 17x750 mm. was prepared as described in part a. The chromatogram was developed with benzene. Only one band appeared. This portion of the column was removed and eluted with boiling benzene. The urethane was recrystallized once from benzene. M.P. 103.5-104.5° C.

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1 The 3,5-dinitrobenzazide was prepared by the method of Smith and Sprung (21). 10 g. of 3,5-dinitrobenzoyl chloride were dissolved in 30 ml. of glacial acetic acid. 3.0 g. of sodium azide were added and the mixture was shaken for 30 minutes. The temperature of the mixture was kept below 45°. The solid was filtered off, and washed with 50 ml. cold water. The product was dried in a vacuum desiccator. After drying, it was digested for a few minutes in 150 ml. of low-boiling petroleum ether. The solid was filtered and dried. Yield: 6.0 g. M.P. 105° C. with decomposition.

Found:		C 68.17	H 10.30	N 6.85
		68.43	10.31	6.77
		68.25	10.18	7.09
				6.91
				6.84
		68.28	10.08	7.29
		68.40	9.98	7.17
		68.31	10.17	6.99
Calculated:	$C_{34}H_{60}N_3O_6$: ($C_{27}H_{56}O$)	C 67.24	H 9.97	N 6.97
	$C_{35}H_{62}N_3O_6$: ($C_{28}H_{58}O$)	67.66	10.07	6.81
	$C_{36}H_{64}N_3O_6$: ($C_{29}H_{60}O$)	68.08	10.14	6.65
	$C_{37}H_{66}N_3O_6$: ($C_{30}H_{62}O$)	68.46	10.25	6.48
	$N_{38}H_{68}N_3O_6$: ($C_{31}H_{64}O$)	68.83	10.34	6.34

h. Preparation of p-phenylazobenzoic acid, p-phenylazobenzoyl chloride, and p-phenylazobenzazide.

34.0 g. of nitrosobenzene were dissolved in 250 ml. glacial acetic acid. 34.5 g. of p-aminobenzoic acid were added. The mixture was stirred and warmed to 50° C. with a water bath. The precipitate was filtered off and washed with cold glacial acetic acid. The azo acid was recrystallized twice from 95 per cent ethanol.

Yield: 30 g. reddish-orange leaflets. M.P. 237-238° C.

The p-phenylazobenzoyl chloride was prepared in the manner described by Ladenburg, Fernholz, and Wallis (16).

10 g. of p-phenylazobenzoic acid were thoroughly mixed with 25 g. of anhydrous sodium carbonate. 125 ml. of thionyl chloride were added and the mixture was refluxed for one and one-half hours on a steam bath. The condenser was replaced by a calcium chloride tube and the mixture evaporated to dryness. The residue was taken up in petroleum ether, and the sodium carbonate removed by filtration. The p-phenylazobenzoyl chloride was twice recrystallized from petroleum ether.

Yield: 9.0 g. of red crystals. M.P. 93-94° C.

The p-phenylazobenzazide was prepared according to the method of Smith and Sprung (21).

3 g. of p-phenylazobenzoyl chloride were dissolved in 10 ml. of glacial acetic acid. 1 g. of sodium azide was added, and the mixture shaken for one-half hour. The mixture was then filtered and washed with 150 ml. of cold water. The residue was dried in a vacuum desiccator. The solid was digested in 150 ml. of petroleum ether, filtered, and dried in vacuo.

Yield: 2.2 g. of red needles. M.P. 116-117° C. with decomposition.

1. Preparation, chromatographic analysis, and analysis of the p-phenylazophenyl urethane.

500 mg. of the compound in 6 ml. of dry p-xylene were refluxed with 330 mg. of p-phenylbenzazide for one hour. The solution was cooled and the solvent removed in vacuo at room temperature. The residue was taken up in benzene and recrystallized four times from this solvent. Yield: 430 mg. of orange needles. M.P. 106-107° C.

250 mg. of the p-phenylazophenyl urethane were dissolved in 100 ml. of benzene and adsorbed on a column, 17x750 mm., of aluminum oxide (Activated Alumina, grade- F-20, mesh- minus 80, Aluminum Ore Company, East St. Louis, Illinois) and celite (Johns-Mansville) (3:1). The chromatogram was developed with benzene. Only one band appeared. This portion was removed and eluted with boiling benzene. The eluted urethane was recrystallized once from benzene. M.P. 106-107° C.

		C	H	N
Found:		77.81	11.14	6.84
		77.93	11.06	6.92
Average:		77.87	11.10	6.88
Calculated:	$C_{40}H_{65}N_3O_2$ ($C_{27}H_{56}O$)	77.48	10.57	6.78
	$C_{41}H_{67}N_3O_2$ ($C_{28}H_{58}O$)	77.66	10.66	6.63
	$C_{42}H_{69}N_3O_2$ ($C_{29}H_{60}O$)	77.84	10.73	6.49
	$C_{43}H_{71}N_3O_2$ ($C_{30}H_{62}O$)	78.01	10.81	6.35
	$C_{44}H_{73}N_3O_2$ ($C_{31}H_{64}O$)	78.16	10.88	6.22

j. Determination of terminal methyl groups.

The number of terminal methyl groups of the compound was determined by the oxidation method of Kuhn and Roth (14), as modified by Ginger (7). The apparatus used was similar to that of Roth and Daw (19) except that it was made of pyrex glass instead of quartz.

10-20 mg. of the compound were dissolved in 2 ml. of concentrated sulfuric acid by gentle warming. After the solution had cooled to room temperature, a few drops of 5 N chromic acid solution were added to start the reaction. The solution was then cooled in an ice bath and 5 ml. of 5 N chromic acid were added. The solution was then refluxed for 90 minutes. After the solution had been cooled to room temperature, the inside of the condenser was carefully washed in the reaction flask with carbon dioxide-free water. The excess of chromic acid was destroyed by the addition of 12 drops of 20 per cent hydrazine hydrate. The acetic acid was distilled off and the distillate collected in 5 ml. portions. Each portion was titrated with 0.0128 N sodium hydroxide, using phenolphthalein as indicator. 5 ml. of water were added to the reaction flask for each 5 ml. of distillate collected. The distillates were tested with barium chloride and were found free from sulfuric acid. For comparison, an oxidation of stearic acid was carried out in the same

manner.

TABLE IV

Substance	Weight sample mg.	Millimoles acetic acid: Calc. for 1 CH_3 group	Millimoles acetic acid: Found	Moles acetic acid found per mole of cpd.
Stearic acid	22.0	0.0775	0.0603	0.79
Low-melting fraction	20.0	0.0474	0.0317	0.67[a]
Acetate	19.2	0.0414	0.0645	1.60[b]
Acetate	18.1	0.0390	0.0585	1.50[b]

a. The molecular weight of the compound was assumed to be 424, $C_{29}H_{60}O$.

b. The molecular weight of the acetate was assumed to be 466, $C_{31}H_{63}O_2$.

From the above data, it would appear that the compound does not have a branched carbon chain.

k. Oxidation with chromic acid.

1 g. of the compound was dissolved in 100 ml. of phosphoric acid (d = 1.7) with gentle warming. The solution was allowed to cool to room temperature. 5 N chromic acid was added dropwise until the reaction began.

The flask was then cooled in an ice bath and 240 ml. of 5 N chromic acid were added slowly. The mixture was refluxed for four hours, while oxygen was bubbled through the solution. The solution was cooled to room

temperature and 3.5 ml. of 20 per cent hydrazine hydrate were added slowly. The reaction mixture was extracted with 400 ml. of ethyl ether in a liquid-extractor for 6 days. The contents of the extractor were filtered. The residue was dissolved in hot benzene and recrystallized four times from benzene.

Yield: 250 mg. of colorless leaflets. M.P. 82.0-82.6° C.

A mixture (1:1) of the oxidation product and the starting material melted from 79.0-80.5° C.

This material did not exhibit any of the characteristic reactions of a carbonyl group. Since it was slightly soluble in 10 per cent potassium hydroxide, it was tested for the presence of a carboxyl group.

200 mg. were dissolved in 30 ml. of chloroform and 10 ml. of an ether solution of diazo methane[1] were added.

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1 The diazo methane was prepared as follows:

The nitrosomethylurea was synthesized by the method of Arndt (2). 10 g. of methylamine hydrochloride were dissolved in 40 g. of water, and 30 g. of urea were added. The mixture was refluxed gently for 2.75 hours. The solution was cooled and 11 g. of sodium nitrite were added with shaking until the sodium nitrite had dissolved. The solution was then cooled to 0° and poured slowly into 10 g. of concentrated sulfuric acid and 60 g. of ice. The mixture was stirred with a mechanical stirrer, and cooled with an ice-salt mixture. The nitrosomethylurea was filtered off and washed with 15 ml. cold water. It was dried over phosphorus pentoxide in vacuo. Yield: 9.5 g.

The diazo methane was prepared by the method of Noller and Bergsteinsson (18). 15 ml. of 50 per cent aqueous potassium hydroxide and 50 ml. of ethyl ether were cooled to 5°, and 5.15 g. of nitrosomethylurea were added with shaking. The mixture was distilled with a water bath at 50° into 40 ml. of ethyl ether, cooled to 0°. The distillate was used immediately.

No reaction was apparent until some of the material began to crystallize, then nitrogen was evolved. The mixture was left standing overnight. The chloroform was evaporated in vacuo. The residue was insoluble in ethyl ether, but soluble in petroleum ether. The product was recrystallized from petroleum ether.

Yield: 35 mg. of white leaflets. M.P. 65.8-67.0° C.

Found:		C 80.56	H 13.39[a]
		80.41	13.64[a]
		79.34	12.97[b]
Average:		80.10	13.33
Calculated:	$C_{27}H_{55}COOCH_3$	C 79.38	H 13.33
	$C_{28}H_{57}COOCH_3$	79.56	13.37
	$C_{29}H_{59}COOCH_3$	79.74	13.40

From the above results, it would appear that one of the oxidation products was montanic acid, $C_{27}H_{55}COOH$.

The ether extract was fractionated, but only a mixture of formic and acetic acid was present.

1. Dehydration with anhydrous zinc chloride.

435 g. of the compound were heated with 526 mg. anhydrous zinc chloride for 3 hours in an oil bath at 180°. The mixture was cooled to room temperature and the

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a. Analyses by Dr. A. J. Haagen-Smit, California Institute of Technology.
b. Analyses by Dr. H. L. Hunter, Eli Lilly and Company.

zinc chloride dissolved in acidified water. The solution was extracted with ethyl ether. The ether layer was washed with water, and dried over anhydrous sodium sulfate. The ether was distilled off, and the residue was taken up in petroleum ether. This solution was boiled with Norit, and filtered. The material was recrystallized twice from petroleum ether.

Yield: 100 mg. of colorless leaflets. M.P. 82-83° C.

A mixed melting point with the starting material melted from 81-82° C.

Therefore, it was assumed that there was no dehydration.

CHAPTER VI

DISCUSSION

Carbon-hydrogen analyses of different samples of the low-melting fraction showed some variation. However, these analyses indicated that the material has 27-31 carbon atoms. The carbon-hydrogen analysis of the acetate derivative does not definitely fix the number of carbon atoms either, but it does place the number of carbon atoms in the same range. The nitrogen analyses of the 3,5-dinitrophenyl urethane and the p-phenylazophenyl urethane would indicate a 27 carbon atom structure. The carbon-hydrogen analyses of these derivatives are in close agreement with the theoretical percentages of carbon and hydrogen in a C_{29} compound.

The presence of a hydroxyl group was proven by the formation of the acetate and the urethane derivatives. From the results of the dehydration with anhydrous zinc chloride and the chromic acid oxidation, it would appear that the compound is a primary alcohol.

The methyl-group determination indicates a linear grouping of the carbon atoms in this compound, for a branched carbon chain would have shown a higher ratio for the moles of acetic acid per mole of compound.

Hydrogenation of the compound and its acetate

showed no uptake of hydrogen, even after extended periods of hydrogenation. From this, the conclusion may be drawn that the compound is saturated.

One of the products found on oxidation of the compound with chromic acid is apparently montanic acid. This would indicate a carbon chain of at least 28 carbon atoms.

The low-melting active fraction from cane juice could, therefore, consist of nonacosanol-1, although the possibility is not excluded that this fraction represents a solid solution of closely related compounds, such as heptacosanol-1, octacosanol-1, and nonacosanol-1. This would account for the varying results of the carbon-hydrogen analyses. Other investigators (4,6,12,13,14,19) have found that the separation of similar compounds differing by only one or two methylene groups is extremely difficult.

Heptacosanol-1, octacosanol-1, and nonacosanol-1 were synthesized by Dr. Rueben G. Jones of Eli Lilly and Company. These compounds were tested in dosages of one gamma. The greatest activity was shown by nonacosanol-1. The octacosanol-1 was slightly less active while the heptacosanol-1 showed very little activity.

CHAPTER VI

SUMMARY

On the basis of the carbon-hydrogen analyses of the compound, its acetate, 3,5-dinitrophenyl urethane, and p-phenylazophenyl urethane, the hydrogenation, the dehydration, and oxidation experiments, it can be concluded that the low-melting fraction isolated from cane juice consists of either nonocosanol-1, or a mixture of closely related compounds.

TABLE I

ACTIVITY TESTS

Material	Activity
	units per gram
Cane molasses	10
Cane juice	100-1000
Crude ethanol precipitate	1,000,000
Fraction melting from 81.5-82°	1,000,000
Fraction melting from 90-130°	1,000,000
Fraction melting from 130-150°	1,000,000
Heptacosanol-1	±10,000
Octacosanol-1	700,000
Nonacosanol-1	1,000,000

PLATE I

RECRYSTALLIZATION OF CRUDE PRODUCT FROM 95 PER CENT ETHANOL

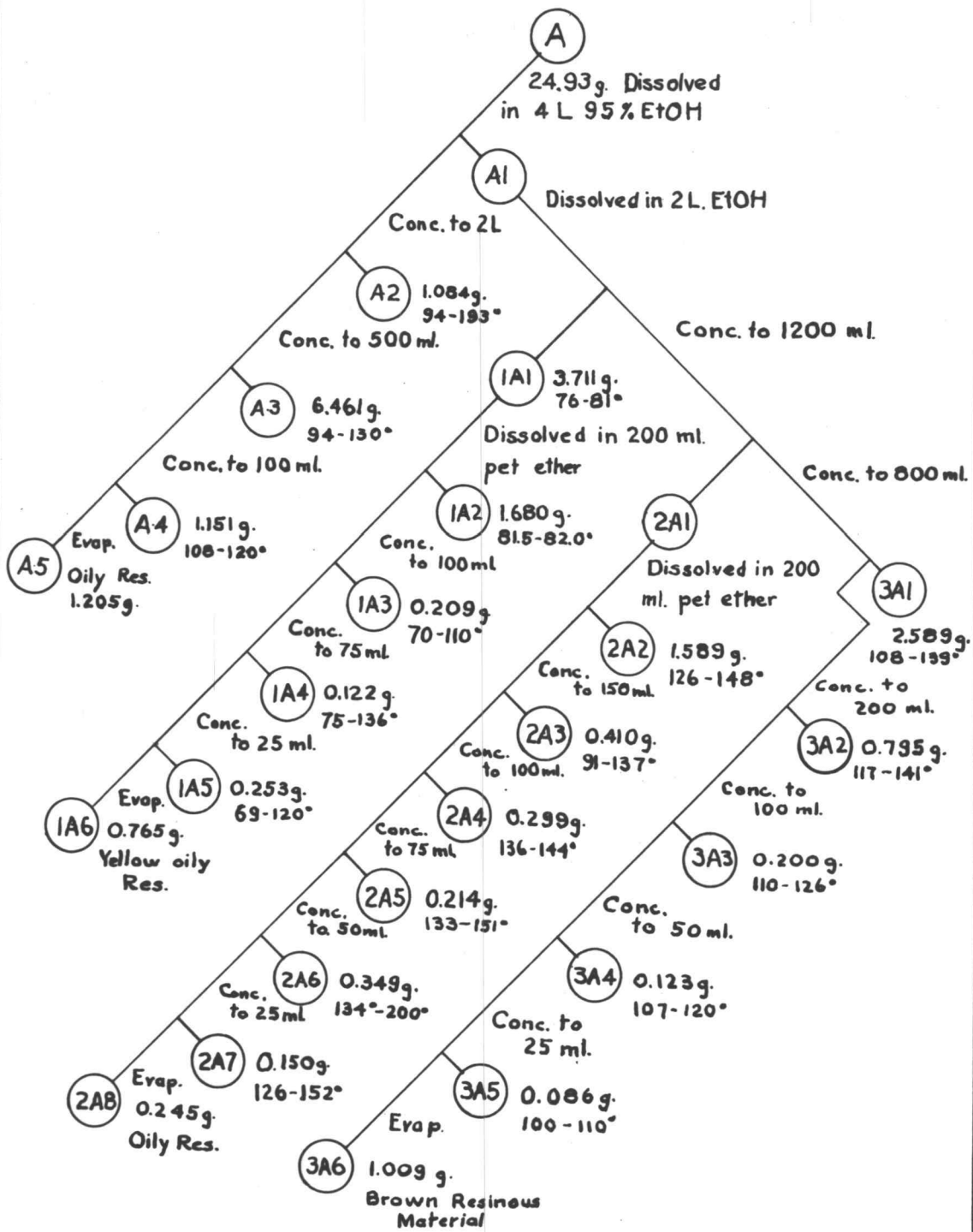
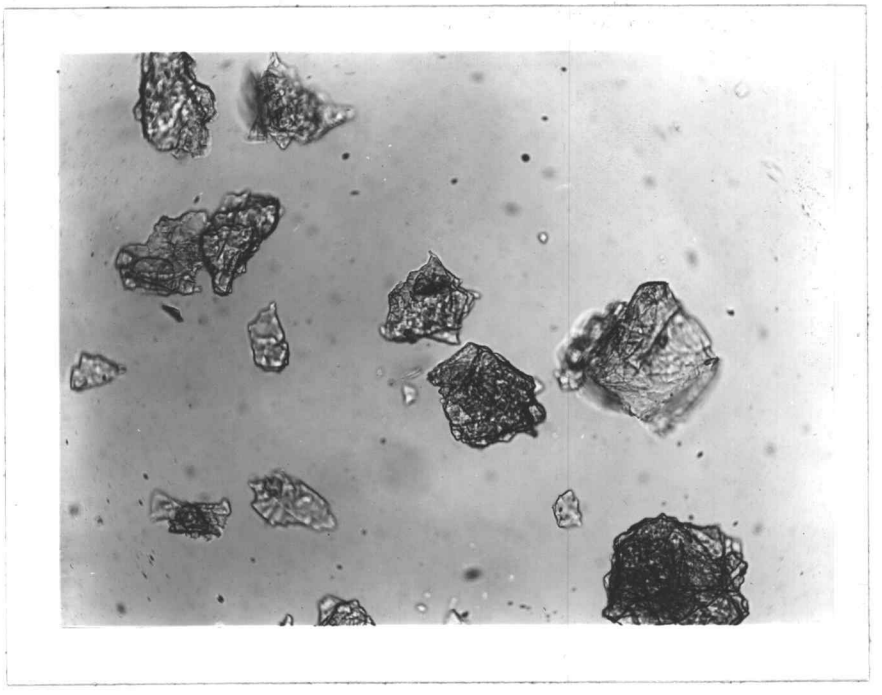


PLATE II



Microphotograph of the fraction melting from 81.5-82° C., isolated from cane juice. 80 x magnifaction.

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