The analogy between the kinetics of the autoxidation of benzoin and desylamine thus is, within the scope of our experiment, complete.

The determination of the rate of autoxidation of benzoin monomethyl ether was made in 45.5% ethanol, employing 0.4 *M* mole of the ether. The initial oxidation rates at two alkali concentrations are given in Table III. The data, substituted into the first-order equation yielded a satisfactory constant.

TABLE III

0.4 *M* MOLE OF BENZOIN MONOMETHYL ETHER

KOH, *M* mole	V_1 cc./mm.	V_1/KOH
3.00	0.00395	0.0013
10.00	.0140	.0014

The analysis of a control with nitrogen showed that the absorption of oxygen was not due to previous saponification of the ether under formation of benzoin which then autoxidized.

Summary

The mechanism of the oxidation of an α-amino ketone is closely analogous to that of the α-hydroxy ketones. The reaction proceeds at a rate which is proportional to the hydroxyl-ion concentration, with formation of the diketone, ammonia, and—with molecular oxygen—of hydrogen peroxide. A similar mechanism also applies to the oxidation of an α-methoxy ketone. The speed-limiting process in all these reactions is obviously the enolization, *i. e.*, the slow dissociation of the hydrogen ion from the carbon bearing the OH, NH_2, or OCH_3 group. The significance of this common behavior for the oxidation proper is discussed.

ROCHESTER, N. Y. RECEIVED JULY 6, 1937

[CONTRIBUTION FROM SEVERANCE CHEMICAL LABORATORY, OBERLIN COLLEGE]

The Isolation of Crystalline Vitamin A[1]

BY HARRY N. HOLMES AND RUTH E. CORBET[1a]

The existence of vitamin A was proved as early as 1913 yet up to 1937 its isolation was not accepted by workers in this field.

One of the first recorded efforts to prepare a rich concentrate was made by Takahashi and Kawakami[2] in 1923 using cod liver oil as the source. They called their product "crude biostearin" and reported a biologic activity of 0.08 mg. daily dose per rat. In 1925 Takahashi[3] and others further concentrated their crude biostearin by solvent partition and high vacuum distillation methods. In fact they claimed isolation of the pure vitamin, a claim later challenged by Drummond, Channon and Coward.[4] The latter workers distilled the nonsaponifiable portion of cod liver oil (previously freed from its cholesterol) at 2–3 mm. and found that the vitamin passed over at 180–220°. The distillate, however, contained several impurities.

British workers in their very thorough reports such as that edited by Hume and Chick[5] in 1935 set up a "provisional standard" of a concentrate with an extinction coefficient of $E^{1\%}_{1\text{ cm.}} = 1600$. This was based upon the work of Carr and Jewell[6] who in 1933 prepared by distillation under very high vacuum a material reading $E^{1\%}_{1\text{ cm.}} = 1600$ as measured by a quartz spectrophotometer. They also reported a feeding level of about 2,000,000 international units per gram for this preparation.

In 1933[7] Karrer and his associates, at Zürich, prepared a similar concentrate from fish liver oils but not by the vacuum distillation method. They used freezing methods and Tswett column adsorption by aluminum oxide, followed by differential adsorption on a column of calcium hydroxide. The extinction coefficient claimed was about 1700 and the rat feeding level reported by Euler, Karrer and Zubrys[8] was very low, about 0.3 gamma. This value was not reported directly in international units, as is the present custom.

Methods Used in This Research

Saponification and Extraction.—At the suggestion of Dr. C. E. Bills we used isopropanol as solvent for the saponifica-

(1) A thesis based upon this research was submitted by Ruth Corbet in partial fulfilment of the requirements for the Doctor of Philosophy degree at The Pennsylvania State College, through a coöperative agreement between The Pennsylvania State College and Oberlin College. Paper read at the Chapel Hill meeting of the A. C. S., Division of Biochemistry, April 15, 1937.—(H. N. H.)

(1a) Research Assistant, Oberlin College, 1935–1937.

(2) Takahashi and Kawakami, *J. Chem. Soc. Japan*, **44**, 590 (1923).

(3) Takahashi, Nakamiya, Kawakami and Kitasato, *Sci. Papers Inst. Phys. Chem. Res.*, **3**, 81 (1925).

(4) Drummond, Channon and Coward, *Biochem. J.*, **19**, 1047 (1925).

(5) Hume and Chick, Medical Research Council Report IV. "The Standardisation and Estimation of Vitamin A," 1935.

(6) Carr and Jewell, *Nature*, **131**, 92 (1933).

(7) Karrer and Morf, *Helv. Chim. Acta*, **16**, 625 (1933).

(8) Euler, Karrer and Zubrys, *Helv. Chim. Acta*, **17**, 24 (1934).

tion process, introducing certain modifications in technique developed in our own laboratory.

Fourteen grams of potassium hydroxide, ground to a coarse powder, is warmed on the water-bath with 103 cc. of pure isopropanol [in an Erlenmeyer flask with a smaller flask inverted in the neck as a condenser]. After cooling, the solution is decanted from any carbonate residue.

To 25 g. of fish liver oil (richest source of vitamin A) in a nitrogen-filled flask is added 100 cc. of the above potassium hydroxide solution, although 80 cc. is sufficient for 25 g. of ishinagi oil due to its high percentage of non-saponifiable matter. Frequent agitation with a gentle rotary motion (not shaking) for about one and a half hours is sufficient to complete saponification.

The jelly-like mass of soap is then dissolved in 150 cc. of recently boiled water. To this solution are added 265 cc. more water and 530 cc. of petroleum ether with gentle rotation of the flask to avoid frothing and emulsification. Layering and extraction are greatly improved by addition of 100 cc. of 95% ethanol immediately following the agitation with petroleum ether.

Usually the soap solution is extracted four times more with separate additions of 500 cc. of petroleum ether in order to remove practically all the vitamin. The combined extraction solutions containing vitamin A and the unsaponified material are evaporated to 40–50 cc. on the water-bath and the concentrated solution pipetted into crystallizing dishes and evacuated to dryness in a vacuum desiccator. A preliminary rinsing of these dishes with methanol displaces some of the air adsorbed on the glass. The residue is dissolved in methanol and transferred to an alcohol-rinsed bottle filled with nitrogen for the freezing process.

Filtration.—A Dewar funnel (10 cm. wide) is fitted with a filter paper moistened with methanol and filled with solid carbon dioxide for about five minutes. The funnel cover must be adjusted so that it too is chilled. Immediately before filtering the vitamin solution the carbon dioxide snow is removed quickly. The solution is poured into the funnel, the cover adjusted and very slight suction, if any at all, applied. Evaporation of such solid carbon dioxide particles as cling to the filter paper maintains an inert atmosphere unless too much suction is used in filtration. If the filtration continues for several minutes it is advisable to add a few particles of solid carbon dioxide to keep out air and to chill the solution.

The necessity of very low temperatures during this and later filtrations is plainly evident. Solid material frozen out of solution at $-50°$, for example, would redissolve if the temperature of the filter were allowed to rise many degrees.

Whenever the physical character of the precipitate permits, decantation is preferable to filtration.

When crystals are very small as much as 97% solvent is held by 3% of solid giving the appearance of a "false gel." In such an event it is impossible to decant the liquid and the entire mass, transferred to a cold filter, must be stirred with a cold spatula while very slight suction is applied.

Freezing.—In preparation for fractional freezing the methanol solution of non-saponifiable matter is adjusted to a concentration of about 0.08 g. per cc. for concentrates from ishinagi and California jewfish or 0.06 g. per cc. for products from oils containing a higher percentage of sterols.

Any material undissolved by methanol at room temperature is removed by filtration and discarded as very poor in vitamin A.

As the first step in freezing, the solution is kept for several hours in an ice–salt bath, any precipitate forming being filtered off and usually discarded. This filtrate, containing nearly all of the vitamin, is transferred to a nitrogen-filled bottle and placed in contact with solid carbon dioxide for several days. Precipitation may begin in one or two days or require three or four days. The solution above the precipitate clears and, if not filtered off, in about a week another type of precipitate forms on top of the old. Formation of this second crop of crystals is to be carefully avoided as it carries down much vitamin.

The filtrate is concentrated by distillation on the water bath at atmospheric pressure to about one-half its former volume and transferred to a nitrogen-filled bottle for further freezing in the solid carbon dioxide refrigerator. Usually a dark precipitate, quite rich in vitamin A, appears in a day or two; this should be removed immediately by filtration or more of the vitamin becomes mixed with it.

Fig. 1.—Crystals of vitamin A in a common test-tube obtained from methanol solution at low temperatures.

Water Additions.—A vital step at this stage is the addition of about 1% of water before returning the above filtrate to the refrigerator. If no precipitation occurs in a few days another per cent. of water is added without stirring since the crystalline nuclei forced out by excess water at the top of the solution aid greatly in the growth of a larger mass of crude vitamin A.

Recrystallization of Crude Vitamin A.—Up to this point the filtrates usually contain most of the vitamin although some of the precipitates may be more than one-half as potent.

The crude crystalline mass is dissolved in absolute methanol, cooled under nitrogen in the solid carbon dioxide refrigerator with occasional additions of a few drops of water, if necessary to start crystallization. The concentration of solid for these recrystallizations should be from 50 mg. to 100 mg. per cc. By recrystallizing the precipitates containing 50% or more vitamin A the yield of pure crystals from ishinagi oil could be brought to about 30% of the vitamin originally available in the oil.

Properties of Crystalline Vitamin A.—The vitamin, when crystallized at very low temperatures from methanol solution, appears in beautiful rosets or radiating clusters of pale yellow needles. The separate crystals are easily seen with the naked eye. They are optically inactive as predicted by other workers. S. E. Sheppard of the Eastman Kodak Company reports them to be isotropic, interesting because very few organic substances belong to this class.

Analysis.—The formula $C_{20}H_{30}O$ suggested by Karrer indicates 83.84% carbon and 10.56% hydrogen for pure

vitamin A. We report five quantitative determinations made in our laboratory.

	Carbon, %	Hydrogen, %
	83.07	10.49
	83.35	10.51
	83.45	10.47
	83.63	10.25
	82.88	10.48
Average	83.28	10.44

There are two important sources of error in analysis of crystalline vitamin A; failure to remove the last traces of solvent (methanol), and addition of oxygen during preparation of the sample for combustion.

Melting Point.—The melting point of the pale yellow crystals was determined by evacuating to remove the solvent, first at low temperatures, and then very gradually warming the cooled bath surrounding the thin-walled melting point tube. To retard this rise in temperature the bath liquid was placed in a Dewar flask (transparent). The melting point determinations ranged from 7.5 to 8.0°, a rather satisfactory range since the resulting yellow liquid, or melt, is very viscous even at room temperatures. It is obvious that great accuracy is difficult because of this high viscosity of the melt, and the problem of low-temperature removal of an adsorbed film of solvent, greater for small than for large crystals.

Crystals from	Melting Point, ° C.		
Ishinagi	7.5	7.5	
Mackerel	8.0	8.0	
Oil "1364"	8.0	8.5	7.5

The temperatures given above represent the initial softening at edges and ends of the needle crystals. The close agreement in melting points of crystals from three radically different fish liver oils is a strong indication that the same substance in pure form was isolated from these three sources.

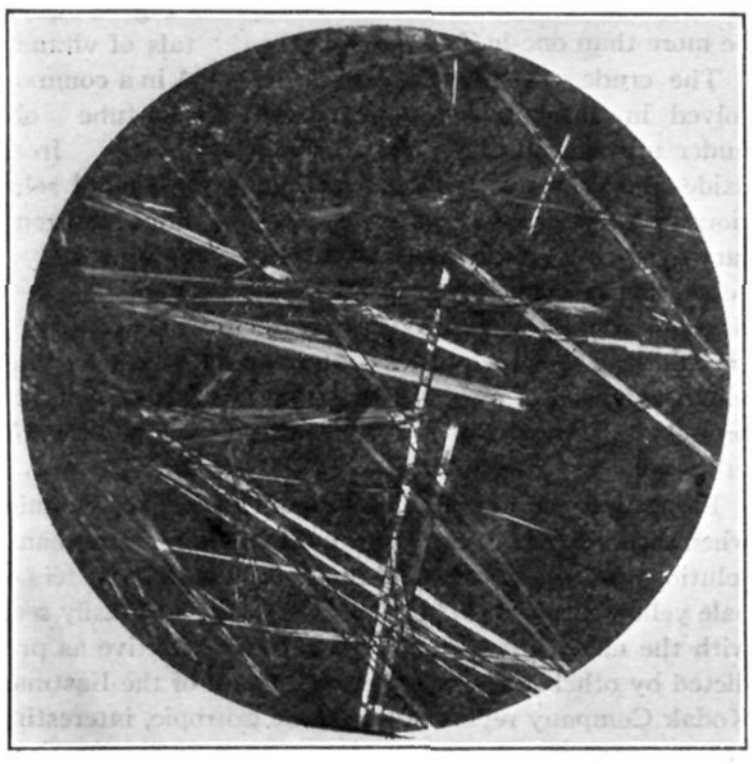


Fig. 2.—Yellow crystals of vitamin A photographed through the microscope by polarized light.

In an earlier preliminary statement we reported a lower melting point but we have since learned to prolong considerably the evacuation process for removal of traces of solvent from the surface of the crystals. Furthermore, we have obtained larger crystals which have less specific surface and hence less adsorbed film.

From consideration of the seven melting point determinations given above it seems probable that crystalline vitamin A melts at 7.5–8.0°.

Molecular Weight.—Efforts to determine the molecular weight by freezing point lowering of benzene solutions met with disaster. A rapid change in values took place with every few minutes of time. Purified cyclohexane was then tried with much better results yet even here the utmost speed was demanded because of a slower but steady change in the molecular condition of the vitamin.

We report four values, still rather preliminary, of 290, 290, 295 and 300, averaging 294. Since some time is required for the process of solution, it seems probable that the correct molecular weight must be a little less than 294. Karrer's formula calls for 286, a difference of less than 3%.

Evaluation.—There are three measures of vitamin A potency: chemical, physical and biological. The chemical test using a Lovibond tintometer, or similar instrument, measures the intensity of blue color developed by the anhydrous antimony trichloride solution in chloroform, a test which, however, also gives a strong blue color with certain impurities in the oils and even in some rich concentrates. Furthermore, there is discrepancy between the blue glasses of different instruments.

This test is therefore only roughly approximate. For example a preparation which in our hands gave a blue value of about 100,000, yielded in another laboratory a value of about 80,000. Chemical evaluation is now generally regarded as merely preliminary.

The physical test is based upon the ability of vitamin A to absorb ultraviolet light of wave length 328 mμ and is very accurate indeed under the proper conditions. The so-called "extinction coefficient" $E^{1\%}_{1\ cm.}$ is a direct measure of the concentration of vitamin A in solution—if there are present no other substances capable of absorbing light of 328 mμ wave length, substances present, unfortunately, in the non-saponifiable fractions of certain oils.

We are prepared to believe that one may, in certain cases, actually get a higher $E^{1\%}_{1\ cm.}$ reading with a slightly impure vitamin A than with a 100% product.

For more accurate readings than were possible

with our Hilger vitameter we secured the generous coöperation (with spectrophotometers) of W. R. Brode at Ohio State University, A. D. Emmett of Parke, Davis and Company, J. H. Waldo of the Eli Lilly Company, S. E. Sheppard and N. D. Embree of the Eastman Kodak Company, and H. T. Clarke and H. H. Darby of Columbia University Medical School. Dr. Emmett used a vitameter carefully standardized against spectrophotometric readings.

Due to too long storage at room temperature in one instance and in others to very natural delays in reading after dilution these readings of the extinction coefficient varied from about 1600 to 1800—and finally to the very remarkable value of $E^{1\%}_{1\,cm.} = 2100$ (or a molecular extinction coefficient of 60,000).

The last value, reported by Darby, is undoubtedly accurate as indicated by a fortunate incident. Dr. Darby, after diluting our 2.5% methanol solution with 95% ethanol, secured the same spectrophotometric reading as others in our coöperating group but when, three hours later, he again read $E^{1\%}_{1\,cm.}$ for the old diluted solution the value of the extinction coefficient had fallen to an astonishing degree.

He repeated the time study with fresh solutions, reading instantly and after some time of standing. In every instance the extinction coefficient at 328 mμ decreased sharply and indications of a new absorption band appeared at 360 mμ.

Without doubt the sensitive vitamin A molecule changed chemically in dilute ethanol solution and, as Darby warns us, everyone making this determination should take care to read instantly after dilution. Since the necessity for this precaution was unknown before, all previous $E^{1\%}_{1\,cm.}$ values should be reconsidered in the light of the Darby experiment.

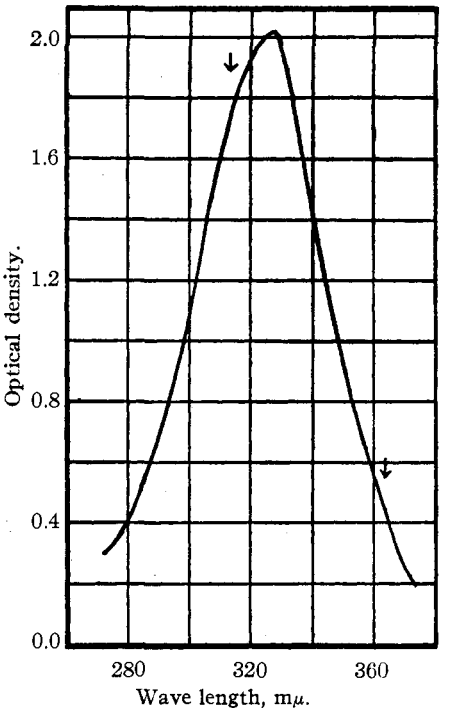


Fig. 3.—Curve of results by H. H. Darby, Columbia University: depth of cell, 2 cm.; temp., 23°; concn. of soln. in ethanol, 0.476 mg. per 100 cc.; read immediately after dilution.

For the ultimate decision as to the identity and purity of a vitamin A preparation we must resort to the biologic test. Fortunately for us Dr. R. Adams Dutcher and Dr. N. B. Guerrant, of The Pennsylvania State College, generously coöperated and depleted nearly sixty white rats for the testing of our crystalline concentrate from ishinagi liver oil. They used a control group of nine rats

STATISTICAL REPORT BY DUTCHER AND GUERRANT OF THEIR BIOLOGICAL ASSAY OF THE HOLMES-CORBET VITAMIN A CONCENTRATE, FEBRUARY, 1937

Animal group number	Number of animals considered	Average initial weight, g.	Average weight at end of depletion period, g.	Amount of concentrate fed daily, ml.	Average gain in weight (g.) during 1st week	2nd week	3rd week	4th week	5th week	Total, g. 4 weeks	5 weeks
0	6	46	105	0.029	8.2	3.5	7.3	2.8	1.8	21.8	23.6
1	6	44	104	.043	12.7	14.7	6.6	6.6	1.7	40.6	42.3
2	6	48	103	.057	12.0	21.8	14.3	14.3	8.5	62.4	70.9
3	6	45	102	.084	11.0	27.2	21.2	16.5	9.0	75.9	84.9
4	6	46	100	.129	15.5	25.2	24.3	19.8	14.5	84.8	99.3
5	6	45	108	.214	20.0	21.0	29.5	23.5	16.2	94.0	110.2
6	5	45	105	.321	23.2	30.4	30.0	21.8	14.0	105.4	119.4
7	6	45	103	.429	27.8	34.2	25.3	24.7	19.8	112.0	131.8
8	9	48	108	1 Unit	5.2	11.0	5.0	3.3	3.1	24.5	27.6

"Assuming that the solutions received contained 0.0103 mg. of the active material per ml., the results of the biological assay indicate that this material had a biological potency considerably greater than 2,265,000 International units per gram, but a potency somewhat less than 3,400,000 units per gram."

fed a definite weight of pure beta carotene (for vitamin A activity) and fed our concentrate at various levels to eight groups of six rats each.

We quote from Dr. Dutcher's letter. "This preparation assayed considerably above 2,265,000 international units per gram and somewhat below 3,400,000."

An examination of the feeding curves leads one to believe that the biological value is approximately 3,000,000 I. U. per gram.

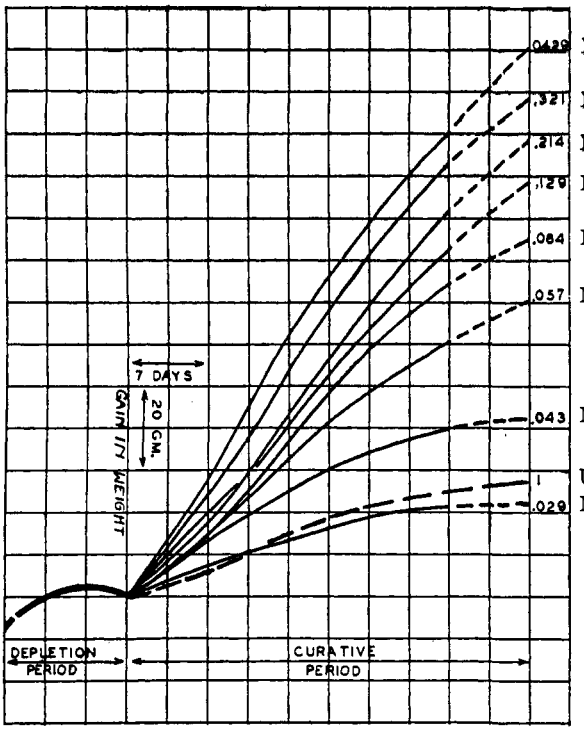


Fig. 4.—Dutcher and Guerrant's graphic record of different feeding levels and comparison with the international unit. Apparently the potency is nearer the lower level corresponding to 3,400,000 units per gram than to the next higher level corresponding to 2,265,000 units per gram.

This work by Dutcher and Guerrant is confirmed by a series of biological tests carried out by Dr. A. D. Emmett, of Parke, Davis and Company, on another crystalline concentrate that we isolated from the liver oil of a totally different fish. We quote from Dr. Emmett's letter.

"This preparation when fed to groups of rats assayed better than 2,535,000 International units per gram. The various data indicate that your concentrate is extremely high in vitamin A and probably has a value of 3,000,000 I. U. per gram."

It is interesting to note here that Dr. Emmett found a trace of vitamin D—only one part in about 9000—adsorbed on the surface of the vitamin A crystals. Doubtless this could be completely removed by further recrystallizations. No vitamin D assays have been run on the crystals from ishinagi oil.

Materials

Methanol.—A Commercial Solvents Corporation product, 99.8–100% pure, containing not more than 0.0025% non-volatile matter, distilled before use.

Ethanol.—The purest made by the United States Industrial Alcohol Company.

Isopropanol.—Eastman Kodak Company's best grade.

Cyclohexane.—Eastman Kodak Company's best grade. Further purified by freezing.

Petroleum Ether.—A low-boiling petroleum fraction obtained from the Viking Distributing Company, Charleston, W. Va.

Nitrogen.—Purchased from the Ohio Chemicals Corporation and further purified by passage through three bottles of alkaline pyrogallol, one of concentrated sulfuric acid and a tower containing glass wool and anhydrous calcium chloride.

Solid Carbon Dioxide.—Purchased from Dry Ice, Inc., Cleveland. Occasional samples contained a little ash of calcium, aluminum and iron compounds, probably due to the occasional use of lime-kiln gases as source.

Fish Liver Oils.—Stereolepis ishinagi from Mead Johnson and Company; mackerel and an unknown oil termed "No. 1364" from the Atlantic Coast Fisheries Company.

Summary

1. Crystalline vitamin A in the form of pale yellow needles has been isolated from the liver oils of three very different species of fish, *Stereolepis ishinagi*, Atlantic mackerel and "No. 1364" (an oil kept secret for commercial reasons). Refinements in the technique of fractional freezing and cold filtration, and a detailed study of water additions to a methanol solution of the vitamin were necessary.

2. Crystals from three species of fish melted at 7.5–8.0°, a close agreement considering the high viscosity of the melt and the very great difficulty of removing the last traces of solvent.

3. The extinction coefficient of crystals from ishinagi was found by Darby of Columbia University to have the extraordinary high value of $E^{1\%}_{1\,cm.} = 2100$, equivalent to a molecular extinction coefficient of 60,000. Darby discovered new precautions absolutely necessary to accuracy of measurement.

4. The biologic assay of crystals from ishinagi was made by R. Adams Dutcher and N. B. Guerrant of The Pennsylvania State College, indicating

a potency of "considerably above 2,265,000 and somewhat below 3,400,000 International Units per gram." A similar biologic assay of crystals from oil No. 1364 made by A. D. Emmett of Parke, Davis and Company indicated that this preparation "probably has a value of 3,000,000 international units per gram."

5. The molecular weight of the vitamin isolated from ishinagi oil was determined (by freezing point lowering of purified cyclohexane) as 294, less than 3% above the weight corresponding to Karrer's formula. The rate of change in this solution indicates that the actual value is a little lower than 294.

6. The average of five analyses in our own laboratory is 83.28% carbon and 10.44% hydrogen, slightly below the Karrer theoretical 83.84% carbon and 10.56% hydrogen. Oxidation in analytical handling and difficulty in removal of final traces of solvent tend to give low results.

OBERLIN, OHIO RECEIVED JULY 31, 1937

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

The Ring Structure of Alpha-Methyl-*l*-sorboside

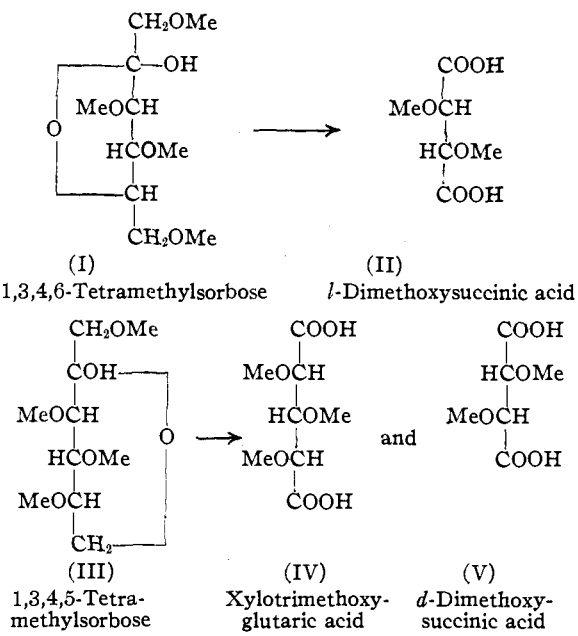
BY ROY L. WHISTLER AND R. M. HIXON

By employing mild conditions of glycoside formation Fischer[1] was able to isolate crystalline α-methyl-*l*-sorboside. This sorboside is the only one so far recorded in the literature. Arragon[2] has reported the preparation of two forms of tetramethyl-α-methyl-*l*-sorboside and of tetramethyl-*l*-sorbose, which can be obtained as colorless sirups.

An attempt to ascertain the ring structure of α-methyl-*l*-sorboside by the usual procedures of methylation and oxidative degradation had been started prior to Arragon's publication. Optical rotations were taken in chloroform but were repeated in methanol in order to compare them with the rotations given by Arragon in this solvent. Confirmation of Arragon's rotations was obtained.

The tetramethyl-*l*-sorbose resulting from the hydrolysis of the tetramethyl-α-methyl-*l*-sorboside in hot 2% aqueous hydrochloric acid has been oxidized by concentrated nitric acid to *d*-dimethoxysuccinic acid. The latter product was obtained in good yield. No intermediate products of oxidation corresponding to those of fructose[3] have, as yet, been isolated. However, the presence of *d*-dimethoxysuccinic acid alone suffices for the certain allocation of the lactol ring if consideration of an ethylene oxide structure is foregone.

If the oxygen bridge engaged carbon 5, a large yield of *l*-dimethoxysuccinic acid (II) would be expected in the oxidation products of 1,3,4,6-tetramethylsorbose (I).



No *l*-dimethoxysuccinic acid could be isolated. If the oxygen bridge engaged carbon atom 6 (III) the oxidation products would be expected to yield xylotrimethoxyglutaric acid (IV) and *d*-dimethoxysuccinic acid (V). Probably due to an insufficient amount of material, no trimethoxyglutaric acid could be separated. *d*-Dimethoxysuccinic acid was, however, isolated in good yield. It is seen at once that the isolation of this product, since it asserts methylation of carbon atom 5, alone suffices for the elimination of the fura-

(1) Fischer, *Ber.*, **28**, 1145 (1895).
(2) Arragon, *Bull. soc. chim. biol.*, **18**, 1336 (1936).
(3) Haworth, Hirst and Learner, *J. Chem. Soc.*, 1040 (1927).