the case of enzyme reactions, specifically in the case of invertase where the temperature coefficients of velocities cannot be correlated to the temperature coefficients of fluidities. It has been found by other workers[13] that no essential difference exists between invertase action *in vitro* and *in vivo* or whether in solution or absorbed.[14] Thus viscosity cannot be the determining factor in the rate of invertase action in biological enzyme reactions. It is plausible to presume a similar behavior of other enzymes.

Experimental Part

The reaction mixture used in all experiments contained 4.72% sucrose, 0.2% invertase solution ("Difco" standardized), and 20% 0.2 *N* acetate buffer (*p*H 4.5). Great care was exercised to adjust the *p*H of the pectin solutions to 4.5 before mixing with the other components of the reaction mixtures. The monomolecular constants were calculated from polarimeter readings made on samples from the reaction mixtures clarified by lead acetate and alcohol. The values used for "*k*" in Fig. 1 are the averages of at least six individual constants, but any other way of expressing the velocity would give a figure of similar shape. The apparent viscosity was determined by the use of the Ostwald viscosimeter, the value for water at 20° being 1'9.5".

After this paper was submitted for publication, a paper by M. Niculescu appeared [*Bull. soc. chim. biol.*, **16**, 903 (1934)] giving evidence that changes produced in the apparent viscosity of the medium by the use of gelatin or salep is without effect on the fermentation of glucose by yeast.

Summary

Neither changes in the apparent viscosity of the medium caused by added colloidal materials nor alterations in the viscosity brought about by temperature changes show a linear relationship to the velocity of hydrolysis of sucrose by invertase. Similar behavior of other enzymes may be presumed. It is concluded that the rate of biological enzyme reactions is not directly proportional to the viscosity of the medium.

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(13) Willstätter *et al.*, *Z. physiol. Chem.*, **115**, 180 (1921); v. Euler, *ibid.*, **105**, 187 (1919); Nelson, *et al.*, *J. gen. phys.*, **15**, 491 (1932); **16**, 571 (1933), etc.

(14) Griffin and Nelson, THIS JOURNAL, **38**, 772 (1916).

[CONTRIBUTION FROM THE GEORGE HERBERT JONES CHEMICAL LABORATORY, UNIVERSITY OF CHICAGO]

A Rapid and Accurate Quantitative Method for the Determination of the Common Carotenoids; Analyses of Beta-Carotene and Leaf Xanthophyll in Thirteen Plant Tissues[1]

BY ELMER S. MILLER[2]

This paper presents a rapid quantitative method for determining carotenoids without separating the components of a plant extract. The spectrophotoelectric method described by Zscheile, Hogness and Young[3] and the analytical procedure devised by Miller[4] were employed in this investigation. The analyses presented in Table III show the accuracy that may be attained by this method. In view of the similarity between the absorption curves of alpha carotene and leaf xanthophyll,[5] it is necessary to separate the carotenes from the xanthophyll before analyses can be made for the respective components. Hence, the writer has made a brief study of the Willstätter and Stoll[6] methanol–ligroin partition method.

Experimental Part

6.4 Mg. of beta carotene and 4.9 mg. of leaf xanthophyll were placed in a flask containing 400 cc. of 89% methanol and 400 cc. of ligroin (b. p. 30–35°). After the carotenoids had dissolved, the solution was transferred carefully to a separatory funnel. The ligroin solution was extracted 5 times with 300-cc. portions of methanol (89% by volume). The last two extractions were colorless. After the ligroin solution had been transferred to a 1-liter volumetric flask and made up to volume, a 10-cc. aliquot portion was placed in a 100-cc. volumetric flask. *In vacuo*, the solution was evaporated almost to dryness (0.5 cc.)—keeping the temperature of the flask below 30° during evaporation. When the vacuum was released, the carotenoids were dissolved in 80 cc. of absolute ethanol and 20 cc. of ether. Analyses of the aliquot portions are given in Table I.

(1) Presented at the Cleveland meeting of the American Chemical Society, Sept. 11, 1934.

(2) National Research Council Fellow.

(3) Zscheile, Hogness and Young, *J. Phys. Chem.*, **38**, 1 (1934).

(4) Miller, *Plant Physiology*, **9**, 693 (1934).

(5) (a) Miller, *Bot. Gaz.*, March issue (1935). (b) Miller, Mackinney and Zscheile, to appear in *J. Biol. Chem.* (1935).

(6) Willstätter and Stoll, "Untersuchungen über Chlorophyll" Berlin, 1913.

TABLE I

ANALYSES OF THE LIGROIN SOLUTION AFTER EXTRACTION WITH 89% METHANOL

Sample	1	2	Av.
Initial total β-carotene, mg	6.4	6.4	6.4
Experimentally determined total but calculated as β-carotene, mg	7.05	7.00	7.025
Percentage of total found by analyses to be leaf xanthophyll: λ 4585	10.0	10.5	
λ 4635	8.3	9.0	
Average	9.1	9.7	9.4
β-Carotene after correction for leaf xanthophyll, mg	6.47	6.375	6.422
Error, %	1.1	0.3	0.7

In a manner similar to that described above, a ligroin solution containing 4.9 mg. of leaf xanthophyll and 9.2 mg. of beta carotene was extracted six times with 300-cc. portions of 92% methanol. The last extraction was colorless. The analyses of aliquot portions of ligroin solution after extraction with 92% methanol are summarized in Table II.

TABLE II

ANALYSES OF THE LIGROIN SOLUTION AFTER EXTRACTION WITH 92% METHANOL

Sample	3	4	Av.
Initial total β-carotene, mg	9.2	9.2	9.2
Experimentally determined total but calculated as β-carotene, mg	9.75	9.7	9.72
Percentage of total found by analyses to be leaf xanthophyll: λ 4635	5.0	5.3	
λ 4904	6.6	7.0	
Average	5.8	6.1	6.0
β-Carotene after correction for leaf xanthophyll, mg	9.22	9.14	9.18
Error, %	0.2	0.6	0.4

The residual ligroin solution after extraction with 89% methanol was extracted ten times with 95% methanol. Visually, the tenth extraction appeared of the same color as the first one. Analyses showed that 10.0–11.5% beta carotene had dissolved in the methanol. Consequently, it is impossible to employ this concentration of methanol for the separation of the carotenes from the leaf xanthophyll.

Extraction of the Carotenoids

History of the Material.—The grasses listed in Table III (except the sugar canes) were grown in the green house on adjacent plots. When the grasses were 10–15 cm. tall, the samples were collected in four different groups. The sugar cane samples were dehydrated below 45°, ground to a powder, and extracted with ether.

Size of Samples.—The results tabulated in Table III show that 5-g. (green wt.) and 0.050–0.500-g. (dry wt.) samples are sufficient for analyses. Immediately after collecting and weighing the samples, they were placed in acetone to minimize enzyme action.

Extraction.—The samples are placed in a mortar containing 25 cc. of acetone and 25 g. of quartz sand. The extract from the macerated tissue is decanted into a 250-cc. Erlenmeyer flask; 20 cc. of ethanol (95%) saturated with potassium hydroxide is added to the extract. The alternate maceration and extraction is repeated three times with 25-cc. portions of acetone and twice with 35-cc. portions of ether. The extraction requires twelve to fifteen minutes. The pulp and sand is now transferred to a Soxhlet extractor. The extraction is completed by placing the extractor on a steam-bath for thirty to sixty minutes. Analyses of this ether extract show that it contains 30 gamma or less of carotenoids.

The combined extracts are transferred to a 3-liter separatory funnel containing 1.5 liters of distilled water. After the separatory funnel is whirled gently and allowed to set for five minutes, the water is drained off into the second separatory funnel. If the saponification is not complete, an addition of 10 cc. of ethanol (saturated with potassium hydroxide) is made. The contents of the second separatory funnel is extracted with 100 cc. of ether. These ether washings are added to the first separatory funnel. The ether extracts are washed four times with 500-cc. portions of distilled water. After each washing, the separatory funnel is stoppered and allowed to set for three to four minutes, except five to eight minutes before the last water extract is drained off.

The ether solution (150–200 cc.) is transferred to a 500-cc. balloon flask and, *in vacuo*, is evaporated to 50–60 cc. The carotenoid solution is transferred to a 100-cc. graduate, and after the solution has been measured, it is made up to volume. If the solution should be slightly turbid, usually sufficient time elapses for the solution to become clear before the final dilutions are made. The solutions are analyzed on the same day that they are prepared.

In 1931, Kuhn and Lederer[7] observed that no alpha carotene occurs in grasses. Their observations were based on optical rotation studies. Since they failed to state which grasses were analyzed, it was necessary to determine the amount of alpha carotene in the grasses mentioned in Table III. The writer employed the Willstätter and Stoll partition method for separating the leaf xanthophyll from the carotene solution in aliquot portions of the respective plant extracts.

This was performed by evaporating *in vacuo* one-half the original sample to approximately 10 cc. The concentrated solution is diluted by the addition of 250 cc. of ligroin and 250 cc. of 92% methanol. As shown in Table II approximately 94% of the xanthophyll is removed by the methanol extraction of the ligroin. The ligroin solution is transferred to a 250-cc. volumetric flask and made

(7) Kuhn and Lederer, *Z. physiol. Chem.*, **200**, 246 (1931).

TABLE III

QUANTITATIVE ANALYSES OF BETA CAROTENE AND LEAF XANTHOPHYLL (IN PARTS PER MILLION)

Plant		Sample, g.	Total carotenoids	Beta carotene	Leaf xanthophyll	Ratio Beta carotene to L. xanthophyll
Setaria stramineofructa		5.0	383 ± 3.0	74.6 ± 0.6	308.5 ± 2.5	1:4.10
Setaria italica		5.0	302.5 ± 1.0	83.0 ± 1.0	220.5 ± 0.0	1:2.65
Hordium sativum (Wis. Ped. No. 38)		5.0	115.4 ± 0.5	31.7 ± 1.0	84.0 ± .0	1:2.65
Dactylis glomerata		10.0	191.0 ± 2.0	42.5 ± 1.7	148.6 ± .4	1:3.50
Bromus inermis		10.0	285.0 ± 5.0	51.3 ± 2.0	238.5 ± 2.5	1:5.55
Zea maize L. (Country gentleman)		5.0	263.0 ± 2.0	46.2 ± 0.8	216.3 ± 3.3	1:4.68
Zea maize L. (Golden bantam)		5.0	288.5 ± 0.5	37.3 ± 1.0	221.7 ± 3.0	1:3.29
Sudan grass		5.0	445.0 ± 2.0	91.1 ± 1.3	354.0 ± 1.0	1:3.88
Festuca elatior		5.0	290.0 ± 2.0	61.4 ± 1.1	223.5 ± 1.0	1:3.63
Sugar cane hybrids	Hybrid H109	1.0	455.5 ± 1.5	131.2 ± 1.2	324.3 ± 0.3	1:2.47
	Hybrid 38–1389	1.0	665.0 ± 2.5	183.0 ± 4.0	481.7 ± .7	1:2.69
	Hybrid 30–2417	1.0	646.0 ± 2.0	183.5 ± 3.2	462.5 ± 1.2	1:2.52
	Hybrid P.O.J. 2878	1.0	478.0 ± 3.5	140.0 ± 0.2	338.7 ± 3.5	1:2.42

up to volume. An aliquot portion is evaporated to 0.5–1.0 cc. and made up to volume in 20% ether and 80% ethanol. After making the correction for the xanthophyll present, the analyses showed that the grasses contained none or less than ±0.7% alpha carotene.

Errors.—The analyses summarized in Table III possess the following errors. The instrumental error in the apparatus was ±0.7% or less. The factor which affected the error in the analyses the most, arose from the heterogeneous nature of the material, especially the green weight samples. Although the magnitude of error was only ±2.0 or less in the determinations of the total carotenoids, it became larger in the analyses for beta carotene, which formed only 15–42% of the total carotenoid concentration. The error in over one-half of the leaf xanthophyll analyses was less than 1%.

Discussion of Results.—The analyses presented in Table III are the averages of the determinations on duplicate samples. From 2–5 wave lengths were employed in determining the ratio of beta carotene to leaf xanthophyll. It is interesting to note the large variation in the ratio of carotene to leaf xanthophyll. The variation in this ratio may be greater between plants within the same genus or even species than between different genera. Among the sugar cane hybrids the amount of total carotenoids varies considerably, but the ratio of beta carotene to leaf xanthophyll does not. By comparison (visually) hybrids H109 and P. O. J. 2878 contained less than one-half the amount of chlorophyll that was present in hybrids 38–1389 and 30–2417. Preliminary analyses show that it is possible to determine the carotenoid components and the vitamin A content (directly) in the same solution.[8]

The writer wishes to thank Prof. T. R. Hogness of the Department of Chemistry, University of Chicago, for suggestions and criticism during the progress of this investigation.

Summary

1. Evidence is presented indicating that the ligroin–methanol (89 and 92%) partition method is quantitative only when corrected.

2. A rapid method is presented for the quantitative extraction of the carotenoids from green weight samples. This method is one-third as long as the older methods.

3. Quantitative analyses of beta carotene and leaf xanthophyll present in thirteen plants have been made by this method.

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(8) Miller, unpublished data.