RAPID DETERMINATION OF GLYCOGEN AND SUGARS IN MOSQUITOES[1]

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ABSTRACT. A method is presented for rapid determination of glycogen and sugars in individual mosquitoes. It is suitable for large scale field studies of correlations between nutritional status and behavior. The method also describes a one step procedure for total carbohydrates and the determination of nectar sugars and the hemolymph sugar trehalose.

INTRODUCTION

The flight energy of mosquitoes is derived from the polysaccharide glycogen stored in fat body and flight muscle and the disaccharide sucrose (or its components glucose and fructose) obtained from nectar and fruit juices and stored in the crop. Earlier methods to determine the small amounts of glycogen that occur in mosquitoes were unreliable. A system that made it possible to determine microgram quantities of glycogen, while saving the sugars and lipids for separate analysis in the same mosquito (Van Handel 1965) has found considerable application in mosquito physiology (Van Handel 1984) but is too time-consuming to analyze thousands of individual mosquitoes collected in the field. In order to establish correlations between nutritional status and such parameters as host attractancy, seasonality, time of day, availability of nectars, etc., the original separation was simplified while maintaining specificity and accuracy. The present system reduces the time for analysis of a group of 12 individual mosquitoes from at least a day to less than one hour.

MATERIALS

Anthrone reagent. Pour 150 ml water into a 1 liter Erlenmeyer flask. While cooling add carefully 380 ml concentrated sulfuric acid. Dissolve about 750 mg anthrone in this diluted sulfuric acid. When kept in a refrigerator, this yellow reagent is stable for many weeks.

Standard solutions. Glucose, trehalose, sucrose (1mg/ml) in 25% ethanol. These solutions are stable indefinitely. Glycogen solution in water (1 mg/ml) is stable for a limited time in a refrigerator.

Sodium sulfate. 2% solution in water.

Methanol.

Centrifuge.

16 × 100 mm culture tubes. Marked at 5 ml level.

Tube heater. An aluminum block provided with 17 × 80 mm holes to accommodate the culture tubes is mounted on a laboratory hot plate kept at 90–92°C. A thermometer in a tube containing a few ml glycerol is placed in the tube heater to monitor temperature.

Vortex-genie for mixing.

Spectrophotometer. Optimal wave length for the green-blue reaction product is 625 nm.

PROCEDURE

CALIBRATION. Place 25, 50, 100, 150 and 200 μg (= μl) glucose solution (1 mg/ml) in tubes, fill with anthrone reagent to the mark, mix, and heat for 17 min in the tube heater. Let cool and read directly at 625 nm. When the optical density (OD) is higher than 1, transfer 1 ml with a glass syringe to a clean tube and fill to the 5 ml mark with anthrone reagent. The original OD is 5X the diluted value. Instead of diluting, the OD can be read against the reagent blank at 555 (or 560) nm where the OD is approximately half that at 625 nm. Plot μg glucose vs OD to form a calibration line at both wavelengths.

SEPARATION AND DETERMINATION OF GLYCOGEN AND SUGARS. Place a mosquito in a culture tube, add 0.2 ml sodium sulfate solution and crush the mosquito with a glass rod. Add 1 ml methanol, mix (vortex) well and centrifuge for about 1 min. Poor mixing causes incomplete precipitation and prolongs centrifugation time. Instead of 1 ml, one can add 0.5 ml of methanol. This shortens evaporation time in the next step to about 10 min, but leaves a slightly higher percentage of the sugar with the glycogen fraction. Decant the supernatant (containing the sugars) to a second set of tubes and evaporate solvent in the tube heater down to 0.1–0.2 ml. Glycogen remains behind in the first tube, adsorbed on the precipitated sodium sulfate, along with mosquito tissue. (When sugar greatly exceeds glycogen, e.g. when a starved mosquito is analyzed soon after a large sugar meal, it is advisable to wash the glycogen precipitate with a few ml of 80% methanol.) Fill both tubes to the mark with anthrone reagent, mix, heat for 17 min., cool, mix and determine OD at 625 nm. Dilute the high OD's as above. Glycogen and sugar content per mosquito can

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be read directly from the glucose calibration line.

QUANTITATIVE DETERMINATION OF TREHALOSE AND SUCROSE. Mosquitoes captured in the field usually do not contain enough total sugar to determine trehalose and sucrose quantitatively and individually. Therefore, the method is described for a pool. Homogenize 10 mosquitoes in 0.3 ml 2% sodium sulfate, add 1 ml methanol, mix well and centrifuge. Transfer the supernatant, which contains sugars, to another tube. Add 0.3 ml water to the tube containing the precipitate, vortex, add 1 ml methanol, vortex, centrifuge and combine the two supernatants (sugar fraction). Concentrate this fraction to 0.5 ml. The glycogen is in the precipitate and can be dissolved in 1 ml water and assayed by reacting 0.1 ml (the equivalent of 1 mosquito) with hot anthrone as described above.

SUCROSE. Transfer 0.2 ml (the equivalent of 4 mosquitoes) of above sugar fraction to one tube and 0.05 ml (0.05 mg) of the sucrose standard to another tube. Add reagent to the 5 ml mark, mix and hold both tubes at 40°C for 20 min or at room temperature for 1 to 1½ hr. Calculate nectar sugars from the OD of the sucrose standard. If the standard has the same OD as the unknown the sucrose (or its constituents glucose and fructose) content of the mosquito was $\frac{1}{4} \times 50 = 12.5\ \mu g$.

TREHALOSE. Transfer 0.1 ml (the equivalent of 2 mosquitoes) of above sugar fraction to one tube and 0.05 ml (0.05 mg) of the trehalose standard to another one. Add to both tubes 0.05 ml 1N HCl and heat at 90°C for 7 min. HCl hydrolyzes sucrose to form glucose and fructose, but leaves trehalose intact. Add 0.15 ml 1N NaOH to both tubes and heat at 90°C for 7 min. NaOH destroys the anthrone reactivity of glucose and fructose but leaves trehalose intact. Add anthrone reagent to the 5 ml mark and heat at 90°C for 17 min. Determine the OD. Calculate trehalose per 2 mosquitoes from the standard reading. This reaction is specific for trehalose. The aliquots are suggested from actual findings of the amounts of sucrose and trehalose in mosquitoes collected in the field. The user may find different aliquots more practical.

DETERMINATION OF TOTAL CARBOHYDRATE CONTENT. Fill tube to the mark with anthrone reagent. With a glass rod, crush a mosquito in the reagent, heat for 17 min, let cool, mix and determine OD at 625 nm. For OD's higher than 1, transfer 1 ml to a new tube, dilute with reagent to 5 ml, mix and multiply this OD by 5. Alternatively one can use the shorter wavelength. The carbohydrate content can be read from the calibration line determined above. Mosquito tissue, including the chitinous exoskeleton, dissolves during heating. The amount obtained from a population starved to death is considered to be of no nutritional value and should be deducted. The apparent carbohydrate content of such a population has been consistently lower than 5 μg/mosquito (Table 1).

RESULTS

CALIBRATION. Standard values for glucose, trehalose and glycogen are so constant that recalibration is needed infrequently, provided the heating temperature and time remain constant. The OD is linear from 10 to 200 μg for glucose, trehalose, sucrose and glycogen. Measured in a Coleman Junior IIA spectrophotometer with linear absorbance, the OD at 625 nm increased 0.16 (0.15–0.17) for each 10 μg of carbohydrate. There was no difference between precipitated glycogen and the standard solution measured directly. It is sufficient to use a standard glucose solution to calibrate both the glycogen and the sugar determination.

SEPARATION OF MIXTURES. The efficiency of the method to analyze carbohydrate mixtures is presented in Table 2. The slight overestimation of glycogen is due to residual sugar adsorbed on sodium sulfate and on the tube wall. It does not exceed 10% of the sugar fraction and can be corrected by increasing the value found for sugars by 10% and reducing the glycogen value by the same amount. The mixtures were chosen because they represent values often found in mosquitoes captured in the field.

Table 1. Comparison of two methods of analysis for glycogen and sugars in *Aedes aegypti*.

	Glycogen			Sugars	
	Present	Previous	(Chitin)	Present	Previous
Starved to death	3.5 ± 0.7	1 ± 1	(2)	1 ± 0.3	1.5 ± 0.5
At adult emergence	8.5 ± 3	9 ± 2	(2)	3.5 ± 0.3	5 ± 1
Three days on 10% sucrose	55 ± 5	54 ± 4	(3)	55 ± 6	63 ± 4
Seven days on 10% sucrose	42 ± 4	38 ± 3	(2.5)	19 ± 2	24 ± 3

Mosquitoes were analyzed (μg/♀ ± S.E.) as in text (present) and according to Van Handel (1965) (previous).

Table 2. Separation of glycogen from trehalose and sucrose.

Added (μg)			Recovered (μg)	
glycogen	sucrose	trehalose	as glycogen	as sugars
50	100		60	94
50	50		55	50
25	75		30	76
25		75	30	70
75		25	72	24
50		50	54	50
50		100	55	95

Mixtures were treated as in text. Each component was assayed against its own standard (glycogen, sucrose, trehalose). Average of three replicates. The standard error of each mean was less than 3%.

COMPARISON BETWEEN THE PREVIOUS AND THE PRESENT SEPARATION PROCEDURE. Mosquitoes from a laboratory colony were either starved to death after adult emergence or maintained on a 10% sucrose solution. They were then analyzed individually for sugars and glycogen by the present method and the separation described previously (Van Handel 1965). The results (Table 1) demonstrate close agreement between the methods and a low value for starved mosquitoes. This base line (3.5 μg apparent glycogen) includes about 2 μg caused by a reaction of anthrone with the integumental chitin.

TEST FOR NECTAR. The presence of nectar in the crop of a mosquito can be demonstrated by its reaction with cold anthrone reagent (Van Handel 1972). However, the amount present in field populations is so low that treating an individual mosquito with 5 ml cold reagent would underestimate nectar feeding. It is possible to combine the qualitative nectar test with a quantitative assay of total carbohydrate. Crush a mosquito in about 0.3 ml reagent. After 30–60 min at room temperature, note a positive sucrose reaction (a color change to green), add reagent to the mark, heat, mix and determine total carbohydrate.

DISCUSSION

In the present method, anthrone positive chitin and glycogen are determined jointly, but the overestimation of glycogen is negligible and eliminated by establishing a baseline for starved mosquitoes. In the previous method, glycogen was separated from chitin with hot KOH, and reprecipitated with ethanol. The contribution of chitin can then be separately determined by washing the KOH insoluble residue and reacting with anthrone. A comparison between the two procedures demonstrates sufficient agreement whereas time saving is very significant. The contribution of chitin is no more than 2–3 μg per mosquito and independent of nutritional status (Table 1). This simplification is suitable for mosquitoes but not necessarily for other flies and insects, where the ratio integumental chitin to glycogen is usually much larger and the chitin reaction yields greenish-brown colors that interfere with and augment the glycogen assay.

STORAGE. Trapped mosquitoes, while alive, continue to absorb sugars. Dead mosquitoes are sensitive to enzymatic degradation of carbohydrates. After death, trehalose is rapidly, and glycogen more slowly converted to glucose. It is therefore essential to store specimens at −20°C or lower as soon as possible after capture. When taken from the freezer the enzymes are still active. A recommended alternative is to dry the mosquitoes at 90°C (45–60 min). This inactivates the enzymes and dried specimens may be stored at room temperature in a tightly closed container. The dry material may have to be homogenized a bit better than fresh mosquitoes, but even after 18 hr at 90°C, there was no difference in glycogen, trehalose and sucrose content.

LARVAE AND PUPAE. The same method can be used to analyze pupae and larvae. A piece of microscope cover glass added to the tube prior to grinding facilitates extraction. Larvae reared on starchy laboratory diets may contain some extraneous carbohydrate in the gut. This would be less likely to interfere in larvae collected from a natural habitat.

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