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HEMOGLOBINOMETRY AS A METHOD FOR MEASURING BLOOD MEAL SIZES OF MOSQUITOES (DIPTERA: CULICIDAE)[1]

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Abstract: A standardized clinical method for measuring hemoglobin has been applied to the measurement of the quantity of blood ingested by *Aedes aegypti* feeding on a vertebrate host. The midgut of a blood-fed mosquito is added to a reagent which converts all hemoglobin to hemiglobincyanide (HiCN) which can be read spectrophotometrically. A sample of the host's blood is required as a standard. We established the validity of this method by comparing hemoglobin determinations with the size of blood meals measured by weight as well as with blood meals administered as enemas. The method is independent of urination and digestion by the mosquito for at least 12 h after feeding (at 27°C) and can be applied to females feeding on an unrestrained host during crepuscular or dark periods. A disadvantage of this method is that females must be killed in order to determine the volume of blood they ingest. Once converted to hemiglobincyanide, the blood meal can be frozen and stored for later analysis.

The blood meal is not only essential for reproduction in most mosquitoes, but in addition, the quantity of blood ingested determines the number of eggs produced (Woke et al. 1956), biting and host-seeking behavior (Edman et al. 1975, Klowden & Lea 1978), and the extent to which pathogens are acquired from an infected host (Hovanitz 1947, Kershaw et al. 1955, Jeffrey 1956). Existing methods of measuring the quantity of blood ingested require either weighing the insect before and immediately after feeding or feeding the female on radioisotope-labelled blood, through a membrane or on a live host (Boorman 1960, de Freitas & da Silveira Guedes 1961, Bennett 1965, Redington & Hockmeyer 1976). When using the gravimetric method, any delay in reweighing after feeding causes an underestimation of the size of the blood meal to the extent of the urine lost. Isotopically labelling the blood eliminates this error, but only small hosts can be labelled with a physiologically safe dose.

The defensive behavior of an unrestrained host can exert a significant influence on the feeding success of mosquitoes and can often delay the completion of feeding (Edman & Kale 1971, Lenahan & Boreham 1976). Furthermore, if experiments are performed during a crepuscular and/or dark period, observation of the onset and termination of feeding is difficult, making the gravimetric method particularly unsuitable. Our interest in studying the blood-feeding behavior of mosquitoes led us to investigate chemical methods for determining blood consumption by a single female.

In this paper, we describe modifications of a standard cyanide method used in human hemoglobinometry. The method is based on the complete conversion of all hemoglobin from lysed erythrocytes to a stable complex, hemiglobincyanide (HiCN; for nomenclature see Van Assendelft 1970). Both lysis and conversion are initiated in a single reagent solution, and the absorbance of the complex is read in a spectrophotometer.

MATERIALS AND METHODS

The material

Aedes aegypti (Linnaeus) were kept at 27°C, 85% RH, under a 16:8 h day:night cycle. Heparinized blood (5 units/ml) was drawn from the heart of anesthetized rats, the wing vein of a pigeon, or the ear vein of a rabbit. Mosquitoes were anesthetized with ether and weighed on a Cahn electrobalance (Model 7500) before, and in less than 60 s after, taking blood from the host, or they were given measured blood meals by enema (Briegel & Lea 1975).

The reagent

We used Drabkin's solution as the reagent, modified according to Legowski & Boroviczeny (1962): 1.0 g sodium bicarbonate ($NaHCO_3$), 0.1 g potassium carbonate (K_2CO_3), 0.05 g potassium cyanide (KCN), 0.2 g potassium ferricyanide [$K_3Fe(CN)_6$], and distilled water to make 1 liter. The solution retains its stability for 1 month when stored in a dark bottle at room temperature. Working with cyanide calls for some caution, although the toxicity is very low at this concentration. Reagents other than Drabkin's, including Van Kam-

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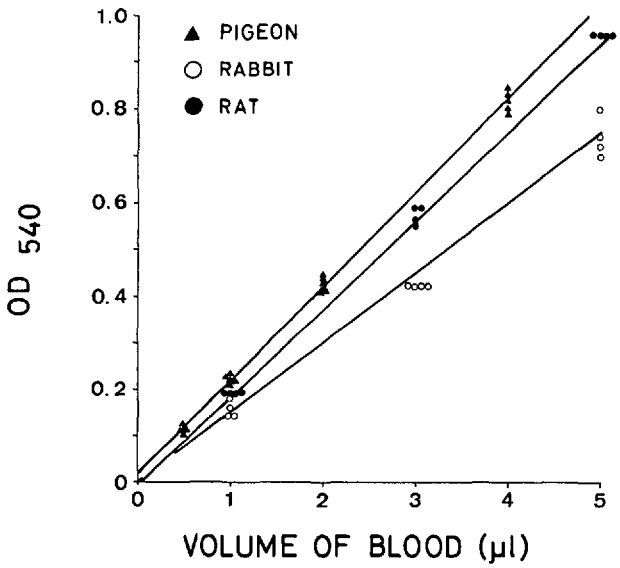

FIG. 1. Linear regression of HiCN (OD_{540}) and amount of blood from a pigeon ($Y = 0.20X + 0.02$, $r = 0.99$, $n = 19$), a rabbit ($Y = 0.15X$, $r = 0.99$, $n = 13$) and a rat ($Y = 0.19X - 0.02$, $r = 1.00$, $n = 13$) added to Drabkin's reagent.

pen & Zijlstra's reagent (Van Assendelft 1970) and Hycel No. 116C (Hycel Inc., Houston, Texas), a commercially available reagent, were tried and found to be less satisfactory. Detergents in these reagents caused foaming during homogenization of the blood meal, and they appeared to increase interference in the chloroform procedure (see below).

The HiCN method

Blood-fed females are dissected, and the blood meal within the midgut is placed into a test tube containing Drabkin's reagent (0.5 ml for *Ae. aegypti*; 1.0 ml for larger mosquitoes). The Malpighian tubules should be removed to reduce possible interference from uric acid. The midgut, blood, and reagent are thoroughly ground with a loose-fitting motorized teflon pestle to assure the complete elution of hemoglobin, and the samples are incubated at room temperature (15°–23°C) for at least 20 min. An aliquot (200 µl) is transferred to a microcuvette and the optical density (OD) read at 540 nm in a spectrophotometer. The complex is stable for several hours, so it is not necessary to read the results immediately.

Calibration

To assess the relationship between OD and blood volume, a calibration curve must be prepared for each host. Since the hemoglobin titer of animals fluctuates periodically, these curves should be performed for each experiment. Various amounts of host blood (e.g., 1, 2, 4, 6 µl) are added to the reagent and the OD of each volume determined. A linear relationship between OD and blood volume is obtained, and the regression line is used to equate the OD of an unknown sample with its blood volume.

Alternative method of preparation

We developed a chloroform procedure for preparing blood-fed females which does not require removal of the midgut. When using this method, the whole abdomen is removed and added to Drabkin's solution and triturated. Complete tissue disintegration is important to insure that all hemoglobin will react with the reagent. After 20 min an equal volume of chloroform is added, and the test tube is then vigorously shaken on a vortex mixer and centrifuged in a horizontal rotor (2000 *g*, 5 min) to separate the chloroform-soluble components. The HiCN remains in the aqueous phase and should be sampled soon after centrifugation to minimize the leaching of components at the interface. A 200-µl aliquot of the aqueous supernatant is transferred to a microcuvette and read at 540 nm as in the standard procedure above. When using this chloroform procedure, the OD of control abdomens without blood must be subtracted from that of a female abdomen with a blood meal, since its components contribute slightly to the readings. We processed abdomens from unfed females of different physiological conditions (starved for 4 days, 20% sugar-fed for 4 days, or gravid) in Drabkin's solution plus chloroform to determine which could be used as controls, and found that the difference in the optical densities of these abdomens was negligible, ranging from 0.04 to 0.05 OD units. Consequently, when using this alternative procedure, the optical density of any of these abdomens may be subtracted from values of test abdomens with blood. Whole mosquitoes may be processed by this method also, but the head and thorax contribute to higher and more variable optical densities. In the standard procedure only blood and midgut tissues are assayed, and it is not necessary to account for the OD of the abdomen.

RESULTS

Quantitative relations between the blood meal size and HiCN

A linear correlation was obtained between optical density and blood volume when increasing amounts of heparinized blood from rat, pigeon, or

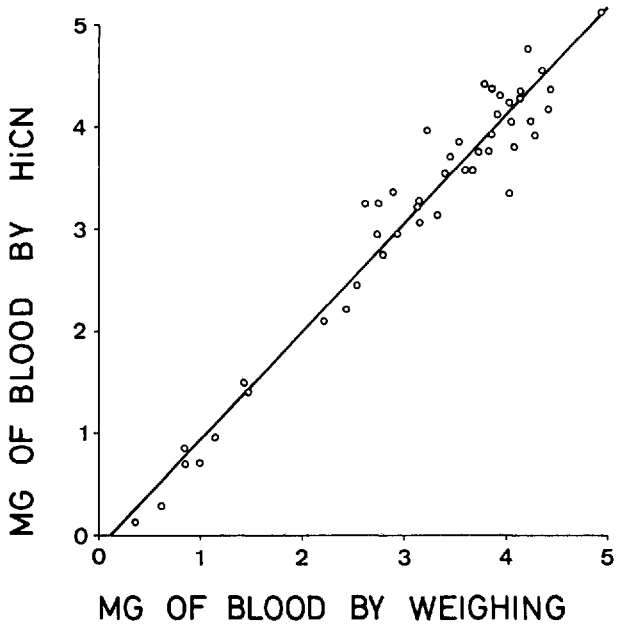

FIG. 2. Linear regression of weight of blood ingested (gravimetric determinations) and weight of blood calculated from HiCN readings in *Aedes aegypti* (Y = 1.06X − 0.13, r = 0.97, n = 47).

rabbit were added to the reagent (FIG. 1). The slopes of the regression lines vary depending on the hemoglobin titer of the particular host.

The validity of the HiCN technique was tested by giving *Ae. aegypti* females measured amounts of rat blood by enema and analyzing them immediately afterwards. A linear correlation between blood volume and OD was evident, and a straight regression line was computed (Y = 0.13X, r = 0.91, n = 58). Hemoglobinometric data were compared with gravimetric determinations when female mosquitoes were individually fed on a human arm and the size of their blood meals determined by weighing each female before and immediately after feeding. The size of the meals was then also determined by the HiCN method, and the absorbancies were converted to mg of blood (specific gravity = 1.05). Again, a linear relationship with a high correlation coefficient was obtained (FIG. 2), showing that both gravimetric and HiCN methods gave similar estimates of the blood ingested.

Effects of blood digestion on the HiCN determination

To determine how long after feeding the hemoglobin analysis could be delayed and still accurately reflect blood meal size, we gave 1- and 4-μl enemas of rat blood to mosquitoes which were held in individual containers at 27°C and provided with 10% sucrose. At specified times, 6 or 7 females were analyzed (TABLE 1). The HiCN values remain relatively constant for a period of 12 h, while digestion of protein, absorption, and excretion of uric acid are actively taking place (Briegel 1975). This indicates that it is possible to allow a biting experiment to continue overnight and still determine the total amount of blood ingested during this period.

TABLE 1. Effect of digestion on HiCN determinations in *Aedes aegypti* given blood enemas of 1 or 4 μl.

TIME AFTER ENEMA (H)	MEAN BLOOD VOLUME (± SE) BY HiCN METHOD	
	1 μl*	4 μl**
0–1	1.0 ± 0.1	4.0 ± 0.1
12	1.0 ± 0.1	3.8 ± 0.1
18	0.6 ± 0.2	3.5 ± 0.1
24	0.5 ± 0.2	3.4 ± 0.3

* n = 7 per interval.
** n = 6 per interval.

When the chloroform procedure is used with whole abdomens, uric acid in the Malpighian tubules might interfere with HiCN blood volume determinations. Using the method of Van Handel (1975) we found that the uric acid content of whole females was less than 7 μg during a sampling period of 48 h following a 3-μl meal of rat blood. The effect of adding uric acid (1 to 25 μg) to Drabkin's reagent alone was negligible ($\bar{x}$ OD = 0.025 ± 0.001 SE, n = 12), and the addition of up to 10 μg of uric acid to 1 or 3 μl of rat blood was also without effect. Although uric acid was of no consequence in HiCN determinations of *Ae. aegypti*, it might be in larger vectors which take more blood, because 20 μg or more of uric acid increased absorbancies of 1- and 3-μl blood samples up to 20%.

Freezing females after the blood meal

When it was necessary to analyze many mosquitoes in a single experiment, we found it convenient to freeze them, allowing the analysis to be spread over several days. Females can be frozen for up to 1 week at −27°C without any appreciable effect on their subsequent hemoglobin analysis, but they become difficult to dissect if stored further. However, if the dissected blood meal is frozen as hemiglobincyanide it may be stored for a much longer period. After the midgut containing blood is incubated in Drabkin's solution for 20 min, during which time the hemoglobin is converted to hemiglobincyanide, the solution can be frozen for a minimum of 40 days at −27°C without losing its stability. In this way, mosquitoes can be dissected

in the field, and the blood meal frozen and sent to a distant laboratory for analysis.

DISCUSSION

We have shown that measurement of hemiglobincyanide is a valid technique for determining the size of the mosquito's blood meal. The HiCN method is unaffected by urination or salivation as long as the insect does not excrete erythrocytes, as in the case of *Anopheles stephensi* Liston. The advantages of the HiCN method over weighing are that an unrestrained host can display its normal defensive behavior and that the feeding process need not be observed if a sample of the host's blood is available. The advantage over isotope labelling is that HiCN can be used with any vertebrate, whereas the blood of only small animals can be labelled, and as Redington & Hockmeyer (1976) have reported, the isotope may not remain evenly distributed throughout the circulatory system. The HiCN method is simpler and more reliable than the pyridine-hemochromogen method of Sutton & Arthur (1962), which was modified by Bursell (1966) for tsetse. The color of their derivative was unstable, and a rigid time schedule was required. The disadvantage of the HiCN method is that once analyzed, the mosquito cannot be used for further experimentation.

We recommend that mosquitoes be dissected, as this avoids the analysis of unfed females and does not require the use of a centrifuge, which may not be available in the field. With the chloroform procedure, the background variation of the unfed abdomen sometimes leads to false positives, but it may be preferable when large numbers of fully engorged mosquitoes are analyzed, or when the technical ability required for dissection is not available.

The stability of the hemiglobincyanide derivative and the simplicity of the HiCN determination provides a convenient method with which to investigate various questions relating to blood-feeding behavior of vectors. In the laboratory, we have used this technique to study the effect of prior blood meals on refeeding by mosquitoes (Klowden & Lea 1978), and in the field, to determine the quantity of blood ingested by wild populations of mosquitoes entering a stable trap and feeding on a specific host (Klowden & Lea 1979).

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