

BLOOD VOLUME AND WATER CONTENT OF THE MALE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* L. —METHODS AND THE INFLUENCE OF AGE AND STARVATION

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Abstract—The water per cent changes little, if at all, with the age of the adult insect. The blood volume per cent decreases markedly during the first 4 days and only slightly thereafter. There was no correlation between the daily fluctuations of blood volume per cent and those of total water per cent. Whereas the total water per cent remained relatively stable in the fed insect after the first week, it *increased* to a significant degree in the starved insect, especially after the sixth day of starvation in both the insects examined whole and eviscerated.

The blood volume per cent *decreased* as a result of starvation. The blood volume per cent of 36.3 ± 0.68 (for 2- to 3-week-old males) is much higher than previously reported. Within fourfold limits (15,000–62,000 cpm) the dose of C^{14} inulin injected had no significant effect on the blood volume. Heating the insect at 50 or 55°C did not alter the blood volume, but heating at 60°C for 15 sec caused an *increase*. Heating at 60°C for 1 min coagulated the outer tissues of the insect and made bleeding difficult. Chilling, which retarded coagulation, failed to change the blood volume. Starvation reduced coagulation. It also reduced the blood volume which nevertheless stayed at a high level.

Age and starvation are discussed with respect to their influence on water balance. The findings are also discussed with respect to the techniques employed by ourselves and other investigators.

INTRODUCTION

IN STUDYING the effect of exposure to a lethal dose of beta radiation on the composition of the blood of the American cockroach, *Periplaneta americana* L., we observed that many of the insects failed to bleed when punctured and appeared to be dehydrated even though apparently healthy and active. The thought that radiation or the ensuing starvation might have caused changes in the water balance led us to investigate the effect of these influences and of age on total water and blood volume of the adult male insect.

GUNN (1931) had previously observed a water content of 70.4–74.8 per cent of body weight in the cockroach, while MERRILL *et al.* (1946) found an average of

68.9 per cent in wild adults of mixed sexes and an insignificant reduction after 48 hr of starvation in a 'moist' atmosphere. Increases in haemolymph water and tissue water in muscle and nerve cord and sheath had been observed by TOBIAS (1948) in cockroaches shifted from a diet of Purina Dog Chow to lettuce, but no change was seen in the water content of nerve cord and sheath of cockroaches starved for 24 hr. WHEELER (1963) found that the blood volume of *P. americana* heat-fixed at 60°C for 1 min ranged from 14 to 21 per cent of the body weight and was highest in adults at ecdysis. His values, obtained by modification of YEAGER and MUNSON's technique (1950), were in substantial agreement with theirs except with their higher value of 27.5 per cent in males. YEAGER and MUNSON (1950) had also concluded that the prolonged withholding of food and water from larvae of the American cockroach failed to change blood volume per cent, and BUXTON (1930) and FRAENKEL and BLEWETT (1944) obtained similar results with the immature forms of certain beetles. On the other hand, LEE (1961) has shown that age and diet affect the water balance of the desert locust, *Schistocerca gregaria*, and recognized, as have HOWDEN and KILBY (1960), the limited usefulness of haemolymph values without reference to the age and physiological state of the insect.

LEVENBOOK's recent use of C¹⁴ carboxyl-labelled inulin (1958) indicated that it was probably superior to the dye injection, sodium chloride, or cell dilution techniques used by Yeager and Munson, as it was not bound by the blood proteins or metabolized or excreted, and could be recovered quantitatively from the blood of the mature *Prodenia eridania* larva. C¹⁴ inulin was also used by BRICTEUX-GRÉGOIRE and FLORKIN (1959) in the study of body fluid distribution and nitrogen and amino acid concentration in the larva of *Bombyx mori*, and has been put to similar use more recently by LEVENBOOK (1962) in the study of amino acid distribution in *P. eridania*. Since C¹⁴ inulin might be acted upon differently in different species of insects, we first examined its disposition in the cockroach and its suitability for the present study of the effect of age and starvation on the water content and blood volume of the American cockroach.

MATERIALS AND METHODS

Virgin adult male cockroaches from our colony were used throughout the work. In each experiment the insects were of the same age except in the experiment dealing specifically with the effect of age. The fed insects were maintained on a diet of Purina Laboratory Chow and water throughout, while the starved ones were denied food at the start of the experiment or some days before but always had water available. Each insect was isolated in its own 250 ml beaker at the beginning of an experiment.

C¹⁴ carboxyl inulin containing 2.15–3.0 $\mu\text{c}/\text{mg}$ was diluted in aqueous solution so that 5 μl emitted counts of 15,000–62,000/min by liquid scintillation counter. These solutions were used for the evaluation of dosage effects and blood-volume determinations. Solutions emitting about 8600 cpm were used for the metabolism studies with a gas flow counter. 5 μl of solution was injected with a 10 μl syringe

fitted with a 28 gauge needle midlaterally between the sixth and seventh sternites so as to reduce the risk of haemorrhage on retraction of the needle and avoid the danger of puncturing the crop. This procedure also avoided contaminating the coxal area from which the blood sample was to be drawn. Before the cockroaches were injected they were chilled for about 25 min at refrigerator temperature (4.5°C) to lessen their activity and relax them in order to reduce the chance of haemorrhage and retard the coagulation of the blood. $10\ \mu\text{l}$ samples of blood were taken from the insects 4 hr after injection. The exudate from a slight incision in the soft cuticle of the coxal joint was taken up into a calibrated drawn glass pipette and transferred immediately, with repeated washing, into a vial containing 1.0 ml of water to which 5 ml of scintillation fluid of the following composition was then added for counting:

5 parts dioxane

1 part cellosolve

1.0% PPO

0.05% POPOP

5.0% naphthalene (BRUNO and CHRISTIAN, 1961).

An alternative procedure was to add the sample to a planchet containing a drop of water and counting the dispersed mixture by gas-flow counter. A separate pipette was generally used for withdrawing each sample. The quenching effect of blood was measured by comparing the counts of a $5\ \mu\text{l}$ dose of C^{14} inulin alone and mixed with $10\ \mu\text{l}$ of blood.

The loss of inulin by excretion, absorption, or metabolism was determined. The excreta of groups of insects which had been injected were collected over varying periods of days and counted for their radioactivity. Insects were immobilized by cold, the fat body and gonads removed and weighed before drying in planchets, and the intestines, with Malpighian tubes attached, were clamped at both ends and severed, freed of haemocoel fluid in water, and dispersed as a mince in planchets. In some experiments the contents were removed from the gut and counted separately. We are not sure that the counts obtained in the faeces were given by C^{14} carboxyl inulin or by some other form of combined C^{14} . It may be pertinent to note, however, that in numerous investigations of the fate of inulin or carboxyl inulin in man and other animals the material has been recovered in its injected form.

To determine the C^{14} inulin oxidized, the CO_2 from different groups of insects injected with C^{14} inulin was trapped in a solution of NaOH or $\text{Ba}(\text{OH})_2$ by an adaptation of LEVENBOOK's procedure (1958) and assayed for C^{14} .

For determining changes in body water and weight of the intestines, a certain number of the insects was chloroformed and eviscerated at daily intervals and the intestines of each insect, stripped of fat body and Malpighian tubes, placed on a weighed glass slide, dried at 112°C overnight, and weighed to obtain the dry weight. The eviscerated body of each insect was weighed immediately, dried at 112°C overnight, and weighed again the following morning. In addition, total body water was determined in several groups of whole insects.

Heat-fixation, when used, was done by trapping the cockroach in a large pre-heated test-tube which was then capped with wire gauze and completely submerged in a large basin of water at the desired temperature for the required length of time. The tube was then drained promptly and the insect dried and bled.

RESULTS

C¹⁴ in the excreta and gut tissue

One-week-old adult males which had been maintained on a regular diet were segregated into two groups: fed, and starved. They were injected with C¹⁴ inulin and placed in individual beakers, and after 8 days four specimens from each group were dissected and the intestinal contents removed for counting. The average per cent of the injected counts recovered in the intestinal contents was found to be 4.3 ± 2.07 S.D. and 5.3 ± 1.14 S.D. for the fed and starved, respectively (Table 1).

TABLE 1—PER CENT OF INJECTED C¹⁴ RECOVERED IN FAECES AND TISSUES AFTER INJECTION OF C¹⁴ CARBOXYL INULIN

Days after injection	Material	Number of cockroaches	Fed	Number of cockroaches	Starved Mean \pm S.D.
8	Intestinal contents	4	4.3 ± 2.07	4	5.3 ± 1.14
9	Intestinal contents and gut			4	5.1 ± 0.75
6	Accumulated faeces, intestinal contents, and gut tissue	9	7.1 ± 2.3	10	9.4 ± 4.7
4 hr	Intestinal contents	6	7.8 ± 2.11	6	7.8 ± 2.16
6	Fat body	9	1.9 ± 1.11	9	1.8 ± 0.69
6	Gonads	9	0.2 ± 0.37	9	0.4 ± 0.32

Counts of the intestinal contents and gut tissue made on four specimens of the starved insects on the ninth day showed that the gut tissue absorbed no inulin. The C¹⁴ in the accumulated faeces, intestinal contents, and gut tissue in cockroaches 6 days after injection was found to be 7.1 ± 2.3 and 9.4 ± 4.7 per cent of the amount injected for the fed and starved, respectively. Values for the intestinal contents of corresponding groups of insects dissected 4 hr after being injected were 7.8 ± 2.11 and 7.8 ± 2.16 , respectively. These values indicate that there is no significant difference in the excretion of inulin between the groups and that most of the inulin that is excreted enters the intestinal lumen within a few hours after being injected (Table 1). The table also shows that there is little accumulation of inulin by the fat body and less by the gonads. The values are undoubtedly exaggerated, especially for the fat body, by virtue of the fact that the organs were not washed to remove the blood with which they were bathed. The conclusion seems warranted that the inulin is completely distributed in 4 hr, and that none, or little, of it is accumulated by the tissues.

Oxidation of C¹⁴ inulin

In duplicate experiments, ten fed cockroaches were put into jars for a period of 19 hr during which they distributed themselves at rest on the wall and floor of the jars and remained at rest the greater part of the time and without violent activity at any time. At the end of this period they were found to have produced 0.45 mg CO₂ per gramme of cockroach per hour. After 5 days of starvation the same insects produced 0.36 mg CO₂ per gramme per hour. In duplicate experiments ten cockroaches of the same age group were starved for 6 days and then injected with C¹⁴ inulin. After 20 hr the CO₂ was precipitated and counted as BaCO₃ in the first experiment and as Na₂CO₃ in the second. In neither instance did the count exceed that of the background radiation (Table 2). It may therefore

TABLE 2—RESPIRATION OF INJECTED C¹⁴ LABELLED INULIN

Number of cockroaches	State of cockroach	Time of injection	MgCO ₂ per g of cockroach per hr	C ¹⁴ in CO ₂
10	Fed		0.454	
10	Starved 5 days		0.363	
10	Starved 6 days	Sixth day		0 (BaCO ₃)
10	Starved 6 days	Sixth day		0 (Na ₂ CO ₃)

be concluded that C¹⁴ inulin did not enter into the metabolism of the male cockroach. These results thus parallel the observation of LEVENBOOK (1958) that C¹⁴ inulin was not metabolized by the army worm, *Prodenia eridania*. It is evident that C¹⁴ inulin is neither absorbed by the cells nor respired and that in a period of 4 hr only the 7.8 per cent excreted (Table 1) need be considered in evaluating the count. Since this loss causes a low blood count, and hence too high an indicated blood volume, it must be subtracted from the original count injected to give the true blood volume.

Quenching effect of blood sample

Ten μl of cockroach blood was found to reduce the count of 5 μl C¹⁴ inulin solution by 13.2 per cent in the gas flow counter and by only 0.27 per cent in the scintillation counter. The error of the dose count of C¹⁴ inulin was less than 1 per cent by the scintillation counter when 30,000 cpm were injected and 3 per cent by the gas flow counter when 8600 cpm were injected. It is evident that while the quenching effect in the scintillation fluid is insignificant and can therefore be ignored in the calculation of blood volume, the quenching effect in the planchets is large and must be used to obtain correct values. Since about 7.8 per cent of inulin was lost through the intestine and 10 μl of blood reduced the count of inulin by 13.2 per cent when measured in planchets, these are the values that must

be taken into account in calculating the blood volume. Thus, the formula for blood volume in μl (with counts corrected for background)

$$V_b = \frac{V_s(C_o - 0.078 C_o)}{C_b} - V_i$$

where V_b = blood volume

V_s = volume of blood sample (i.e. 10 μl)

C_o = count of original injection

V_i = μl of solution injected (i.e. 5 μl)

C_b = count of blood sample

which is applied to samples counted by scintillation must be modified by the quenching factor for samples counted in planchets, thus:

$$V_b = \frac{V_s(C_o - 0.078 C_o)}{C_b/0.868} - V_i$$

The term blood volume per cent is used in the sense of YEAGER and MUNSON (1950) to designate the volume in μl per 100 mg body weight. Converting μl of blood of sp. gr. 1.0149 (YEAGER and FAY, 1935) into mg would permit the presentation of the data in terms of blood weight per cent.

The great difference between our values for blood volume and Yeager and Munson's, and Wheeler's, has led to an examination of techniques with a view to uncovering the cause of these differences and establishing correct values. Three possible sources of error are examined: (1) the effect of dose (cpm) of C^{14} carboxyl inulin; (2) the effect of heating the insect at different temperatures before bleeding to eliminate coagulation; and (3) the effect of other means of eliminating coagulation.

Effect of dose of C^{14} inulin on the blood volume of the insect

Table 3 shows that there was little difference in the blood volume whether the cockroaches were injected with 15,000, 30,000, or 62,000 cpm of C^{14} inulin.

The effect of heating the insect at different temperatures

Heat-fixation at 60°C for 1 min was used by Wheeler to eliminate coagulation of the cells and also for determining blood volume. We found that the effect of heating the insects even at 50°C for 30 sec was lethal. Some seemed dead at the time of bleeding, while after 24 hr a few others were responsive to touch and one or two had managed to regain their feet but were quite lethargic. All the insects heated at higher temperatures or for longer periods of time were dead before the end of treatment. Clotting continued to occur in some of the insects heated at 50°C for 2 min. Although clotting was rare in the insects heated at 55°C for 30 sec, less and less blood was available as the temperature or length of treatment rose. Except with the insects heated at 50°C for 30 sec, it was necessary to enter the haemocoel to obtain blood. The cockroaches heated at 60°C for even 15 sec were difficult to bleed and the softer outer tissues appeared coagulated and shrunken, as if cooked. The condition was intensified by heating at 60°C for 1 min and it

TABLE 3—DOSE EFFECTS OF C¹⁴ INULIN ON BLOOD VOLUME PER CENT ($\mu\text{l}/100\text{ mg}$) IN 2-WEEK-OLD MALE COCKROACHES

15,000 cpm		30,000 cpm		62,000 cpm	
Blood volume μl	Blood volume %	Blood volume μl	Blood volume %	Blood volume μl	Blood volume %
189	28.5	234	39.7	183	32.4
233	38.2	201	34.4	217	35.5
230	33.7	164	28.2	271	43.2
236	35.1	200	34.2	220	29.2
235	39.6	179	30.3	219	36.4
195	28.2	194	29.5	225	33.7
234	38.4	219	38.1	191	33.0
261	34.5	241	38.4	231	40.8
218	33.4	159	32.7	180	32.0
220	35.7	198	34.8		
273	45.9	183	32.5		
220	29.9	243	33.8		
228	37.8				
321	45.5				
230	40.4				
Mean \pm S.E. 36.3 \pm 1.38		Mean \pm S.E. 33.9 \pm 1.03		Mean \pm S.E. 35.1 \pm 1.51	

TABLE 4—EFFECT OF HEATING ON BLOOD VOLUME IN 2-WEEK-OLD MALE COCKROACHES

Untreated	Blood volume per cent ($\mu\text{l}/100\text{ mg}$)				
	50°C		55°C		60°C
	30 sec	120 sec	30 sec	120 sec	15 sec
39.7	29.5	30.7	34.6	34.2	34.8
34.4	36.5	29.1	35.8	36.5	37.8
28.2	35.8	38.6	40.1	29.7	33.1
34.2	29.6	30.0	32.6	32.8	33.7
30.3	45.3	33.6	34.3	39.7	49.3
29.5	35.6		31.4		52.8
38.1	39.4		30.7		51.3
38.4	30.8				39.5
32.7	34.3				39.1
34.8					
32.5					
33.8					
Mean \pm S.E. 33.9 \pm 1.03	34.1 \pm 1.57	32.4 \pm 1.7	34.2 \pm 1.2	34.4 \pm 1.7	41.3 \pm 2.6

was then unprofitable to work with the animals. Table 4 shows that heating the cockroaches up to a temperature of 55°C for 2 min did not change the blood volume. On the other hand, heating at 60°C for 15 sec caused a pronounced *increase in the blood volume* and at the same time a greater variance. It will be shown that destroying the clotting properties of the blood could not have brought about this increase.

The effect of other means of avoiding coagulation of the blood (starvation, chilling) on the blood volume

Starvation reduces the clotting of blood and tends to decrease the blood volume significantly but not drastically, as we have shown (Table 6, Fig. 3). Chilling at 38–40°C for about 25 min retards coagulation; nevertheless the blood volume of chilled insects was not significantly different from that of untreated insects (Table 5). Thus, neither destroying, nor attenuating, nor retarding the clotting of blood in the insect reduced its volume to the levels reported by Yeager and Munson and by Wheeler.

TABLE 5—EFFECT OF CHILLING ON BLOOD VOLUME PER CENT ($\mu\text{l}/100\text{ mg}$) IN 6-WEEK-OLD MALE COCKROACHES

Untreated	Chilled
37.7	32.7
30.6	42.2
30.6	36.8
31.6	32.2
31.1	31.3
30.5	31.3
31.9	30.6
31.9	30.6
30.4	31.8
32.7	32.4
34.5	31.2
26.7	34.2
36.4	32.1
38.2	29.4
	34.7
	33.1
	32.1
Mean \pm S.E.	
32.5 \pm 0.84	32.9 \pm 0.69

The effect of age on total water and blood volume per cent in adult males

Fed adult cockroaches of different ages were weighed and injected with C^{14} inulin; 4 hr later 10 μl of blood was withdrawn for counting and the insect killed with chloroform and dried. Observations over an age period 1–31 days showed no

significant trend in the total water content, which was 71.3 ± 2.10 per cent of the total weight (Fig. 1). The blood volume per cent, on the other hand, showed a very different picture. While the overall slope was minus 0.20, there was an especially pronounced drop in blood volume per cent during the first 4 days of age,

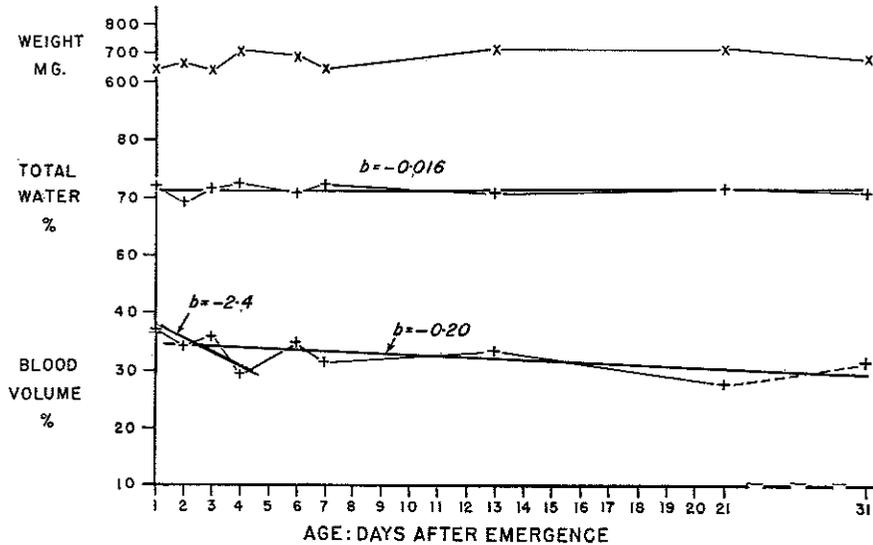


FIG. 1. The effect of age on per cent of blood volume and total water of adult male cockroaches. b is the slope of regression.

with a slope of minus 2.40 (Fig. 1). The slope diminished rapidly between the seventh and the thirty-first day, being only minus 0.10. The effect of age on blood volume per cent, particularly during the first 4 days following emergence, necessarily affects the quantitative evaluation of haematological changes and therefore requires the selection of insects of appropriate age for the purpose in view. The relatively stable state after the first week enables the investigator to establish comparable conditions over a wide range differing from several days to some weeks, and has accordingly warranted our use of insects ranging in age from 1 to 3 weeks for most of our experiments.

The effect of starvation on water content

Fed male cockroaches were divided into two groups, one of which was starved for 5 days. On the fifth day and at daily or frequent intervals thereafter a number of each group, after being weighed and bled for the determination of blood volume, was killed and dried. The data show that while the fed cockroaches maintained a fairly constant water content, that of the starved cockroaches increased significantly (Fig. 2A; Table 6).

To obviate any possible effect of the intestinal contents on the weights, several hundred insects were divided into two groups, one of which was then starved. At

daily intervals representatives of each group were eviscerated and water content determined. In every experiment the water content of the starved insects was higher than that of the fed, with a significant rise ($P = 0.01$) occurring at about the same time after the beginning of starvation as it did with the whole insect (Fig. 2B). Although the total water per cent is slightly lower for both the fed and the starved

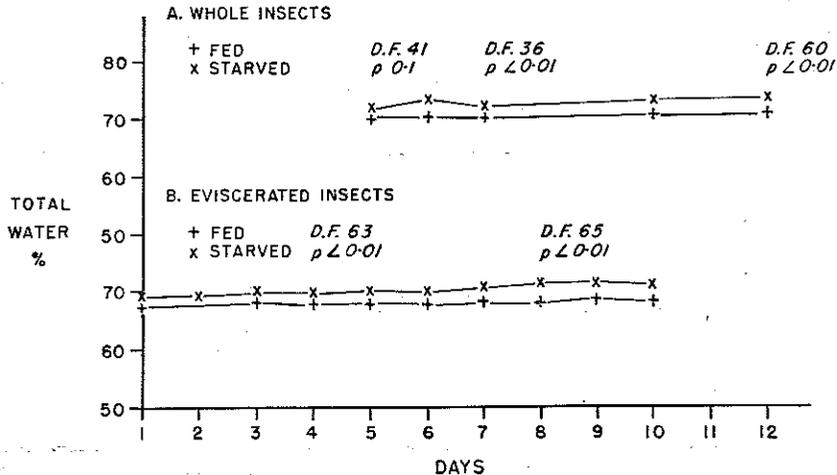


FIG. 2. The effect of starvation on per cent of total water. Insects examined or eviscerated. D.F. = degrees of freedom; P = probability statistic. With whole insects (A), day 1 is actually fifth day of starvation.

groups of the eviscerated, by virtue of the removal of the intestinal water and contents, the results are otherwise similar to those obtained with the whole animal. The intestines themselves could have had little effect on the results for they constituted but 5.6 per cent of the dry weight of the insect.

The effect of starvation on blood volume

We have seen that starvation increases the total water content of the cockroach in relation to its weight. Determination of blood volume per cent should indicate any shift in the distribution of water between the blood and the tissues. The insects used for these determinations were the same as those used for recording total water in Fig. 2A and a second separate group. The starved insects had therefore been without food for 5 days prior to the first bleeding.

The results show that the blood volume per cent of the fed cockroaches, aged 2-3 weeks, was relatively stable with a mean value of 36.3 ± 0.68 (S.E.) during the 8 days of testing. They show especially that the blood volume per cent of the starved insects was significantly less than that of the fed (Table 6, Fig. 3). Confirmatory differences have been obtained in another experiment using the gas flow counter for determining differences in blood volume per cent on the sixth and eighth days of starvation.

Our results lead us to conclude that: (1) the per cent water changed little, if at all, with the age of the adult insect; (2) the blood volume per cent decreased markedly during the first 4 days and only slightly thereafter. There was no correlation between the daily fluctuations of blood volume per cent and those of total water per cent;

TABLE 6—THE EFFECT OF STARVATION ON BLOOD VOLUME PER CENT ($\mu\text{l}/100\text{ mg}$) AND TOTAL WATER OF ADULT MALE COCKROACHES

Day of starvation		No.	Weight, mg Mean \pm S.E.	Blood volume % Mean \pm S.E.	P	No.	Total water % Mean \pm S.E.	P
Fifth	Fed	23	724.8 \pm 14.3	34.9 \pm 0.63	<0.20	15	70.5 \pm 0.41	<0.2
	Starved	24	678.3 \pm 19.9	33.7 \pm 0.61		28	71.8 \pm 0.43	
Seventh	Fed	22	708.5 \pm 15.1	38.6 \pm 0.77	<0.05	13	70.3 \pm 0.47	<0.01
	Starved	15	682.6 \pm 15.7	36.1 \pm 0.84		25	72.4 \pm 0.51	
Twelfth	Fed	24	688.7 \pm 13.7	35.2 \pm 0.65	<0.01	29	71.0 \pm 0.57	<0.01
	Starved	26	662.4 \pm 14.2	32.5 \pm 0.75		33	74.1 \pm 0.51	

(3) whereas the total water per cent remained relatively stable in the fed insect after the first week, it *increased* to a significant degree in the starved insect, especially after the sixth day of starvation in both the insects examined whole and

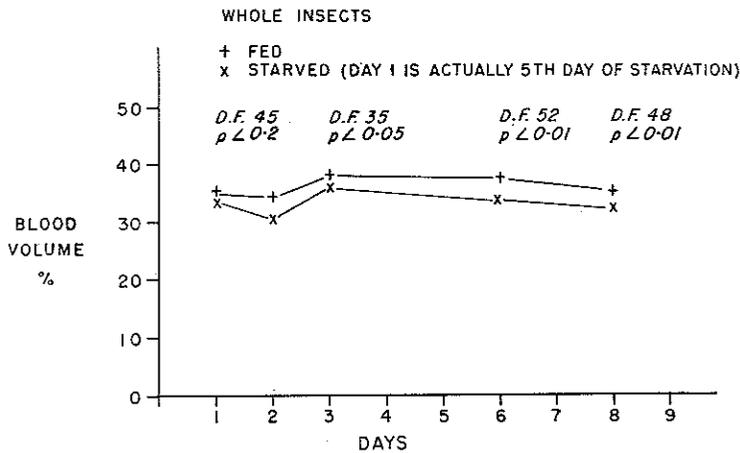


FIG. 3. The effect of starvation on per cent of blood volume.

eviscerated; (4) the blood volume per cent *decreased* as a result of starvation in the adult male cockroach; (5) the blood volume per cent is much higher than reported by YEAGER and MUNSON (1950) and by WHEELER (1963). The higher value of 36 per cent for the blood volume in the young fed cockroaches may be attributed to the improved technique permitted by the availability of C^{14} inulin and scintillation counting. This technique is also probably responsible for the recognition of a

significant change in blood volume per cent induced by starvation; (6) heat fixation (WHEELER, 1963) and other means of eliminating or controlling blood agglutination failed to cause any, or a major, reduction in blood volume. On the contrary, *heat fixation increased the blood volume.*

DISCUSSION

Our results show that the adult male cockroach does not merely live out its long life in physiological decline, but rather is in a more fluid state in the first few days after emergence. Apart from the change in respect to water, we have evidence of this in the fact that it is physiologically incapable of mating for several days after emergence. Young females remain virginal for several days, while males are unresponsive to the sex pheromone for the first 3 or 4 days and an increasing number become responsive during the next 2 weeks (WHARTON *et al.*, 1954). Thus it takes the adult some days to mature physiologically. These findings support the growing realization (ROCKSTEIN, 1959) that the young adult insect has not attained maturity full blown but is still in a state of development and subject to great change, as emphasized by LEE's findings (1961).

Wheeler has shown that the blood volume per cent of the cockroach varies with the phase of the last moulting cycle and that it falls during the first 24 hr following the last ecdysis, after which it returns to 'normal'. Our own observations show that the drop continues precipitately for the first 4 days following emergence and then tapers off in very much the same manner as shown by LEE (1961, Fig. 1) with *Schistocerca gregaria*. Our observations were made during a period for which Wheeler had no comparable data, for after the first 24 hr he used 'old random adults' that were not more than 21 days old. Our data show how greatly different the blood volumes are in adult males between the first to the fourth and the twenty-first day of age.

Heating the cockroach in water at 60°C for 1 min apparently destroys the coagulating system of the blood as it relates to the haemocytes, thereby permitting the cells to be examined individually. However, the treatment promptly kills the insect, its tissues appear cooked, and the blood ceases to flow freely or to be available in quantities comparable to the yield in the live cockroach. We could seldom obtain more than 1 or 2 μ l of blood from such animals. Such conditions and such small quantities must present difficulties in determining blood volume, as we have experienced, and as indicated by the high standard error of ± 2.6 for the blood volume per cent obtained by us with animals heated at 60°C for 15 sec, and by Wheeler with 'old random adults' heated at 60°C for 1 min. Nevertheless, even though Levenbook did not publish variances of blood volume per cent, and with no evidence of his own, Wheeler states that his own method gives repeatable and far less variable results than the C¹⁴ inulin technique as used by Levenbook with *Prodenia*, and now by ourselves with a standard error of ± 0.68 in the cockroach.

Our data show conclusively that heating the entire cockroach in a water bath at 60°C for 15 sec increases the blood volume while incurring a greater variance than with the live insect. Heating at 60°C for 1 min inhibited free bleeding and

made it almost impossible to obtain a desirable quantity of blood for evaluation. Heating under less rigorous conditions—which also, however, were lethal—permitted adequate sampling of the insects, but bleeding was somewhat encumbered and higher standard errors obtained without appreciably changing the final values. In fact, although chilling was effective as a retardant of coagulation and was seemingly harmless, no device was superior to direct, deft bleeding with a suitable pipette.

Wheeler's modification of Yeager and Munson's technique and use of heat-fixation, the latter of which was designed to destroy the cell agglutinants in blood, were not tested for their possible effect on blood volume. Is the error of estimating the intensity of colour of a blood sample from a cockroach injected with 0.25% amaranth less or more than with 1%? The standard error of ± 2.6 , compared with Yeager and Munson's of ± 1.87 , for male cockroaches suggests that it is. Heating the injected cockroach at 60°C, apart from causing a shift in body fluid, as we have shown, may cause an intensification of the dye colour. Lee's finding that it was sometimes necessary to use pressure to send the injected dye into the leg of the desert locust gives reason to believe that in the cockroach the dye may not be uniformly distributed and hence gives misleading values of blood volume. Possibly, as suggested by the shift of fluid from the tissues to the haemocoel which we have demonstrated, a concentration of the dye may occur in the antennae on heating. It also seems extraordinary that by using heat fixation which is claimed to be superior, Wheeler should obtain results that agree so closely with those of Yeager and Munson for all but the males which they injected with dye. Compared with our data of Table 6 for the fed cockroaches, or with any other set of our data except those obtained with insects heated at 60°C, his results with S.E. of ± 2.6 were not 'far less variable'.

The rapid changes which occur in the blood volume of the young male cockroach while the total body water is relatively constant show that important shifts are taking place in the water balance of the fed insect. Equilibrium is never fully attained even many days later. Since this is true of the routinely fed animals, one may consider a fluid state to be the norm. In view of the fact that in the starved condition there is an increase in total water per cent but a decrease in blood volume per cent, it is evident that food intake changes the balance, presumably by the assimilated solutes and the products of metabolism causing a shift of water from the tissues to the blood. There is thus a continuous change in water distribution in the routinely fed animals, with phases of gradient concentrations or regressions approaching or receding from equilibrium as the animal eats or fasts.

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REFERENCES

- BRICTEUX-GRÉGOIRE S. and FLORKIN M. (1959) Contribution à la biochimie du ver à soie. II. Teneurs d'un tissu de *Bombyx mori* en eau intracellulaire et en eau extracellulaire, et repartition de l'azote dialysable dans ces deux fractions. *Arch. int. Physiol. Biochim.* **67**, 29-34.

- BRUNO G. A. and CHRISTIAN J. E. (1961) Determination of carbon-14 in aqueous bicarbonate solutions by liquid scintillation counting technique. Application to biological fluids. *Anal. Chem.* **33**, 1216-1218.
- BUXTON P. A. (1930) Evaporation from the mealworm (*Tenebrio*: Coleoptera) and atmospheric humidity. *Proc. roy. Soc. (B)* **106**, 560-577.
- FRAENKEL G. and BLEWETT M. (1944) Utilization of metabolic water. *Bull. ent. Res.* **35**, 127-139.
- GUNN D. L. (1931) Temperature and humidity relations of the cockroach. *Nature, Lond.* **128**, 186.
- HOWDEN G. F. and KILBY B. A. (1960) Biochemical studies on insect haemolymph. I. Variations in reducing power with age and the effect of diet. *J. Ins. Physiol.* **4**, 258-269.
- LEE R. M. (1961) The variation of blood volume with age in the desert locust (*Schistocerca gregaria* Forsk.). *J. Ins. Physiol.* **6**, 36-51.
- LEVENBOOK L. (1958) Intracellular water of larval tissues of the southern army worm as determined by the use of C¹⁴ carboxylinulin. *J. cell. comp. Physiol.* **52**, 329-339.
- LEVENBOOK L. (1962) The distribution of free amino acids, glutamine, and glutamate in the southern army worm, *Prodenia eridania*. *J. Ins. Physiol.* **8**, 559-567.
- MERRILL R. S., SAVIT J., and TOBIAS J. M. (1946) Certain biochemical changes in the DDT poisoned cockroach and their prevention by prolonged anesthesia. *J. cell. comp. Physiol.* **28**, 465-476.
- ROCKSTEIN M. (1959) Metachemogenesis-postemergence biochemical maturation in insects. *Smithson. misc. Coll.* **137**, 263-288.
- TOBIAS J. M. (1948) Potassium, sodium and water interchange in irritable tissues and hemolymph of an omnivorous insect, *Periplaneta americana*. *J. cell. comp. Physiol.* **31**, 125-142.
- WHARTON D. R. A., MILLER G. L., and WHARTON M. L. (1954) The odorous attractant of the American cockroach, *Periplaneta americana* L. I. Quantitative aspects of the response to the attractant. *J. gen. Physiol.* **37**, 461-469.
- WHEELER R. E. (1963) Studies on the total hemocyte count and haemolymph volume in *Periplaneta americana* (L.) with special reference to the last moulting cycle. *J. Ins. Physiol.* **9**, 223-235.
- YEAGER J. F. and FAY R. W. (1935) Micromethod for determining insect hemolymph specific gravity (*Periplaneta americana* Linn.). *Proc. Soc. exp. Biol. N.Y.* **32**, 1667-1669.
- YEAGER J. F. and MUNSON S. C. (1950) Blood volume of the roach *Periplaneta americana* determined by several methods. *Arthropoda* **1**, 255-265.