

## IONIC CONTENT OF HAEMOLYMPH IN THE COCKROACH, *PERIPLANETA AMERICANA*

### A CRITICAL ANALYSIS

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(Received 13 March 1970)

#### INTRODUCTION

With the development of fine microanalytical techniques, a rather considerable amount of information has been collected concerning the ionic content of the blood in insects (Bishop, Briggs & Ronzoni, 1925; Brecher, 1925, 1929; Drillhon, 1934; Babers, 1938; Boné, 1944, 1946; Tobias, 1948*a, b*; Clark & Craig, 1953; Duchâteau, Florkin & Leclercq, 1953; Ramsay, 1953; Hoyle, 1952-4, 1956; Asperen & Esch, 1956; Clark, 1958; Treherne, 1961; Sutcliffe, 1962, 1963; Pichon, 1963; Pichon & Boistel, 1963*a, b*; Brady, 1967*a, b*, 1968; Wall, 1970; and others). There are great differences between different orders of families, some having a relatively high Na/K ratio as in vertebrates, whereas this same ratio can be far lower than unity in others (0.12 in the haemolymph of *Melolontha vulgaris*: Boné, 1944). Another particularly striking feature of this aspect of haemolymph composition in insects is that it is extremely variable. The Na/K ratio in the blood of cockroaches reared on a mixture of tap water and milk powder may vary between 1 and 14 (Pichon, 1963; Pichon & Boistel, 1963*b*). Furthermore the mean value of the Na/K ratio for a large batch of animals can be changed from 6.2 for insects reared on a standard mixed diet to 4.4 for insects reared on an exclusively vegetarian diet (Tobias, 1948*a*, Pichon, 1963). It can be further decreased to 2.7 with oral administration of a concentrated solution of KCl (Tobias, 1948*a*) and to 2.5 if the animals are left without food and drink for 10 days (Pichon, 1963; Pichon & Boistel, 1963*a*).

Similar variations of the Na/K ratio with diet have been observed in other insects, for instance the stick insect, *Carausius morosus*, (Ramsay, 1952), in the locusts *Locusta migratoria* (Ellis & Hoyle, 1954; Hoyle, 1954) and *Schistocerca gregaria* (Phillips, 1961) or the larvae of *Drosophila melanogaster* (Croghan & Lockwood, 1960).

The possible effects of such changes need no emphasis because of the importance of Na<sup>+</sup> and K<sup>+</sup> ions to excitable tissues (see Hodgkin, Huxley & Katz, 1952; Boistel & Coraboeuf, 1958; Yamasaki & Narahashi, 1959; Narahashi, 1963; Pichon, 1968).

We have shown in a recent paper (Pichon & Boistel, 1968) that, in *Periplaneta americana*, the resting potential of giant axons recorded *in vivo* in the haemolymph

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was low ( $-43.0$  mV). This low value could be related to the relatively high potassium concentration in the haemolymph in 'normal' insects. It seemed particularly interesting to perform a series of experiments of this kind on cockroaches whose haemolymph had been previously modified, for instance with high  $K^+$  diets, and see whether these changes were followed by alterations in the membrane characteristics of excitable tissues. The purpose of the experiments described in this paper was to provide the biochemical basis for this more complete study of nervous excitability and its relation with blood ions in the cockroach *Periplaneta americana*.

The experiments attempt to answer the following questions: (1) How stable are the ionic concentrations in the haemolymph during the larval life of this insect and how far are they regulated? (2) Is the ionic distribution in the haemolymph homogenous? The results deal with  $Na^+$ ,  $K^+$  and  $Ca^{2+}$  ions. They describe (i) the changes in the ionic content of haemolymph throughout the larval stages; (ii) the kinetics of ionic regulation after changes in the intake of ions in the food; (iii) multiple measurements in the haemolymph of the same insect, (iv) serial measurements of blood ions from different regions of the animal.

#### METHODS

##### *Rearing*

The cockroaches were reared at  $26-28^\circ C$  in glass aquaria. Each aquarium contained no more than 30 adult animals. The standard diet consisted of a mixture of milk powder and wheat flour (5/6 flour and 1/6 milk powder), lettuce leaves and tap water. The altered diets could be derived from this standard one by suppressing any of the constituents, and by adding distilled water or sucrose or high  $K^+$  solution ( $25$  mM- $Na^+$ ,  $1.75$  mM- $Ca^{2+}$  and  $265$  mM- $K^+$ ).

##### *Collection of blood samples*

Except for the last series of experiments, (iv) the procedure for collecting samples has already been described (Pichon, 1963). The insects were anaesthetized with sulphuric ether. The dorsal articular membrane between the first and second thoracic ganglia was punctured by means of a glass needle and the droplet of haemolymph which appeared at this level was drawn into a calibrated glass micropipette ( $10-20 \mu l$ ). The sample was then discharged into a 10 ml beaker and weighed.

##### *Analytical procedure*

The samples were diluted in distilled water and the proteins were precipitated by adding a small amount of a 20% solution of trichloroacetic acid.

The ionic content of these samples was analysed by flame photometry (flame photometer Coleman model 21). The standard solution consisted of solutions of NaCl, KCl and  $CaCl_2$  in distilled water at concentrations close to those normally found in haemolymph samples; the errors due to radiation interferences were thus nearly eliminated and the same series of standards could be used for the three ions. This technique allowed very small concentrations of cations to be measured with a precision approaching 2% for  $Na^+$  and  $K^+$  and 5% for  $Ca^{2+}$ .

The results will be expressed in mM/kg  $\pm$  the standard error: E.

## RESULTS

A. *Changes of blood ionic content during larval life*

The first thing to do for these experiments was to isolate the different stages starting from the ootheca. New-born insects were transferred to a rearing aquarium. This aquarium was then checked daily to find out which animals had just moulted (as judged by their colour and size). Such second-stage animals were removed from the first aquarium and put in a second one. By following the same procedure for each stage it has been possible to obtain 15 different stages (including the imago). Imagos can appear as early as the 13th stage, and in this case they were put directly into the 15th stage aquarium. The diet of these insects consisted exclusively of lettuce leaves, for this seems to be one of the only foodstuffs that first instar animals can use properly. The results of our analyses are shown in Table 1 (each mean result corresponds to more than 20 insects of a given stage).

Table 1. *Ionic content of haemolymph during the larval life of Periplaneta americana*

Stage	Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>2+</sup>	Na/K (mM/kg of haemolymph)
o (ootheca)	38.4	63.5	4.3	—
1	114.8	61.8	0.9	1.85
2	143.7	56.2	1.6	2.55
3	81.8	32.7	0.6	2.35
4	95	72.6	6.3	1.3
5	93.3	22.2	0.6	4.2
6	102.5	27.7	3.2	3.7
7	125.7	44.4	3.7	2.83
8	133.3	3.8	8.15	3.5
9	102.0 ± 15.9	69.4 ± 18.4	10.3 ± 4.4	1.4
10	134.2 ± 18.4	33.4 ± 0.5	2.3 ± 0.5	4.0
11	127.4 ± 13	23.1 ± 1.8	3.5 ± 1.5	5.5
12	144.7 ± 22.5	34.7 ± 8.4	5.05 ± 0.75	4.1
13	104.5 ± 20	46.9 ± 8	6.2	2.2
14	125.3 ± 25.5	52.1 ± 29.6	5.75	2.4
15	119 ± 28	28 ± 20	3.1 ± 1.1	4.3

The evolution of cationic blood content during the larval life is illustrated in Fig. 1. One can see that despite large variations from the mean (proportionally much larger for K<sup>+</sup> than for Na<sup>+</sup>), the Na/K ratio remains fairly constant during the larval life of the cockroach. A careful analysis of the results shows that there is a slight increase in the Na<sup>+</sup> and Ca<sup>2+</sup> concentrations and a slight decrease in the K<sup>+</sup> concentrations. These changes are not significant, however, and one can say that the ionic characteristics of blood are fixed from the first stage of the larval life.

B. *Kinetics of ionic regulation after change in the ionic content of the food*

These experiments were initially carried out in an attempt to obtain different batches of insects with a given haemolymph concentration of Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup>. They were based on previously reported observations that blood potassium could be raised by a lettuce diet (Tobias, 1948; Pichon, 1963, Pichon & Boistel, 1963*b*) or by food deprivation and dehydration (Pichon, 1963; Pichon & Boistel, 1963*a*) or decreased by food

deprivation without dehydration (Pichon, 1963). In order to obtain more homogenous results and to avoid variation due to previous feeding, the animals were starved for 4 days and then given one of the following diets:

Diet 1. Standard (mixture of lettuce leaves, milk powder, wheat flour and tap water).

Diet 2. Lettuce leaves

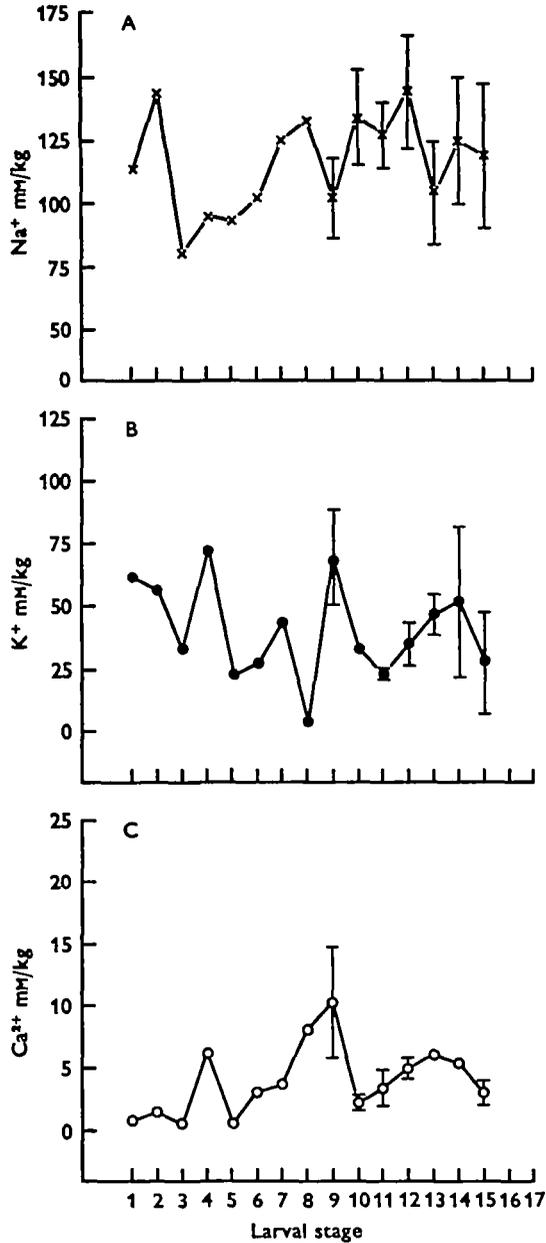


Fig. 1. Evolution of the cationic concentration in the haemolymph of *Periplaneta americana* during larval life. A, Na<sup>+</sup> ions; B, K<sup>+</sup> ions; C, Ca<sup>++</sup> ions. The vertical lines drawn through the points indicate the extent of twice the standard error of the mean.

Diet 3. No food or drink.

Diet 4. Lettuce leaves and glucose.

Diet 5. No food but distilled water to drink.

Diet 6. No food but high potassium saline to drink.

The ionic content of the blood was then measured after 4, 6, 8, 10, and 30 days on this diet. The results are listed in Table 2 and the variations with time for each individual diet analysed in Fig. 2A-F.

Table 2. *Kinetics of variations in the ionic content of haemolymph in Periplaneta americana reared on different diets.*

Diet	No. of days on this diet					
	4	6	8	10	15	30
(1) Na <sup>+</sup>	155.8	152	151.1	150.2	146.7	—
K <sup>+</sup>	15.6	15.5	11.4	12.6	17.4	—
Ca <sup>2+</sup>	4.9	—	4.1	2.7	3.6	—
(2) Na <sup>+</sup>	142.4	150.0	147.2	126.5	—	119
K <sup>+</sup>	18.4	17.6	13.9	15.2	—	28
Ca <sup>2+</sup>	3.5	—	3.3	4.3	—	3.1
(3) Na <sup>+</sup>	177.4	161	174.1	185.6	125.0	74†
K <sup>+</sup>	28.2	22	22.7	23.7	26.7	19.5†
Ca <sup>2+</sup>	5.2	4.7	3.0	3.1	3.5	0.75†
(4) Na <sup>+</sup>	154.7	124	156.4	134.6	137.3	—
K <sup>+</sup>	18.8	16	14.6	9.4	12.6	—
Ca <sup>2+</sup>	3.7	—	4.2	3.0	3.9	—
(5) Na <sup>+</sup>	150.3	152	151.7	138.8	145.4	—
K <sup>+</sup>	19.7	16.0	16.4	11.6	7.9	—
Ca <sup>2+</sup>	3.6	—	3.4	3.3	3.2	—
(6) Na <sup>+</sup>	157.9	150	152	133.2	153	115.5
K <sup>+</sup>	22.6	41.2	16	14.2	10.7	19.4
Ca <sup>2+</sup>	3.2	—	3.7	3.5	3.7	5.0

\* Each result is the mean value for at least three individuals. † From Pichon (1963).

The first observation that one can make is that regulation of blood ions is remarkably effective in the cockroach, even when the animal is ingesting such varied diets as distilled water or lettuce leaves. If one analyses the results more carefully, one can see that when the insect is fed on a standard diet the mean content of blood ions remains relatively stable (146.7 mM-Na<sup>+</sup>, 17.4 mM-K<sup>+</sup> and 3.6 mM-Ca<sup>2+</sup> after 15 days; Fig. 2A). When insects are fed exclusively with lettuce leaves (Diet 2), the blood Na<sup>+</sup> concentration falls after 8 days whereas blood K<sup>+</sup> increases (142.4 mM-Na<sup>+</sup>, 18.4 mM-K<sup>+</sup> and 3.5 mM-Ca<sup>2+</sup> after 4 days against 119 mM-Na<sup>+</sup>, 28 mM-K<sup>+</sup> and 3.1 mM-Ca<sup>2+</sup> after 30 days on this diet; Fig. 2B). When the insects are left starving without water (Diet 3) the concentrations of blood ions first increase (177.4 mM-Na<sup>+</sup>, 28.2 mM-K<sup>+</sup> and 5.2 mM-Ca<sup>2+</sup> after 8 days) then decrease (74 mM-Na<sup>+</sup>, 19.5 mM-K<sup>+</sup> and 0.75 mM-Ca<sup>2+</sup> after 30 days). The decrease appears only after 14 days. It is much more marked for Na<sup>+</sup> (more than 50%) than for K<sup>+</sup> (30%) (Fig. 2C). For insects fed with lettuce leaves and glucose (Diet 4) one can see a slight decrease in concentrations of Na<sup>+</sup> and K<sup>+</sup>: 154.7 mM-Na<sup>+</sup>, 18.8 mM-K<sup>+</sup> and 3.7 mM-Ca<sup>2+</sup> after 4 days as against 137.3 mM-

$\text{Na}^+$ ,  $12.6 \text{ mM-K}^+$  and  $3.9 \text{ mM-Ca}^{2+}$  after 15 days (Fig. 2D). When insects are starved but given distilled water (Diet 5), there is a slight decrease in blood  $\text{Na}^+$  concentration and a prominent decrease of blood  $\text{K}^+$  concentration:  $150.3 \text{ mM-Na}^+$ ,  $19.7 \text{ mM-K}^+$  and  $3.6 \text{ mM-Ca}^{2+}$  after 4 days against  $145.4 \text{ mM-Na}^+$ ,  $7.9 \text{ mM-K}^+$  and  $3.2 \text{ mM-Ca}^{2+}$

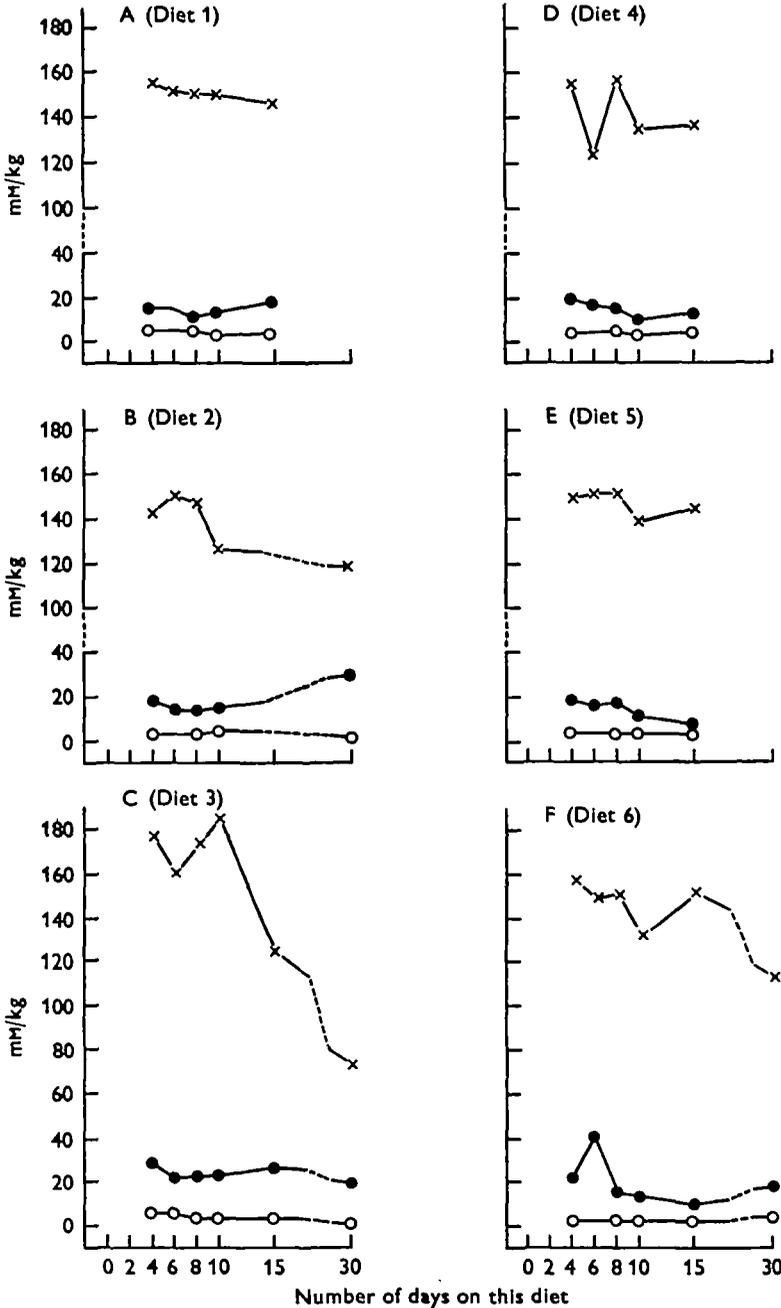


Fig. 2. Kinetics of variations in the blood ionic content following change in the diet. x—x, Na<sup>+</sup> ions; ●—●, K<sup>+</sup> ions; ○—○, Ca<sup>2+</sup> ions.

after 15 days. Blood  $K^+$  decreases by 50% (Fig. 2E). When animals are kept starving with high  $K^+$  saline to drink (Diet 6) there is a rather large decrease in blood  $Na^+$  concentration whereas blood  $K^+$  concentration varies widely between more than 40 mM and 10 mM. After 4 days the ionic composition is the following: 157.9 mM- $Na^+$ , 22.6 mM- $K^+$  and 3.2 mM- $Ca^{2+}$ ; after 30 days it is 115.5 mM- $Na^+$ , 19.4 mM- $K^+$  and 5.0 mM- $Ca^{2+}$ .

A complete analysis of these results would require a better knowledge of the amount of ions, water and nutrients which are ingested by each individual during a given period of time. One can, however, see that the only way to get fairly stable ionic concentration for the three cations is to feed them on a mixed diet such as the 'standard' one. On the other hand if one wants to decrease the  $Na^+/K^+$  ratio the best way is to submit the animals to prolonged starvation and dehydration, whereas an increase in this same ratio can be obtained in giving distilled water to starving insects. Some peak values for  $K^+$  concentrations may be obtained after about one week for starving animals either dehydrated or given high  $K^+$  saline.

Table 3. Ionic content of different samples of haemolymph collected successively in the same insect

(mM/kg of haemolymph)					
Diet	Insect no.	Sample no.	$Na^+$	$K^+$	$Ca^{2+}$
Starving	1	1	84	8	2
		2	87	8	2.2
Tap water to drink	2	1	117	11.1	4
		2	118	11.9	4.5
Lettuce leaves	3	1	102	31	3.3
		2	140	21.1	3.0
		3	143	19.4	3.7
	4	1	128	21.9	3.6
		2	180	24	3.1
		3	109	17.1	2.9
5	2	1	104	11	4.7
		2	123	11.7	5.5
6	2	1	121.4	15.6	3.8
		2	127.5	28.5	2.2
Milk powder	7	1	117	17.3	—
		2	112	9.5	4.2
		3	130	31	4.2
Tap water	8	1	117.2	9.1	6.5
		2	172	20.3	4.3
	9	1	136	35.4	1.6
		2	76	37.4	7.2

### C. Multiple measurements of the haemolymph of the same insect

In view of the very wide variations in haemolymph ionic concentration in different insects living in apparently identical conditions (see Pichon, 1963) the question was raised whether the recorded values were giving a true picture of the whole haemolymph composition, or if they corresponded to the ionic composition of the haemolymph at a given moment in a given place in the insect's open circulatory system.

Using only a few  $\mu$ l of blood for each analysis and selecting insects with a relatively

large blood volume, it has been possible to analyse the ionic content of different samples collected successively during a very short period of time (a few minutes or less) from a given part of the body (dorsal thorax) of individual insects. The results of such analyses for nine animals are shown in Table 3.

From this table one can see that the ionic content is not the same in successive samples, except when the animals are starving (1 and 2). The  $\text{Na}^+$  and  $\text{K}^+$  concentrations are changing quite independently and it seems very difficult to find out if there is or not a general pattern of variations (i.e. an increase or decrease of a given ion), because examples of both can be found in two different animals or even the same one (insect No. 7).

These results are strong evidence against the homogeneity of haemolymph in normally reared animals, as has been pointed out earlier. The following experiments will confirm this evidence.

#### D. *Serial measurements of blood ions in different regions of the animals*

The series of measurements has been carried out on a large batch of insects reared on the 'standard' diet. Small samples of haemolymph (5–10  $\mu\text{l}$ ) were collected from one, or in a small number of cases from two, different parts of the body. (In this last case, the order of the two collections was varied to avoid systematic errors due to the effect of the first collection on the ionic content of the second sample.)

Haemolymph was collected from five different points: (1) in the dorsal vessel between the 1st and 2nd thoracic tergites, (2) at the tip of one antenna, (3) ventrally in the thorax between the prothoracic and mesothoracic legs, (4) in the legs (at the tibia-tarsal joint of the mesothoracic leg), (5) in the abdomen between the 3rd and 4th abdominal sternites. Each measurement corresponds to a mixture of three samples. 80 cockroaches have been used for these experiments. The results are summarized in Table 4 and illustrated in Fig. 3. They show very clearly that the ionic content of the haemolymph collected from different parts of the animals is not the same. If one considers the  $\text{Na}^+$  content, it can be seen that it is higher in the antenna (165.2 mM/kg) than in all other parts of the animals. This differs significantly from the sodium concentration in the dorsal vessel (147.6 mM/kg;  $P = 0.01$ ) or from that of the ventral thorax (144.6 mM/kg;  $P = 0.02$ ).

By contrast the concentration of  $\text{K}^+$  is lower in the antenna (13.8 mM/kg) than in any other part of the body. This differs from the  $\text{K}^+$  concentration found in the dorsal vessel (18.8 mM/kg;  $0.1 < P < 0.2$ ). It is significantly different from figures for the ventral thorax (17.0 mM/kg;  $0.05 < P < 0.1$ ) and the abdomen which contains the highest  $\text{K}^+$  concentration (22.8 mM/kg;  $0.02 < P < 0.05$ ) and is highly significantly different from values for the leg (19.1 mM/kg;  $P < 0.01$ ).

Similar unequal distribution is found for  $\text{Ca}^{2+}$  ions the lowest concentration (3.5 mM/kg) being found in the ventral thorax, and the highest (4.6 mM/kg) in the abdomen. The difference between the  $\text{Ca}^{2+}$  concentrations in the blood from the dorsal vessel (3.8 mM/kg) and from the abdomen is significant ( $0.05 < P < 0.1$ ) as are the differences between the ventral thorax and the antenna on one hand (4.25 mM/kg;  $0.05 < P < 0.1$ ) and the abdomen on the other hand ( $0.02 < P < 0.05$ ). The  $\text{Ca}^{2+}$  concentration in the leg and the abdomen are not significantly different ( $P \approx 0.2$ ).

Table 4. *Ionic content of haemolymph in Periplaneta americana in different regions of the animal*

Serial no.	Region	(mm/kg of haemolymph)			No. of insects sampled
		Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>++</sup>	
1	Dorsal vessel	147.6 ± 2.3	18.8 ± 3.0	3.8 ± 0.13	12
2	Antenna	165.2 ± 6.4	13.8 ± 1.2	4.25 ± 0.36	21
3	Ventral thorax	144.6 ± 6.2	17.0 ± 1.1	3.5 ± 0.25	24
4	Leg	162.1 ± 4.0	19.1 ± 1.5	3.8 ± 0.5	21
5	Abdomen	161.6 ± 5.4	22.8 ± 4.0	4.6 ± 0.42	24

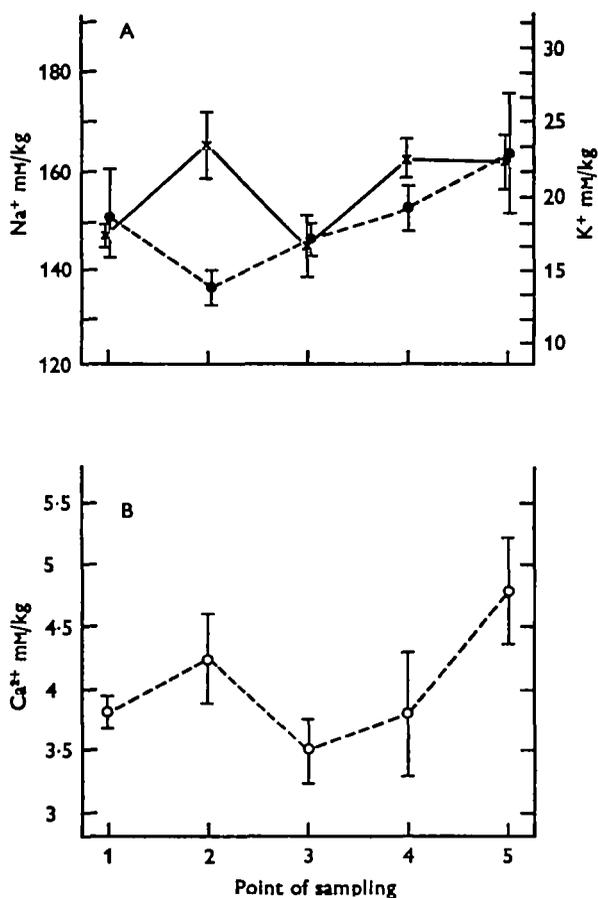


Fig. 3. Differential distribution of ions in blood samples collected in different parts of the body 1: dorsal vessel; 2: antenna; 3: ventral thorax; 4: mesothoracic leg; 5: ventral abdomen. The vertical lines drawn through the points indicate the extent of twice the standard error of the mean.

## DISCUSSION

Our analysis of the haemolymph in the American cockroach, *Periplaneta americana*, has given us a rather complete picture of the ionic environment of the nervous system in this insect.

The ionic composition of the haemolymph is determined from the first larval stage (114.8 mM/kg Na<sup>+</sup>, 61.8 mM/kg K<sup>+</sup> and 4.3 mM/kg Ca<sup>2+</sup>) and remains relatively unchanged throughout the 13 to 15 stages of the animal. This variable number of stages in *Periplaneta americana* might be related to the non-homogeneity of our own strain of insects or to rearing conditions. The effect of manipulating insects might also affect this number.

The high K<sup>+</sup> concentration which is found in the larvae can be partly related to the vegetarian diet (lettuce leaves) which is known to increase the K<sup>+</sup> level in the blood. (Tobias, 1948; Pichon, 1963). A slow increase in this concentration occurs from the 1st to the 15th stage and this might reflect a better regulation in older animals than in younger ones; the difference is, however, not significant. Another explanation of this high K<sup>+</sup> level and of its very important variations either between stages (300% difference between 4th and 5th instar larvae) or within a same stage ( $E > 50\%$  for 14th instar larvae) would be the occurrence of very important changes in the tissues related to the moulting cycle. A more detailed knowledge of the changes in the inter-moulting period would allow a better understanding of this point. It is also possible, as will be pointed out later in this discussion, that some of these variations might also be related to the fact that a variable amount of extra K<sup>+</sup> can leak out of the tissues, when they are injured by the sampling procedure, or out of the haemocytes.

The Na<sup>+</sup> and Ca<sup>2+</sup> levels change much less than the K<sup>+</sup> level. A slight increase (not statistically significant) of these two levels is, however, observed throughout the larval life.

The ionic content of the haemolymph seems to be very effectively regulated when the animals are given various diets. The relative stability of this content in insects reared on the standard diet was to be expected.

The effect of starvation and dehydration, which is first to increase then to decrease the ionic concentration in the haemolymph, is quite consistent with previously reported observations (See Pichon, 1963). The proportionally larger increase in K<sup>+</sup> concentration which falls only slowly with time can be related, as pointed out earlier (Pichon, 1963), to a lysis of the K<sup>+</sup>-rich tissues coupled with a decrease in the active excretion by the Malpighian tubules (see Ramsay, 1953-5; Maddrell 1969) and a reduction of the blood volume in the starving insect (see Pichon, 1963; B. J. Wall, in preparation). Similar increases of the blood K<sup>+</sup> have been observed in the nymphs of *Sphinx ligustri* (Drilhon & Florence, 1946) and *Rhodnius prolixus* (Ramsay, 1952).

When animals are starved but given distilled water, the general tendency is towards a decrease in the concentration of ions with time. This was also to be expected, for the blood volume remains relatively constant. The decrease is, however, very slow for Na<sup>+</sup> and Ca<sup>2+</sup> ions. This observation is consistent with the hypothesis that these ions, and especially Na<sup>+</sup> ions, might be stored somewhere in the tissues and become quickly available again after a haemorrhage (Pichon, 1963) or a large increase in blood volume (Pichon, 1963; Wall, 1970).

If insects are given high  $K^+$  diets orally (lettuce leaves or high  $K^+$  saline) the  $K^+$  level in the haemolymph is higher than normal, but very variable. This variability could be explained by the fact that the ingestion of  $K^+$  as well as its excretion is discontinuous in time, and that the haemolymph concentration at a given moment and in a given part of the animal is the result of at least two antagonistic mechanisms, namely: absorption of  $K^+$  with food and drink and active excretion by the Malpighian tubules. The  $Na^+$  level decreases with time; this can be easily understood in terms of a low  $Na^+$  concentration in the diet. Rather large variations in this level, although proportionally smaller than for  $K^+$  ions, can be interpreted in a similar way, the  $Na^+$  concentration in the haemolymph at a given moment and in a given part of the animal being the result of an equilibrium between absorption of these ions with food and drink, secretion by the Malpighian tubules, rectal reabsorption and a hypothetical tissue storage mechanism. The fact that the variations in  $K^+$  and  $Na^+$  blood levels are independent shows that these variations can hardly be explained by variations in blood volume alone.

Where glucose is added to the lettuce leaves diet, one can observe a slight reduction in the blood  $K^+$  level, which one could expect from a stimulation of the active excretion of  $K^+$  by this highly energetic nutrient. Furthermore, it is known that an increase in glucose in the diet decreases the rate of crop emptying in the cockroach. This could slow down the rate of absorption of potassium by the gut (Treherne, 1957) and thereby result in a reduction in the potassium concentrations in the haemolymph.

Multiple measurements in the same insect have shown quite clearly that, except for starving animals, the ionic composition of the blood is not constant. These results are quite consistent with previous observations that a first sampling may alter significantly the blood ionic content after some hours (Brady, 1967*b*) 1 day (Brady, 1967*b*) or 2 days (Pichon, 1963). The change in the present experiment occurred, however, much faster (minutes) and in quite a random manner. This is a strong argument against the homogeneity of haemolymph ionic composition, for such rapid variations must reflect differences in regions adjacent to the point from where the blood samples were collected.

One can argue that the recorded differences may arise from drastic changes in blood cell density (Brady, 1967*a, b*). If one assumes that the apparent changes in blood potassium are related to changes in the number of haemocytes, these latter variations must be very important to explain the former. For instance, in the case of the insect No. 6 in Table 3, the increase in  $K^+$  concentration is 12.9 mM; on a basis of a figure of 0.83 mM- $K^+$ /l for 10,000 haemocytes/ $\mu$ l (Brady, 1967*b*) this would correspond to 155,000 haemocytes/ $\mu$ l. Taking the figure of 720  $\mu^3$  as a mean for the volume of one individual haemocyte (Brady, 1967*b*) the calculated volume occupied by haemocytes in 1  $\mu$ l of blood would be 0.1116  $\mu$ l, thus more than 10%. This falls within the range predicted by Brady for the increase of blood cell volume after 2 h (4.64–12.18% of total blood volume). If the haemocytes contain no  $Na^+$  this increase in their number would result in a dilution by 11.16%. Thus the blood sodium would fall from 121.4 to 107.8 mM/l. If the haemocytes contain 61 mM/l  $Na^+$  (maximum probable haemocyte concentration; from Brady, 1967*b*), the fall would be reduced to 114.6 mM. The measured change in  $Na^+$  concentration for this same insect, instead of being a decrease, is in fact an increase to 127.5 mM, which is in conflict with the hypothesis that changes

in the number of haemocytes might be responsible in that case for the changes in blood ion concentration. Another observation which is in conflict with Brady's conclusions (that a first sampling results in an increase in the number of blood cells) is that one can find either an increase or a decrease in the  $K^+$  concentration after a first sampling (see also Pichon, 1963).

One must therefore admit that these ions are really not equally distributed within the haemolymph, a fact which is largely confirmed by our serial measurements of ion concentration in blood samples collected in different regions of the animal.

Similar differential distribution of ions within the animal is very surprising if one considers the haemolymph as a circulating fluid analogous to vertebrate blood. But in fact, this assumption is far from being true. In the insect body there is only one tissue fluid which occupies the haemocoel and it freely bathes all the tissues. This fluid then is the equivalent of the blood and the lymph of vertebrates. The circulation is open and there is only one blood vessel, the heart, into which the blood is aspirated through a series of ostia during diastole and driven forward during systole to the head. The haemolymph then percolates slowly backwards through the tissues. Because of this very special organization of the circulatory system, the blood is much less mixed than in the closed circulatory system of vertebrates. Moreover, the circulation being relatively slow the haemolymph may stay in contact with the tissues and have ionic exchanges with them; this may significantly effect the local concentrations of ions within the blood. These phenomena may create rather important gradients of ions between different regions of the body, such as we have recorded in *Periplaneta americana*. One must point out that it is possible that, in our experiments, additional differences might be attributed either to a more or less important lesion at the point of sampling (a larger injury resulting in an increase in the  $K^+$  level) or to a differential distribution of haemocytes in the different parts of the body. Nevertheless, these two phenomena can hardly be responsible for all the differences especially since the changes in  $Na^+$  and  $K^+$  appear to be independent of each other (see above).

At this stage of our experiments it seems premature to draw definite conclusions even when significant differences have been found. For instance, the important  $K^+$  gradient between abdominal (22.8 mM/kg) and dorsal haemolymph (18.8 mM/kg) may be related to the occurrence between the two points of sampling of two already mentioned antagonistic mechanisms: absorption of the  $K^+$  by the gut and excretion by the Malpighian tubules. This difference is, however, far from being significant ( $P > 0.4$ ) because of the large value of the standard deviations in these two regions of the body. A more complete analysis would require a reduction of this standard deviation which might be obtained by a careful selection of the insect strains and a severe control of variables such as time after feeding, nature of the diet, rate of excretion, etc. When the differences are significant, for instance between the leg and the abdomen for  $K^+$  ions ( $P < 0.01$ ) these two points of sampling are too far apart in the blood stream to allow any valuable interpretation to be given. Nevertheless, the fact that ions may be unequally distributed between the different parts of the body is extremely important from the physiological point of view. This is particularly relevant with sensory receptors such as stretch receptors or muscle fibres which are not protected from variations of the surrounding fluid. One can imagine that these differences may be more or less accentuated with feeding and eventually disappear when the

animal is starved for some time (as can be seen from our multiple measurements on starving insects; see Table 3). On the other hand, it may not be fanciful to suppose that the blood stream could carry more or less rapidly some kind of wave of haemolymph containing high  $\text{Na}^+$  or  $\text{K}^+$  after the absorption of high  $\text{Na}^+$  or  $\text{K}^+$  diets, the time constant of the increase or decrease of the concentration of these ions being a function of absorption and excretion rates of these given ions together with changes in blood volume and kinetics of storage mechanisms.

It is not inconceivable that such variations may induce some modifications in the behaviour of the animals like those pointed out earlier in the locust (Ellis & Hoyle, 1954; Hoyle, 1954, 1956) and in the cockroach (Pichon & Boistel, 1963*b*). However, the fact that the nervous system seems to be effectively protected from rapid changes in the ionic composition of the blood (Pichon & Treherne, 1970; Treherne, Lane, Moreton & Pichon, 1970) considerably limits the short-term effects of these variations on nervous activity. Continuous recording of blood ion concentration—for instance, using cation selective electrodes together with an electrophysiological analysis of membrane potentials in the (unprotected) muscle fibres and in the (protected) nerve fibres—will certainly allow some progress to be made in this field of the relations between blood ions and excitability in insects.

#### SUMMARY

1. The haemolymph of the American cockroach, *Periplaneta americana*, has been analysed for  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  ions in different experimental conditions.

2. The ionic content of the haemolymph is maintained constant after the first larval instar. It is characterized by a rather high  $\text{K}^+$  level and a particularly large variability which could be related either to the nature of the diet (lettuce) or to numerous alterations of the blood and tissues in relation to the moulting cycle.

3. The regulation of this ionic content when the animal is given different diets is particularly effective. Starvation results in a decrease in the mean ionic concentration in the blood, this decrease following a pronounced increase of the  $\text{Na}^+$  and  $\text{K}^+$  levels when starvation is associated with dehydration. The blood  $\text{K}^+$  level can be raised artificially with high  $\text{K}^+$  diets (lettuce leaves or high  $\text{K}^+$  saline) but is then very variable. These results are discussed in terms of absorption, excretion and storage mechanisms.

4. The analysis of blood samples collected successively from the dorsal thorax of different individuals shows that, when the animals are fed normally, the ionic composition varies widely, showing that in these conditions the haemolymph is not homogeneous.

5. Serial measurements of blood ions in different regions of the animal show unequal distribution of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$ . This result is discussed in view of the very special circulatory system of insects when compared with that of vertebrates.

6. The overall picture of insect blood which can be drawn from these experiments is that the complexity of the regulatory system together with the fact that the circulatory system is open and lacunous allows relatively wide variations in the ionic concentration to occur on both sides of a genetically fixed mean. This particular feature must be taken into account when one considers the physiological role of blood ions in mechanisms such as excitation, conduction or muscular contraction.

I am grateful to Dr S. H. P. Maddrell for reading and correcting the manuscript and to Mr J. W. Rodford for preparing the illustrations.

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