The processes occurring in the animal body are reflected in the composition of the body fluids. On this principle human medicine has based the rather new science of clinical chemistry. The chemical and cytological analysis of blood, urine, cerebrospinal fluid, bile and several secretions has proved to be of the first importance for a good judgment of the nature of pathological processes and conditions. Deviations from normal metabolism can easily be detected since the concentrations of most chemical substances in the body fluids, in normal circumstances, vary within rather narrow limits. This especially applies to concentrations in the blood, which may be considered to reflect the chemical processes in the human body fairly well. Moreover, the blood constitutes a very important medium for these processes. It contacts, directly or indirectly, all cells of the body and thereby has a very great influence on the course of metabolism in these cells. By far the most investigations in this field have been performed on the human body fluids, but certainly the above considerations hold for the body fluids in other groups of the animal kingdom as well.

The purpose of our investigations has been to obtain an impression of the chemical composition of the haemolymph of Periplaneta americana L. This insect was chosen because it is used in our laboratory as an experimental animal in many of the investigations on the action of insecticides. We did not content ourselves with the determination of the average values but attempted as well to obtain information on the in-

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dividual differences. Moreover, we examined the changes in the chemical composition following disturbances of the normal conditions brought about experimentally. This was expected to give us some information on circulation, tolerance and regulation.

Several investigators collected data on the chemical composition of the haemolymph in insects. These have been summarized e.g. by Buck (1953) Wigglesworth (1950), Bone (1944), and Duchâteau, Florkin and Leclercq (1953). The last authors paid special attention to the mineral constituents and to the relation between mineral composition of the haemolymph and feeding habits or systematic position of the insects. Our work too was mainly concerned with the mineral composition since it is a well known fact that in many animals there are strong relations between mineral composition and excitability of nerves and muscles. To elucidate such relations is of the utmost importance for the physiological investigations of insect nerves and muscles and indirectly for the research on the insecticidal action of several compounds.

I. EXPERIMENTAL TECHNIQUES

Animal material. Our determinations and experiments have all been performed on the American cockroach, Periplaneta americana L. The experimental animals were obtained from the Zoological Gardens “Artis” at Amsterdam. In our laboratory the animals were kept at 29°C. They were fed on a mash, consisting of 10 parts of wheat meal, 9 parts of whole milk powder and 1 part of dried yeast, supplemented with vitamins A and C, mixed in water.

Only adults have been used, except in a few cases, where calcium and magnesium determinations were made in the haemolymph of last instar nymphs. It should be emphasized that the age of the animals on arrival in the laboratory was unknown.

Injection technique. The roaches were injected by inserting the injection needle between the sternites of the 5th and 6th abdominal segments and delivering, by means of an “Agla” micrometer syringe, an amount of fluid varying from 30 to 100 μl. The injection of such an amount of fluid in itself is quite harmless to the insects.

Haemolymph for analysis was collected in several ways. Sometimes antennae and legs were cut off by scissors and the haemolymph appearing on the cut surfaces drawn into a capillary pipette. In other cases haemolymph was obtained by puncturing with a blunt glass needle the thin exoskeleton where the prothoracic leg joins the thorax or by making a little hole at the ventral side of the abdomen between the sternites. In all cases nearly or completely clear haemolymph was obtained. The amounts which could be collected from a single individual varied from 10 to 50 μl.

Methods for the determination of sodium, potassium, and chloride. All methods to be described here, have been checked by performing determinations in solutions of known composition and in normal human sera.

Sodium. After dilution with distilled water the haemolymph was deproteinized with trichloroacetic acid. To the filtrate was added zinc-uranyl-acetate reagent and ethanol to precipitate the sodium as sodium-zinc-uranyl-acetate. The fluid was centrifuged, the supernatant liquid discarded and the precipitate washed three times with acetone saturated with sodium-zinc-uranyl-acetate. The precipitate was then dissolved in hot water and transferred to a small beaker. The amount of sodium-zinc-
uranyl-acetate was then titrated electrometrically with sodium hydroxide solution. References: WEINBACH (1935); GORTER and DE GRAAFF (1947).

**Potassium.** Determinations were all made with a flame-photometer after precipitation of proteins by trichloroacetic acid. The smallest amount of haemolymph permitting reliable measurements of the potassium-concentration was about 100 µl. Therefore, in the case of potassium, no determinations could be performed in single individuals of the insect.

**Chloride.** Two methods were used, which were both quite satisfactory.

1. The haemolymph was deproteinized by addition of 90 per cent ethanol. After centrifugation the supernatant liquid was transferred to a small flask. After washing the precipitate once with 90 per cent ethanol and adding the washing to the flask, the chloride was titrated with silver nitrate using potassium chromate as an indicator. This method is essentially that of MOIR (ref. NOYONS, 1952).

2. A second method which was used successfully is that described in literature as Votoček’s method. The haemolymph was deproteinized with trichloroacetic acid. The chloride in the filtrate was titrated with mercuric nitrate using diphenylcarbazone as an indicator. Ref. GORTER and DE GRAAFF, 1947.

**Methods for the determination of some organic constituents.** All methods, which will be described here, have been checked by performing determinations in solutions of known composition and in normal human sera.

**Total nitrogen and Non-protein nitrogen** were determined by a micro Kjeldahl method. Total nitrogen (T.N.) was determined by complete destruction of a very small amount of haemolymph with sulphuric acid. After addition of sodium hydroxide the ammonia was distilled into a solution of boric acid by way of a Parnas-Wagner apparatus and titrated with 0.02N hydrochloric acid. Non-protein nitrogen (N.P.N.) was determined in the same way after precipitation of proteins with trichloroacetic acid. (Unless large quantities of haemolymph are available, the determinations of N.P.N. are rather inaccurate. The estimation of N.P.N. in single individuals, therefore, is very difficult.)

**Total Reducing Value** was determined by the Hagedorn and Jensen method. The haemolymph was deproteinized with cadmium hydroxide and oxidized by an alkaline potassium ferricyanide solution. The excess of ferricyanide was measured by iodometric titration. This Total Reducing Value (T.R.V.) was expressed as glucose, but probably it is due only in part to glucose or even to sugars. No separate determinations on fermentable and non-fermentable reducing substances have been made. References: ARIENS FUJITA and IWATAKE (1931).

**Methods for the determination of calcium and magnesium.** In a previous paper (VAN ASPEREN and VAN ESCH, 1954) we gave a very short description of a micro method for the determination of calcium and magnesium. As this method has not yet been published in more detail, we shall give a detailed description here. The method is based on the use of a chelating agent as introduced by SCHWARZENBACH et al. (1946) for the determination of several metals. The chelating agent, ethylenediaminetetraacetate (EDTA), has been proved to be very useful for the direct titration of calcium and magnesium, especially in the determination of water hardness and in clinical investigations of blood plasma. As, in our case, only very small amounts of fluid were available, we used a well known Linderstrom-Lang microtitration technique (ref. GLICK, 1949).

**Procedure.** A small amount of haemolymph obtained from the roach as described above and varying from 5 to 20 µl, was drawn into a small glass pipette by capillary forces. The fluid was then blown out into a small titration vessel (capacity ca 500 µl), weighed and diluted with approximately 100 µl of distilled water.

In the case of the calcium titration ca 10 µl 4 N sodium hydroxide and ca 5 µl
0.4 per cent murexide solution were added immediately before the titration which was carried out with 0.004 N ethylenediamine tetraacetate solution (brought to pH 10.8 with sodium hydroxide) from a Linderström-Lang microburette.

In the case of the magnesium titration ca 60 µl 0.1 N sodium hydroxide, ca 25 µl M ammoniumchloride-ammoniumhydroxide buffer solution and ca 5 µl 1 per cent eriochrome black T solution were added immediately before titration, which was performed as above.

The EDTA titration fluid was prepared according to Holtz (1951) by dissolving 1.488 gm of di-sodium ethylene diamine tetraacetic acid-dihydrate in distilled water. The solution was titrated with N sodium hydroxide to pH 10.8 and then made to 1 liter with distilled water. It is stable.

The murexide solution was prepared by dissolving ca 40 mg of murexide in 10 ml of distilled water. Since this material is unstable, the solution should be freshly prepared every few days.

The eriochrome black T solution was prepared as described by Buckley, Gibson, and Bortolotti (1951). This solution is stable for many weeks if kept in a refrigerator.

Discussion of the method. At the pH of the titration the calcium-murexide complex shows a pink shade, whereas the calcium-free murexide solution is lilac blue. Murexide, therefore, is used as an indicator for the presence of unchelated calcium. The indicator for the presence of magnesium is eriochrome black T which forms a bright red complex with magnesium. In the absence of magnesium the dye assumes a blue colour. The rather faint yellow colour of the haemolymph did not interfere, except in a few cases, where dark brown pigments had developed after preservation of the haemolymph for more than one day.

The affinity of EDTA for calcium is much greater than for magnesium. The end point of the murexide titration, therefore, indicates that all the calcium has been chelated, whereas in the eriochrome black titration the colour shift occurs only when both the calcium and magnesium have been chelated. The difference between the amounts of EDTA used in both titrations can thus be used for the calculation of the magnesium content.

Calculations are very simple since one mole of EDTA chelates one mole of divalent cation. As, according to Buckley, Gibson, and Bortolotti (1951), a period up to 45 seconds between drops of EDTA is required for the colour to become stabilized, the end point of the titration should be approached very slowly.

Reliability of the method. Several experiments were performed to test the reliability of the method.

1. Micro-determinations of calcium and magnesium, according to the above description, were carried out on solutions, containing varying amounts of calcium chloride and magnesium sulphate and on human sera, to which varying amounts of magnesium sulphate had been added. The results were compared with those obtained with macro-methods. From Table 1 it may be concluded that there is a good agreement of the data obtained by calculation, macro-determination and micro-determination.

According to Table 1 the standard deviations calculated for most series of parallel determinations are rather small and generally do not exceed 5 per cent of the average value.

2. Haemolymph was collected from several cockroaches and determinations were made in samples of this pooled haemolymph. This procedure was repeated several times and the standard deviations of all series of parallel determinations were calculated. Each series consisted of 2–8 determinations. For eight series of the micro-calcium titration the standard deviations averaged 5 per cent of the mean (18.6 mg calcium per 100 gm of haemolymph). For nine series of the micro magnesium titra-
TABLE I Determination of calcium and magnesium in solutions and human sera

<table>
<thead>
<tr>
<th>Determination of</th>
<th>Results, all expressed as M ± S.D.¹, in milligrammes/100 gram.</th>
<th>Calculated</th>
<th>Macro-EDTA-titr.</th>
<th>Micro-EDTA-titr.</th>
<th>Macro-oxalate prec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂-solution</td>
<td>Ca — 9.2 ± 0.1 (5) 9.4 ± 0.6 (13) 9.1 ± 0.1 (2)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Mg 30.0</td>
<td>30.2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CaCl₂ + MgSO₄-solution</td>
<td>Ca 18.4 19.6 (1) 19.0 ± 1.8 (5)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Mg 30.0</td>
<td>30.2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CaCl₂ + MgSO₄-solution</td>
<td>Ca 18.4 31.7 ± 1.2 (5) —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Mg 15.0</td>
<td>16.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Human serum + MgSO₄</td>
<td>Ca 11.7 19.6 (1) 11.6 ± 0.1 (2)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Mg 36.6²</td>
<td>34.6</td>
<td>—</td>
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<tr>
<td>MgSO₄-solution</td>
<td>Mg 30.0 30.1 ± 0.1 (2) 31.6 ± 1.7 (8)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Human serum + MgSO₄</td>
<td>Mg 17.4² 16.2 ± 0.1 (2) 17.9 ± 0.8 (5)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Human serum + MgSO₄</td>
<td>Mg 17.7² 16.3 ± 0.3 (3) —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

In brackets: number of determinations

¹ M = Mean value; S.D. = Standard Deviation = $\sqrt{\frac{\sum d^2}{n-1}}$

² Calculated assuming that the Mg-content of the plasma is 3 mg/100 gram.

The standard deviations averaged 14 per cent of the mean (13.8 mg magnesium per 100 gm of haemolymph). This last value is rather high, for which two reasons may be produced: a. Magnesium concentrations are calculated from the difference between two titration values and therefore are liable to greater errors than the calcium concentration which can be titrated directly. b. Usually after the haemolymph has been collected some coagulation of cells and proteins occurs. By collecting most of the coagulate in one sample we were able to show that the magnesium content of the coagulate is distinctly higher than that of the clear serum. The calcium concentrations of coagulate and serum were not significantly different.

3. Duplicate determinations of calcium and magnesium were made by taking two samples of haemolymph successively from the same insect. This was repeated several times. On average the duplicate values differed by 9 per cent for the calcium and 12 per cent for the magnesium determinations.

4. Haemolymph was collected and micro- and macro-EDTA-titrations were carried out. There was a very good agreement of the results, the differences amounting to only a few per cent.

5. In two experiments 116 per cent and 89 per cent of magnesium sulphate added to samples of pooled haemolymph were recovered. Taking into account that each experiment includes several manipulations with small amounts of fluid, these results are reasonable.

From the results of the experiments described above, the conclusion can be drawn, that the titration in itself is reasonably accurate. The greatest errors are introduced by the manifold handling of the haemolymph. This, therefore, should be reduced as far as possible.
The calcium concentration being fairly constant, we felt ourselves justified, at least in a number of determinations, in omitting the murexide titration and in calculating magnesium concentrations by subtracting the average titration value for calcium from the value obtained with the eriochrome black titration. This of course introduces an error, but, since the number of manipulations is decreased, the accuracy of the titration is enhanced.

II. THE CHEMICAL COMPOSITION OF THE HAEMOLYMPH

The methods described in the preceding section have all been adapted to the small amounts of haemolymph which could be obtained from individual insects. By performing rather large numbers of determinations in single individuals we have been able to obtain data on the individual variation as well as on the average values. Table II shows the data obtained for sodium, potassium, chloride, calcium, magnesium, total-nitrogen, non-protein nitrogen, and total reducing value. The data for non-protein nitrogen, although giving a good impression of the average concentration are rather inaccurate with regard to the individual variability as expressed by the standard deviation. Apart from many determinations in single individuals several determinations have been made in pooled haemolymph, which are also presented in Table II.

Discussion. If we compare our results with those found in literature, we note a very good agreement in the data for sodium, chloride, and calcium, but large differences in the data for potassium, magnesium, and T.R.V. Differences may be due to several factors, such as differences in the previous history of the insects, environment, feeding conditions, race etc. Whether our data agree with those of literature, in our view therefore, could largely be a matter of chance. This is supported by a number of observations made after the data of the table had been collected. Thus the T.R.V., a year after the data of the table had been obtained, averaged about 500 mg per 100 gm of haemolymph, which is more than three times as much as quoted in the table (range 210-740). The total nitrogen, one year later, on an average proved to be considerably lower than according to Table II. The mean amounted to about 700 mg per 100 gm of haemolymph with very large individual differences. We are not, therefore, inclined to attach too much importance to the mean values obtained in our investigations and to those found in literature. Differences may be caused by the diet. Tobias (1948) proved, that by giving a leaf diet (lettuce) during 12-18 days preceding the chemical analysis of the haemolymph, the potassium concentration of the serum was increased with more than 50 per cent. Oral administration of concentrated potassium chloride solutions resulted in a more than 100 per cent increase of the potassium value. We
<table>
<thead>
<tr>
<th>Sex</th>
<th>Number of determinations</th>
<th>Method</th>
<th>Mean ± S.D.</th>
<th>Range</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>47</td>
<td>single</td>
<td>378 ± 59</td>
<td>247-500</td>
<td>350</td>
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<tr>
<td>female</td>
<td>45</td>
<td>single</td>
<td>332 ± 63</td>
<td>220-528</td>
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<tr>
<td>male + female</td>
<td>18</td>
<td>pooled</td>
<td>370 ± 55</td>
<td>264-540</td>
<td></td>
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<tr>
<td>Potassium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male + female</td>
<td>32</td>
<td>pooled</td>
<td>31 ± 5</td>
<td>25-50</td>
<td>100</td>
</tr>
<tr>
<td>Chloride</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>male</td>
<td>44</td>
<td>single</td>
<td>507 ± 43</td>
<td>423-660</td>
<td>504</td>
</tr>
<tr>
<td>female</td>
<td>49</td>
<td>single</td>
<td>518 ± 45</td>
<td>437-639</td>
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</tr>
<tr>
<td>male + female</td>
<td>19</td>
<td>pooled</td>
<td>512 ± 32</td>
<td>454-588</td>
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<tr>
<td>Calcium</td>
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<tr>
<td>male</td>
<td>43</td>
<td>single</td>
<td>17.4 ± 1.7</td>
<td>14.5-21.6</td>
<td>17 ± 3</td>
</tr>
<tr>
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<td>31</td>
<td>single</td>
<td>18.1 ± 2.3</td>
<td>12.0-25.1</td>
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<td>unknown</td>
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<td>single</td>
<td>18.0 ± 1.6</td>
<td>15.2-21.2</td>
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<tr>
<td>nymphs</td>
<td>2</td>
<td>single</td>
<td>17.4</td>
<td></td>
<td></td>
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<tr>
<td>male + female</td>
<td>54</td>
<td>pooled</td>
<td>16.1 ± 2.1</td>
<td>12.2-23.6</td>
<td></td>
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<tr>
<td>Magnesium</td>
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<tr>
<td>male</td>
<td>40</td>
<td>single</td>
<td>13.3 ± 3.5</td>
<td>7.0-22.8</td>
<td>7</td>
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<tr>
<td>female</td>
<td>43</td>
<td>single</td>
<td>13.7 ± 2.4</td>
<td>10.8-22.6</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>nymphs</td>
<td>4</td>
<td>single</td>
<td>12.5</td>
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<td></td>
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<tr>
<td>Total-Nitrogen</td>
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<tr>
<td>male</td>
<td>49</td>
<td>single</td>
<td>1041 ± 242</td>
<td>647-1664</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>43</td>
<td>single</td>
<td>1054 ± 230</td>
<td>683-1760</td>
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<tr>
<td>male + female</td>
<td>11</td>
<td>pooled</td>
<td>990 ± 250</td>
<td>670-1528</td>
<td></td>
</tr>
<tr>
<td>Non-protein Nitrogen**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>16</td>
<td>single</td>
<td>229 ± 82</td>
<td>140-483</td>
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<tr>
<td>male + female</td>
<td>11</td>
<td>pooled</td>
<td>227 ± 52</td>
<td>126-300</td>
<td></td>
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<tr>
<td>Total Reducing Value** †</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>male</td>
<td>34</td>
<td>single</td>
<td>148 ± 42</td>
<td>80-276</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>36</td>
<td>single</td>
<td>141 ± 32</td>
<td>87-215</td>
<td></td>
</tr>
</tbody>
</table>

* *Single means that each determination was performed on the haemolymph of one individual insect.

Pooled means that each determination was performed on a sample of haemolymph collected from several insects.

† Data for Total Reducing Value expressed as mg glucose per 100 gm of haemolymph.

** In a later series of experiments strongly deviating results have been obtained; see Discussion below.

References: 1 TOBIAS (1948); 2 CLARK and CRAIG (1953); 3 MUNSON and YEAGER (1949); 4 YEAGER and FAY (1935).

Do not know the potassium concentration of the diet given in our laboratory and very little is known about the diet of the insects in the Zoological Gardens at Amsterdam. Therefore, the possibility of the diet being a cause of differences cannot be excluded. Nevertheless we feel that the differences between our values for potassium and those given by TOBIAS probably are too large to be explained by dietal factors.
only. As a result of the very low potassium values the sodium/potassium ratio in the whole haemolymph of our insects was about 20. This very high value, according to Boné (1944), is characteristic of carnivorous and aquatic insects. For a pantophagous insect like Periplaneta americana a much lower ratio would be expected. According to Duchâteau, Florkin, and Leclercq (1953) high values of the sodium/potassium ratio, as occurring in many groups of animals, are mostly found in the primitive groups of insects, whereas low sodium/potassium ratios are to be considered as biochemical specializations.

Diet could have played an important part in the great variation of our T.N.-values too. We observed a tendency of the T.N.-concentrations to increase if the insects were kept in our laboratory for a longer period of time. This, perhaps, may be the result of the rather large amounts of protein-rich food ingested.

The large differences between the T.R.V. given by Yeager and Fay (1935), the data of Table II, and those found in our later investigations (respectively 65, 145 and 500 mg glucose per 100 gm of haemolymph) are likely to be caused by differences in diet and life conditions though we have no definite proof of it.

With regard to the magnesium concentrations our data differ greatly from those given by Tobias (1948), which are much lower, and from those given by Clark and Craig (1953), which are considerably higher. In a former paper (Van Asperen and Van Esch, 1954) we expressed our view that the dry-ashing of the haemolymph, as used by both authors, may lead to erroneous results because by this procedure organic phosphorus is converted to inorganic phosphates which may interfere. However, in the light of the large differences found for potassium and some organic constituents with similar methods, we cannot exclude the possibility that differences in the animal material have played a part.

The values for calcium and chloride in our investigations show relatively small variations as expressed by the rather small standard deviations and moreover they agree quite well with those found in literature.

In a preceding paper (Van Asperen and Van Esch, 1954) calcium and magnesium concentrations were also given for two other species of Blattaria (Periplaneta australasiae and Blabera fusca). From these data it could be concluded that the interspecific differences are rather small and that the calcium/magnesium ratio is about the same in all three species. If both calcium and magnesium concentrations are expressed in molarities, this ratio is 0.79 in Periplaneta americana, whereas Clark and Craig (1953) found a ratio of only 0.37. This, once more, is a warning against drawing far reaching conclusions from such rather uncertain data. In correlating the ionic composition of the body fluids
with feeding habits, systematic position etc. much more attention should be paid to variations resulting from several causes.

From Table II it may be concluded, that in general the individual variability is much less for the mineral than for the organic constituents and especially in the case of calcium and chloride the individual differences are small. This might perhaps indicate the importance of rather fixed relations and stable conditions with regard to the mineral composition of the haemolymph. In the third section of this paper we shall deal with this problem in more detail. The differences between the concentrations found in the two sexes do not appear to be significant.

The ion balance shows a great resemblance to that in human plasma. The total cation titre of 183 milliequivalents (sodium 157, potassium 8, calcium 8, magnesium 10) is 79 per cent balanced by 144 anionic milliequivalents chloride. According to GLASER (1925) the pH of the haemolymph of Periplaneta americana is 7.5–8.0. The total protein content of the haemolymph being rather high, proteinates are very likely at this pH to make a major contribution to the residual 39 negative milliequivalents. Bicarbonates and phosphates probably do not play an important part in the cation-anion balance.

Binding of cations to proteins should be taken into consideration. Sodium and potassium have both been determined after deproteinization of the haemolymph, as is usual in human clinical chemistry. As, however, rather high concentrations of trichloroacetic acid were used it is very unlikely that protein bound fractions of these minerals could have been eliminated in this way. The data obtained for all cations, in our view, represent the total concentrations in the haemolymph of the insects.

The fact should be emphasized, that we did not distinguish between whole haemolymph and haemolymph serum as was done by TOBIAS (1948). The haemolymph always proved to be an almost or completely clear fluid. Some coagulation occurred on standing. This was most probably a clotting of proteins and haemocytes, but the volume of the clot was very small. Haemocyte counts in 32 females and 28 males averaged 67,000 ± 18,000 and 67,000 ± 16,000 per μl respectively. From these data the conclusion can be drawn that the total cell volume of the haemolymph is very small. Therefore, only small differences between whole haemolymph and serum can be expected. In our view the differences up to 40 per cent as found by TOBIAS (1948) are quite inexplicable.

\[ \text{Standard deviation} = \sqrt{\frac{\sum d^2}{n-1}} \]
III. TOLERANCE AND REGULATION

In the preceding section we have dealt with the normal chemical composition of the haemolymph. Special attention has been paid to the individual variability of some organic and the most important mineral constituents. The rather small individual differences with regard to the mineral constituents, especially calcium and chloride, led us to suggest that it is important for the animals to maintain more or less fixed relations and conditions. It will be the purpose of this section to describe a number of experiments, in which these relations and conditions were disturbed in order to get a better insight into the potentialities of tolerance and regulation. The disturbance of normal conditions was achieved by injecting varying amounts of distilled water, simple salt solutions or "physiological" salt solutions of varying composition. In a number of cases insecticidal emulsions were injected to bring the insects into very abnormal physiological conditions.

Experiments were all performed in the following way. The insects were divided into a number of groups, each group consisting of from 6 to 40 animals. The insects of one of these groups were not injected and served as controls. The others were all injected with the same amount of injection fluid. After a certain interval of time haemolymph for analysis was collected from one of the groups and this was repeated with the other groups at later times. Haemolymph was taken only once from each insect.

I. INJECTION OF DISTILLED WATER AND INSECT RINGER SOLUTION; DETERMINATION OF SODIUM, CHLORIDE, TOTAL NITROGEN, AND NON-PROTEIN NITROGEN

The Insect Ringer solution used was composed of 9.32 gm NaCl, 0.77 gm KCl, 0.50 gm CaCl₂, 0.18 gm NaHCO₃, 0.01 gm NaH₂PO₄, and distilled water to 1 liter. The composition of this Ringer solution and that of haemolymph are compared in Table III.

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>The composition of Insect Ringer solution and haemolymph of Periplaneta americana</th>
</tr>
</thead>
<tbody>
<tr>
<td>Element</td>
<td>Insect-Ringer-Solution</td>
</tr>
<tr>
<td>Sodium</td>
<td>162 millimol./liter = 372 mg/100 ml.</td>
</tr>
<tr>
<td>Potassium</td>
<td>10.3 millimol./liter = 40 mg/100 ml.</td>
</tr>
<tr>
<td>Calcium</td>
<td>4.5 millimol./liter = 18 mg/100 ml.</td>
</tr>
<tr>
<td>Magnesium</td>
<td>13 mg/100 ml.</td>
</tr>
<tr>
<td>Chloride</td>
<td>178 millimol./liter = 632 mg/100 ml.</td>
</tr>
</tbody>
</table>
In this series of experiments 77 µl of distilled water or Insect Ringer solution was injected and haemolymph was collected from legs and antennae after varying intervals of time. The results are presented in Table IV, where the concentrations are expressed as percentages of the values found for the haemolymph of control groups.

Table IV: Injection of distilled water and Ringer solution

Concentrations all expressed as percent of the controls

<table>
<thead>
<tr>
<th>Injected 77 µl</th>
<th>Distilled water</th>
<th>Ringer-solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Interval of time</td>
<td>Interval of time</td>
</tr>
<tr>
<td></td>
<td>0-10 min.</td>
<td>60 min.</td>
</tr>
<tr>
<td>Sodium</td>
<td>82 (2)</td>
<td>98 (3)</td>
</tr>
<tr>
<td>Chloride</td>
<td>91 (2)</td>
<td>89 (2)</td>
</tr>
<tr>
<td>Total-Nitrogen</td>
<td>74 (1)</td>
<td>100 (2)</td>
</tr>
<tr>
<td>Non-protein Nitrogen</td>
<td>90 (1)</td>
<td>93 (2)</td>
</tr>
</tbody>
</table>

In brackets the number of observations from which the mean values given in the table are calculated.

1 “Interval of time” is the interval between the injection and the collection of the haemolymph.

No abnormalities in the behaviour of the insects were observed after injection of these fluids.

Discussion. For the interpretation of these results a good knowledge of bloodvolume, circulation time and time necessary for complete mixing of injected fluid and haemolymph is very important. According to Yeager and Munson (1950) the blood volume of adult Periplaneta americana is about 20 per cent, whereas Wigglesworth (1950) gives a value of only 6 per cent. For several reasons we prefer the first value. It should, however, be kept in mind that probably large differences may occur. We attempted to determine the blood volume by injecting Evans' blue solution, but very irregular results were obtained. This was most probably due to adsorption of the dye by the tissues. The mean weight of the cockroaches in our investigations was about 1 gm, so the blood volume may be approximately 200 µl.

If no regulation took place, the injection of 77 µl of distilled water would result in a decrease in concentration to about 75 per cent after complete mixing. The same decrease may be expected for Total Nitrogen and Non-protein Nitrogen after injection of 77 µl of Ringer solution. Such a dilution is indicated only in the case of Total Nitrogen and immediately after injection for sodium and Non-protein Nitrogen. The results suggest strongly the occurrence of regulation processes in the first hours after injection in the case of sodium, chloride and Non-
protein Nitrogen. This regulation is not achieved by removal of water from the haemolymph as in this case Total Nitrogen values would also be restored. As we did not feel these results to be conclusive, further experiments were performed.

2. INJECTION OF ABNORMAL INSECT-RINGER SOLUTIONS; DETERMINATION OF SODIUM AND CHLORIDE

Only a small number of experiments of this kind was performed. In all cases 77 μl was injected and haemolymph collected after one hour from legs and antennae. Injection of Ringer solutions, in which KCl or CaCl₂ had been substituted by an equivalent amount of NaCl did not affect the sodium and chloride concentrations. If NaCl was replaced by an equivalent amount of KCl, the sodium concentration was lowered to 73 per cent, whereas Cl was unaffected. Injection of a Ringer solution, to which 1.28 per cent KCl had been added, brought about only a small increase of chloride concentration. The sodium concentration in one case was unaffected and in another case it was considerably increased.

The injection of Ringer solutions with very high potassium concentrations (670 and 710 mg/100 ml), as described above, generally resulted in paralysis. Movements in the insects became very slow and most animals were lying on their backs for a long time. After 5 to 6 hours recovery started. After a lapse of 24 hours 3 out of 10 insects were dead, the remaining 7 were quite normal. No abnormalities were observed after the injection of potassium free or calcium free Ringer solutions.

3. INJECTION OF DISTILLED WATER AND CONCENTRATED CALCIUM CHLORIDE SOLUTIONS; DETERMINATION OF CALCIUM

Haemolymph was collected from several parts of the insects as described before.

The concentration of calcium in the thoracal haemolymph was only slightly affected by the injection of 80 μl of distilled water. If, however, the haemolymph was collected from the abdomen, an initial decrease of the concentrations to about 55 per cent was observed 7 to 30 min. after the injection. After 60 min. and 145 min. respectively 79 per cent and 83 per cent of the normal concentration was found.

A large number of experiments were performed, in which 30 μl of a concentrated calcium chloride solution, containing 219 mg calcium per 100 ml (54.8 mMol), was injected. Haemolymph was collected from the abdomen or from thorax, legs and antennae. Fig. 1 shows the
results. All plots in this figure are mean values calculated from 3–12 determinations. The Standard Deviations for the individual observations made for each plot averaged 23 per cent of the plotted mean value. The Standard Errors of the plotted mean values ranged from 4 to 15 per cent, averaging 9 per cent.

**Discussion.** Assuming as we did that the total volume of haemolymph is 200 μl, the concentration resulting from the injection of 30 μl of this calcium chloride solution would be about 44 mg per 100 ml. This value would be reached after complete mixing of haemolymph and injected fluid, provided that no regulation took place (we may call this the simple mixing level). From the graphs of fig. 1 it can easily be seen that complete mixing occurs only after about one hour. At that time a calcium concentration of about 27 mg per 100 ml is reached. This means either that calcium has shifted from the haemolymph to the tissues or that a calcium free solution or water has been transferred from the tissues to the haemolymph. In either case some kind of regulatory process has to be present.

In order to find out whether or not the haemolymph could have been diluted by water transport from the tissues, chloride and Total Nitrogen determinations were made in the haemolymph at several times after
injection of the calcium chloride solution. The chloride and T.N.-
values found 60 and 150 minutes after the injection were only slightly
lower than those of the controls (chloride 96 ± 7 per cent; T.N. 96 ±
17 per cent). Most probably, therefore, regulation is achieved by remo-
val of calcium from the haemolymph. We are quite ignorant as to
where and how this calcium regulation is brought about.

Most remarkably the calcium concentration in the thoracal haemo-
lymph is increased to about 27 mg per 100 ml immediately after the
injection and this level is maintained during the whole regulatory
period. The interpretation of these results is very difficult because very
little is known about the circulation and distribution of haemolymph in
the body. We did some experiments, in which dyes (filtered solutions of
Indian ink or Evans’ blue) were injected into the abdomen. They were
seen to spread within a minute through the whole body, but measure-
ment of the colour intensity revealed that there was no uniform distri-
bution, even after a long period of time.

Similar circulatory factors may affect the results obtained after in-
jection of distilled water or salt solutions and could give an explanation
of many of the data obtained in our experiments. It might be suggested
that the rapid spread of salts or dyes in the first minute or even the first
seconds after the injection is caused by an increase of pressure in the
abdomen as a consequence of the injection.

4. INJECTION OF DISTILLED WATER AND CONCENTRATED
MAGNESIUM SULPHATE SOLUTIONS;
DETERMINATION OF MAGNESIUM

Two experiments have been made, in which 80 µl of distilled water was
injected and haemolymph was collected from the abdomen. The results
were somewhat irregular, but there was an indication that the very low
concentration (6.5 mg/100 ml), observed after about 7 min. is increased
during the following 30–60 minutes, without, however, reaching the
normal level.

In several experiments the insects were injected with 30 µl of a
magnesium sulphate solution containing 300 mg magnesium per 100
ml (125 mMol). Magnesium determinations were performed on the
samples of haemolymph taken from the abdomen after varying periods
of time. The results can be found in Fig. 2.

Discussion. The magnesium concentration resulting from simple
mixing of the haemolymph (volume taken as 200 µl) and injected fluid,
can easily be calculated. This value (“simple mixing level”) is about
50 mg per 100 ml. The magnesium concentration actually falls below
this level, but not as rapidly and distinctly as has been found for cal-
cium in the preceding experiments. This may be caused by the very large amount of magnesium injected. It should be emphasized, however, that the animals all behaved quite normally. This, in itself, is very surprising, as in many animals, magnesium concentrations as high as those found in our experiments have strongly narcotizing effects which are antagonized by calcium. Levenbook (1949) injected magnesium into the haemolymph of adult locusts and found that it rapidly pro-

![Graph showing magnesium concentration over time](image)

Fig. 2. Magnesium concentration after injection of 30 μl of magnesium sulphate solution containing 300 mg magnesium per 100 ml.

duced a cataleptic condition. The insects recovered after a subsequent injection of calcium, which, if injected alone, would have been fatal. So, both calcium and magnesium are highly toxic for locusts, but both ions together are non-toxic by reciprocal antagonism. This certainly is not true for Periplaneta americana. No toxic effects of either calcium or magnesium were observed at the concentrations studied or after injection of 60 μl of the concentrated solutions, which is twice as much as used in the experiments.

5. INJECTION OF ETHYLENEDIAMINE TETRAACETATE SOLUTIONS; DETERMINATION OF CALCIUM AND MAGNESIUM

The object of the injection of distilled water described under 3 and 4 was to lower the calcium and magnesium concentrations in the hae-
molymph. We showed, however, that even the injection of rather large amounts of distilled water (80 μl) could not bring about a decrease of more than about 30 per cent. We therefore carried out a number of experiments, in which 30 μl of a 67 mMol. ethylenediamine tetraacetate solution was injected. This amount would be sufficient to chelate all the calcium and magnesium present in the haemolymph of the insect, at least if simple mixing occurred. From the results the following conclusions could be drawn:

a. Immediately after the injection, (determinations made after 15 min.) no free calcium was present in the thoracal or in the abdominal haemolymph.

b. In the following five hours there was a steady increase of the calcium concentrations, but complete recovery did not occur in this period (see fig. 3). After 5 hours approximately 75 per cent of the normal concentration was found. No calcium or magnesium determinations were made after more than 5 hours.

c. The same may apply to magnesium, but here the results did not allow of a quantitative interpretation.

d. The insects were seriously affected by the injection. The symptoms
of intoxication (paralysis, slow movements, the animals lying on their backs) appear mostly within 15 minutes and last for several hours. About 30 per cent of the insects die within 24 hours, the others recover completely.

6. INJECTION OF AND EXPOSURE TO GAMMA BENZENEHEXACHLORIDE

As in our laboratory a study is undertaken of the mode of action of gamma benzenehexachloride (gamma BHC), we were especially interested in the effects caused by gamma BHC intoxication. Injection of large doses of gamma BHC resulting in very severe symptoms, did not alter significantly the concentrations of sodium, chloride, total nitrogen or non-protein nitrogen. Cockroaches exposed to concentrated deposits of gamma BHC causing marked symptoms, showed quite normal calcium and magnesium values. Moreover, the ability to restore nearly normal calcium concentrations after injection of concentrated calcium chloride solutions was unimpaired.

IV. GENERAL DISCUSSION

In the second section of this paper we stated that the variability of the concentrations found for the mineral constituents in the haemolymph of Periplaneta americana was rather small. In the third section we were able to show that the insects had a marked ability to restore normal concentrations after severe disturbances. Moreover, in most cases the insects showed a high tolerance. We should like to stress the possibility that this high tolerance could be to some extent a consequence of the strong regulating ability.

Furthermore it is very important to note that (with one exception - ethylenediamine tetraacetate) injection of large amounts of fluid into the abdomen never caused large changes of the concentrations in the thoracic haemolymph. This possibly can be partly explained by the slow circulation of haemolymph through the body. The small rate of circulation might give the insects an opportunity for regulation before the “disturbance” reaches the thoracic and head parts of the body.

The results of our experiments on regulation point to a transfer of ions rather than to a transfer of water. Removal of ions from the haemolymph might be accomplished by the Malpighian tubes, but no experiments were performed to investigate this point.

V. SUMMARY

The paper deals with the chemical composition of the haemolymph in the insect Periplaneta americana.

The first section gives a description of the experimental techniques.
Special attention is given to the method for the determination of calcium in small amounts of haemolymph. Microtitration with ethylenediamine tetraacetate using a well known Linderstrøm-Lang technique gave very reliable results (see Table 1). The methods for the determination of sodium, potassium, chloride, total nitrogen, non-protein nitrogen, and total reducing value are only briefly described, as they are principally similar to those used in human clinical chemistry. Descriptions are also given of the techniques for injection, and for collection of haemolymph.

The second section deals with the composition of normal haemolymph. A large number of determinations of Na, Cl, Ca, Mg, T.N., N.P.N., and T.R.V. in individual insects were performed to gain a better insight in the individual and sexual differences. Moreover a large number of determinations in pooled haemolymph were performed. The results are summarized in Table II.

The values obtained are compared with those found in literature and discussed.

The third section describes several kinds of experiments to test the regulatory power and the tolerance of the insects. Varying amounts of distilled water or salt solutions were injected and concentrations determined after different intervals of time. It is shown that the insects are able to restore normal concentrations rather rapidly. This especially applies to the results obtained after injection of very concentrated solutions of calcium chloride, magnesium sulphate and ethylenediamine tetraacetate (see figs. 1, 2, and 3). The tolerance proved to be very high if compared with that in mammals. No abnormalities in the behaviour of the insects resulted from the injection of 30 or 60 $\mu$L of solutions containing 200 mg calcium or 300 mg magnesium per 100 ml. The injection of large quantities of potassium or ethylenediamine tetraacetate sometimes caused paralysis and death.

By comparing the concentrations found in abdominal haemolymph and thoracal haemolymph after intra-abdominal injection of a concentrated calcium chloride solution an estimate of the time necessary for complete mixing of the haemolymph could be made. It proved to be in the order of 30–60 minutes.

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