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THE FREE AMINO ACID POOL OF THE HAEMOCYTES OF *CARCINUS MAENAS* (L.)

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The functions of the blood cells of crustaceans have received little investigation. The majority of the work reported in the literature to date has been concerned with the involvement of the haemocytes in the phenomenon of plasma coagulation. In several crustacean species highly fragile blood cells have been described – 'Hardy's explosive corpuscles' – which are generally believed to contain substances which can induce plasma coagulation (Hardy, 1892). This is supported by the observation that materials which can decrease, delay, or prevent alterations in the explosive corpuscles have also been found to interfere with plasma coagulation.

Crustacean blood cells have also been shown to contain a tyrosinase which is liberated during coagulation and which is capable of the oxidation of adrenaline and also of catechol. It is thought to be involved in pigment formation and quinone tanning (Bhaguart & Richter, 1938; Pinhey, 1930; Roche & Latreille, 1934).

Recently, however, a possible hepatic function has been proposed for crustacean blood cells (Johnston, Spencer Davies & Elder, 1971). These investigators found that the haemocytes of *Carcinus* could maintain stores of both glycogen and non-glycogen polysaccharides which were quantitatively more important than those found in the hepatopancreas of the crab. Preliminary biochemical and histochemical analyses have have also shown that the *Carcinus* haemocytes contain the enzyme glucose-6-phosphatase, a pre-requisite for the glycogenolysis of glycogen to glucose, which could then be liberated from the cells.

The present investigation is concerned with the discovery of a large pool of free amino acids within *Carcinus* haemocytes. It sets out to measure the relative contributions of the individual amino acids to this pool and to make a comparison with the results for other tissues from *Carcinus*. Some possible functions for the amino acids are suggested, and the significance of the results is discussed with respect to other investigations concerning the free amino acids of crustacean blood.

MATERIALS AND METHODS

Blood samples of 2·5 ml were obtained from male specimens of *Carcinus maenas* (L.) in the weight range 60–80 g. Two methods of sampling were employed which yielded identical results. Initially samples were obtained by cutting off the dactylopodite of a walking leg and allowing the blood to collect in a vial. However, the method used routinely consisted of the insertion of a hypodermic needle into an arthrodial membrane at the base of a walking leg and removing the required volume of blood

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in a syringe. It was felt that the latter sampling technique caused less damage to the animal and also enabled samples to be taken from the same animal on successive days.

The blood samples were divided into three parts. 1 ml was used for a whole-blood sample and 1 ml was subjected to mild centrifugation (2000 *g* for 5 min) to produce plasma and cell fractions. Light-microscope examination of the plasma fraction revealed it to be free of blood cells and little or no clotting occurred in the sample if the operation was carried out rapidly. The other 0·5 ml of the original blood sample was used for a blood-cell count.

0·25 μmole of norleucine were added to each fraction to provide an internal standard for the extraction procedure. 1 ml of a 0·4 N perchloric acid solution was added, with vigorous agitation, to each fraction in order to precipitate the proteins present. The precipitate was centrifuged off at 24,000 *g* for 10 min. The supernatant was decanted off into a storage tube and the precipitate was washed twice with 0·5 ml aliquots of a 0·2 N perchloric acid solution. The supernatants of the washes were added to that of the original centrifugation. The pH of this sample was adjusted to neutrality, using potassium hydroxide, and stored at 0 °C for 30 min in order for the precipitation of the insoluble potassium perchlorate to reach completion. This precipitate was spun off at 24,000 *g* for 15 min and the sample was dried down under vacuum prior to its application, in a 0·01 N hydrochloric acid solution, to the column of a Technicon Automatic Amino Acid Analyser.

The results were corrected for any loss during the extraction procedure by reference to the norleucine internal standard. They are expressed as μmoles/ml.

Blood-cell counts were performed by diluting the sample of crab blood 50:50 with a solution of 2% EDTA. This solution was then used to load a haemocytometer slide, and estimates of the number of blood cells were made by light-microscope examination using phase-contrast illumination. Estimates of the mean blood-cell diameter were also made, using an ocular micrometer, on a number of blood samples subjected to different treatments, all of which gave essentially similar values. The above information was then used to find the approximate volume of blood cells in the blood sample, and thus to estimate the intracellular amino acid concentration of the blood cells.

RESULTS

The values obtained for the three fractions, i.e. blood, plasma and cells, are shown in Table 1 for three typical samples, each sample being from a different crab. The crabs were kept under identical conditions in the laboratory marine aquarium and were fed for 3 days prior to sacrifice. It can be seen that a considerable percentage of the total free amino acid pool of whole crab blood is present within the cell fraction.

The corresponding blood-cell counts ($\pm$ S.E.) obtained for the above samples were as follows: sample A 32,000 $\pm$ 3000 cells/μl: sample B 33,550 $\pm$ 1200 cells/μl; and sample C 34,445 $\pm$ 835 cells/μl. These values are very much lower than those found for human blood, which contains about 5,000,000 cells/μl, but fall within the usual range of between 100,000 cells/μl and 10,000 cells/μl found in insect blood (Tauber & Yeager, 1935, 1936; Brady, 1967).

In order to obtain an approximate value for the intracellular concentration of the free amino acid pool, it was necessary to estimate the total volume occupied by the

blood cells per unit volume of blood. One method commonly used for such purposes is the haematocrit, but since the results obtained by this method are very variable and depend upon the speed of centrifugation used to pack the cells, it would be difficult to interpret the results obtained from this method in terms of the actual volume of the blood occupied by the cells. Instead, estimates of the diameters of blood cells were made using an ocular micrometer under phase-contrast illumination. The diameters of cells were measured in a number of differing suspension media such as whole blood, whole blood diluted with saline, 2% EDTA, 0·2% EDTA, and 0·2% sodium citrate. The three latter media had anticoagulants present and in all cases the values obtained for the blood-cell diameters lay between 8 and 12 μm. In the first two media the majority of the blood cells were clumped together, but the diameters of certain isolated cells agreed with the above values.

Table 1. *The compositions of the free amino acid pools from whole blood, plasma and cell fractions from three typical specimens of* Carcinus

The results are expressed as μ moles/ml. For the cell fractions the values are in μ moles of amino acid present in cell fraction obtained from 1 ml of crab blood. — = present but not estimated and T = present in trace amounts.

Sample ...	A			B			C		
Fraction ...	Blood	Plasma	Cells	Blood	Plasma	Cells	Blood	Plasma	Cells
Amino acid									
Taurine	1·552	0·338	1·256	2·104	0·229	1·862	2·621	0·409	2·416
Aspartate	0·016	0·008	0·012	0·053	0·025	0·015	0·049	0·016	0·020
Glutamine	—	—	—	—	—	—	1·182	0·632	0·507
Serine	—	—	—	—	—	—	0·135	0·083	0·058
Glutamate	0·225	0·064	0·110	0·238	0·078	0·197	0·207	0·023	0·192
Proline	0·989	0·681	0·186	0·538	0·318	0·187	0·628	0·339	0·315
Glycine	0·473	0·367	0·144	0·560	0·362	0·174	0·807	0·322	0·388
Alanine	0·586	0·519	0·324	0·800	0·364	0·426	1·072	0·394	0·676
Valine	0·061	0·042	0·018	0·064	0·062	0·020	0·042	0·023	T
Methonine	0·029	T	0·006	0·028	0·018	0·006	T	T	T
Isoleucine	0·021	0·012	0·005	0·024	0·017	0·007	0·015	T	T
Leucine	0·044	0·024	0·015	0·042	0·029	0·014	0·026	0·016	T
Tyrosine	0·021	0·014	0·009	0·032	0·025	0·013	0·025	T	T
Phenylalanine	0·015	0·009	0·005	0·017	0·017	0·006	0·017	T	T
Lysine	0·032	0·028	0·014	0·035	0·018	0·008	0·034	0·020	0·022
Histidine	0·029	0·026	0·014	0·049	0·020	0·012	0·034	0·020	0·021
Arginine	0·247	0·171	0·104	0·383	0·208	0·195	0·325	0·112	0·187

It was therefore decided to adopt 10 μm as a mean value for the blood-cell diameter and to use this to find the order of magnitude of the total blood-cell volume. Assuming the cells to be approximately spherical in suspension, a value of 262×10^{-9} μl is obtained for the volume of a single blood cell, and the corresponding percentages of whole blood volume occupied by cells for the blood sample A, B and C are respectively 0·84, 0·88 and 0·91%. This volume of approximately 1% is in the same range as that found by Brady (1967) for cockroach blood (2·6%) but is, of course, considerably smaller than for human blood where the cells occupy nearly 50% of the total blood-cell volume (Tauber & Yeager, 1935, 1936).

The above total blood-cell volumes, although approximate, enable the order of magnitude of the intracellular free amino acid pool of the blood cells to be calculated.

The mean value for the above three samples is set out in Table 2, together with the free amino acid concentrations of the other blood fractions. The values of the free amino acid concentrations present in other tissues of the shore crab, *Carcinus*, are also included for comparison.

Table 2. *The composition of the free amino acid pools from various tissues of* Carcinus

The results are expressed as μmoles/ml blood ± S.E. for the whole-blood and plasma fractions and as μmoles/ml cell water ± S.E. for the cell fraction, peripheral nerve and muscle. The values for muscle are all calculated from Duchâteau *et al.* (1959), except for the taurine value which comes from Chaplin *et al.* (1970), using a correction factor obtained from Florkin & Schoffeniels (1969). — = present but not estimated.

Sample	Whole blood	Plasma	Cells	Peripheral nerve	Muscle
Amino acid					
Taurine	2·090 ± 0·310	0·325 ± 0·050	209·200 ± 33·700	117·30 ± 6·87	50·53
Aspartate	0·039 ± 0·010	0·017 ± 0·005	1·780 ± 0·250	308·90 ± 13·84	5·21
Glutamine	1·180	0·633	55·700	36·90	—
Serine	0·140	0·083	6·330	4·66	—
Glutamate	0·223 ± 0·009	0·055 ± 0·016	18·930 ± 2·930	56·20 ± 3·00	48·13
Proline	0·720 ± 0·138	0·446 ± 0·117	25·990 ± 4·310	37·90 ± 2·06	131·60
Glycine	0·613 ± 0·100	0·350 ± 0·014	26·510 ± 8·100	11·37 ± 2·46	134·60
Alanine	0·819 ± 0·140	0·426 ± 0·047	53·800 ± 10·680	33·65 ± 2·46	27·40
Valine	0·056 ± 0·007	0·042 ± 0·011	2·170 ± 0·050	1·06 ± 0·02	4·41
Methionine	0·029 ± 0·001	0·018	0·739 ± 0·020	1·01 ± 0·06	—
Isoleucine	0·020 ± 0·003	0·015 ± 0·003	0·723 ± 0·112	0·42 ± 0·05	2·27
Leucine	0·037 ± 0·006	0·023 ± 0·004	1·098 ± 0·553	0·72 ± 0·05	3·48
Tyrosine	0·026 ± 0·003	0·019 ± 0·006	1·306 ± 0·196	0·81 ± 0·08	0·40
Phenylalanine	0·016 ± 0·001	0·013 ± 0·004	0·631 ± 0·055	0·26 ± 0·02	0·67
Lysine	0·033 ± 0·001	0·022 ± 0·003	1·699 ± 0·434	0·92 ± 0·06	2·54
Histidine	0·038 ± 0·006	0·022 ± 0·002	1·783 ± 0·289	0·75 ± 0·05	1·33
Arginine	0·318 ± 0·039	0·164 ± 0·028	18·400 ± 3·035	13·99 ± 0·33	48·40
Total	6·392	2·672	426·800	626·80	460·90

DISCUSSION

It can be seen from the above results that the blood cells of the common shore crab, *Carcinus maenas* (L.), like muscle and nervous tissue from the same animal, possess a pool of free amino acids large in comparison to their concentrations in the plasma. This results in the presence of a steep concentration gradient across the membranes of the blood cells.

It would seem likely that the large pool of free amino acids in the blood cells could play an important role in the isosmotic intracellular regulation of these cells in a similar fashion to that suggested for the corresponding free amino acid pools in other invertebrate tissues (Florkin, 1956; Camien *et al.* 1951; see also reviews by Schoffeniels & Gilles, 1970*a*, *b*) since the cells would also have to adapt their internal osmotic pressures if the euryhaline crab moved into a medium of differing salinity which would impose an osmotic stress upon its tissues.

The pattern of the amino acid pool can be seen to vary from tissue to tissue in *Carcinus* (Table 2). Thus glycine, proline and arginine are highly concentrated in muscle tissue, while aspartate and taurine are the most important in peripheral nerve

and taurine, alanine and glutamine are the most concentrated in blood cells. The especially high taurine concentration found in the blood cells is of particular interest. High concentrations of taurine have been reported previously in nervous tissue from several invertebrate species (Tallan, 1962) and Kravitz *et al.* (1963*a*, *b*) have shown that of the inhibitory substances present in an extract of lobster peripheral nerve 30% of the total inhibition could be attributed to taurine molecule. The taurine molecule is a structural analogue of γ-amino-butyric acid, which is generally thought to be the inhibitory transmitter of the crustacean nervous system, but it differs from it by having two instead of three carbon atoms and also in the nature of the acidic moiety (Jacobson & Smith, 1968). In view of this inhibitory effect of taurine on crustacean nerve impulses and of its concentration in *Carcinus* haemocytes, it is interesting to speculate that one possible function of the blood cells of *Carcinus* is to maintain the level of free taurine in the plasma at a low level which will not interfere with the transmission of impulses by the crab nervous system.

It is also of interest to note that in *Carcinus* the concentration of the anionic amino acid, aspartate, is low in haemocytes and muscle cells in comparison to its concentration in peripheral nerve. It has been suggested previously that the anionic amino acids undoubtedly play a great part in the balancing of the high potassium levels of arthropod muscles and nerves (Lewis, 1952; Schoffeniels & Gilles, 1970*b*) and that the retention of potassium ions appears to be largely electrostatic. Thus it would seem possible that in *Carcinus* different anions are being used to balance the high potassium levels in muscle and blood cells from those used in peripheral nerve. In the axoplasm of the giant squid axon the large amounts of isethionic acid, which is the deaminated analogue of taurine and which were discovered by Koechlin (1954), are thought to balance the high internal concentration of cations. Thus it is possible that the high internal concentration of the strongly anionic taurine molecule could play an analogous role in the haemocytes of *Carcinus*.

The discovery of a large free amino acid pool in the blood cells of *Carcinus* provides a possible explanation for the varying values set out for the mean blood amino acid concentrations of *Carcinus* in the literature. Vales equivalent to 11·65 mg N% and 12·0 mg. N% were obtained by Binns (1969*b*) and Delaunay (1931) respectively and these values were considerably greater than the plasma concentration of non-protein α-amino nitrogen equivalent to 2·81 mg N% found by Robertson (1960). Both Binns and Delaunay used whole-blood samples for their estimations whilst Robertson gently centrifuged his blood samples and obtained values corresponding to the plasma fraction used in the present investigation.

The existence of this free amino acid pool in *Carcinus* haemocytes may necessitate the re-interpretation of some existing physiological data. For example, the presence of this intracellular amino acid fraction could affect our conception of the mechanism of reabsorption of amino acids by the antennal gland of *Carcinus* proposed by Binns (1969*b*). The latter investigator's blood concentrations include a proportion of free amino acids which are contained in the blood cells, whereas urine in *Carcinus* is produced by a non-selective movement of a protein-free (and cell-free) filtrate of the blood plasma into the antennal gland (Binns, 1969*a*). It is also unknown if the variation in salinity of the bathing medium has any effect upon the distribution of the blood amino acid pool between the cellular and plasma fractions.

Thus in future investigations of active reabsorption of free amino acids by the antennal gland of *Carcinus* in media of differing salinities care should be taken to ensure that urine concentrations are compared with plasma concentrations of free amino acids, in order to find the degree of reabsorption and are not compared with whole-blood concentrations which contain a pool of free amino acids which does not contribute to the formation of urine from the plasma. The presence of this large intracellular free amino acid pool in the haemocytes should also be taken into consideration in any further studies of amino acids in crustacean blood.

SUMMARY

1. The presence of a large intracellular pool of free amino acids in the haemocytes of *Carcinus maenas* (L.) is described. It was found that 58% of the total free amino acid concentration of a whole-blood sample was present in the cell fraction.

2. The blood-cell count for *Carcinus* was found to be around 33,000 cells/μl which corresponded to 1% by volume of the whole-blood sample. Thus 58% of the total free amino acid concentration of the blood sample is sequestered into 1% of the total volume.

3. The pattern of the amino acid pool of the haemocytes is shown to differ from that of muscle and nervous tissue from *Carcinus*. In particular, the taurine molecule accounted for 50% of the pool in the haemocytes.

4. Possible functions for the amino acids of the haemocyte pool are suggested and the results are discussed in relation to other studies on free amino acids in crustacean blood.

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