applicable whenever functions of known or assumed form are to be fitted to experimental series of values. The calculations required are not unduly time-consuming, usually involving only a fraction of the corresponding time spent in laboratory work and planning.

The statistical methods not only supply more accurate estimates and the necessary measures of precision but have a further advantage, in that subjective biases which might otherwise have arisen are thereby avoided. In fitting by eye, for instance, a series of straight lines to Lineweaver–Burk plots corresponding to a range of substrate pH values, there may be a subjective tendency to fit the lines either steeper or flatter than they should be, giving rise to distortions in the actual trend of K_m with pH. Another kind of bias arises if there is a subconscious tendency to make the trend in slope of the series of lines rather more uniform than it should be. A plot of the K_m determinations against pH would give, as a consequence of the subjective elimination of variability, a misleading visual impression of the accuracy of the experimental work, and certainly significance tests based on such determinations would be invalid.

It should be emphasized that statistical measures of precision supply a gauge for random variation only. An experiment may supply, in this sense, a precise determination, which nevertheless is seriously biased by some defect or limitation in the experimental procedure.

SUMMARY

1. An account is given of the weighted and non-linear regression methods relevant to enzyme kinetic studies, with a brief preliminary outline of statistical terminology and the basic calculus of random variation.

2. Statistical considerations indicate that, for graphical determinations of the parameters K_m and V in the Michaelis–Menten equation, the linear plot of s/v against s is preferable to the double reciprocal plot.

3. A computational procedure is given for estimating K_m, V and the relevant standard errors.

4. The application of regression methods is further illustrated with the estimation of dissociation constants from a series of K_m determinations.

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Free Amino Acids of the Haemolymph of *Schistocerca gregaria* Forsk.

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Insect haemolymph contains free amino acids in higher concentrations than the blood of other animal groups; detailed data have been collected from different species of various orders of insects only in the last ten years, mainly after the development of microbiological and chromatographic methods of analysis.

Some investigators have recently studied the variations of amino-acidaemia during developmental stages of holometabolous insects, in relation to the metabolic processes involved (cf. Florkin, 1959).

As part of a research on amino acid metabolism in Orthoptera, we report here the quantitative pattern of the free amino acids in the blood of a locust, *Schistocerca gregaria* Forsk., at various stages of development. A similar investigation on a grasshopper, *Anacridium aegyptium* L., has already been published (Benassi, Colombo & Peretti, 1959).

MATERIAL AND METHODS

Blood was analysed from 3rd and 5th instar hoppers and from adults of a normal pigmented and of an albino strain of *S. gregaria* Forsk. The strains were kindly supplied by the Anti-Locust Research Centre of London and reared at the Institute of Zoology of Padua University. The albinos are homozygotes for a single recessive gene (Hunter-Jones, 1957). Hoppers and adults of both strains were reared at 30°, under crowded conditions, fed on bran supplemented with dry yeast (10%) and on leaves of chicory.

Samples of blood were taken from cut hind legs of several insects in order to pool at least 0·5–1·0 ml. of haemolymph for each group. The free amino acids from deproteinized whole blood were determined as the 2:4-dinitrophenyl derivatives by the methods of Sanger (1945) and Levy (1954). The ether-soluble dinitrophenyl (DNP) amino acids were separated by two-dimensional paper chromatography according to Biserte & Osteux (1951) and the water-soluble DNP amino acids by one-dimensional chromatography according to Blackburn & Lowther (1951). The DNP derivatives of aspartic acid and glutamic acid, as well as those of asparagine and glutamine, migrate as a single spot in the chromatographic system commonly used. Separation is obtained by using in the second run 2·5 M instead of 1·5 M-phosphate buffer.

Each spot was eluted with 1% $NaHCO_3$ (15 min. at 60°) and readings were done at 360 mμ (385 mμ for DNP-proline) with the Hilger Uvispek spectrophotometer. The amounts of each amino acid were calculated according to Levy (1954). For each sample five to ten chromatographic separations were carried out, and the average and the

Table 1. (*a*) *Free amino acids of haemolymph of pigmented* Schistocerca gregaria

Amino acid values are given as μmoles/100 ml. of haemolymph.

	3rd instar	5th instar		Immature adults		Mature adults	
		Males	Females	Males	Females	Males	Females
Glycine	843±11	4507±139	4259±32	1375±45	1811±101	684±52	703±180
α-Alanine	216±11	804±20	475±19	339±23	347±28	104±10	48±2
Serine	193±9	604±20	376±19	257±32	187±19	170±18	102±4
Threonine	221±9	663±80	423±5	215±13	192±22	76±10	Traces
Valine	263±14	823±34	652±51	357±44	275±29	184±9	69±4
Leucine and isoleucine	201±10	717±27	463±19	225±14	187±23	101±8	57±4
Methionine	123±8	231±15	256±5	140±3	Traces	Traces	93±2
Aspartic acid + Glutamic acid	274±11	533±26	1000±19	820±22	656±23	290±11	219±13
Glutamine	340±7	1656±92	153±9	852±39	671±33	Traces	179±5
Proline	350±11	1147±40	810±40	545±21	807±30	574±58	474±11
Lysine	—	73±40	78±35	140±69	Traces	Traces	Traces
Arginine	83±2	117±3	104±5	53±2	358±19	58±8	Traces
Tryptophan	138±20	170±11	148±7	115±16	96±21	Traces	54±8
Phenylalanine	84±4	57±11	Traces	64±8	Traces	Traces	Traces
Tyrosine	254±21	1583±51	903±21	384±26	51±3	217±16	168±14
Histidine	167±6	269±32	337±6	30±3	32±1	152±5	Traces
Totals	3750	13 954	10 437	5641	5670	2648	2166

Table 1. (*b*) *Free amino acids of haemolymph of albino* Schistocerca gregaria

Amino acid values are given as μmoles/100 ml. of haemolymph.

	3rd instar	5th instar		Immature adults	
		Males	Females	Males	Females
Glycine	2333±75	1671±53	1381±38	1079±41	1541±38
α-Alanine	402±50	247±8	189±9	189±12	198±6
Serine	134±14	217±7	250±32	133±12	139±12
Threonine	214±29	230±5	197±9	154±26	194±8
Valine	191±7	229±7	211±9	217±15	278±9
Leucine and isoleucine	134±29	182±3	153±10	137±12	163±6
Methionine	Traces	Traces	33±10	64±2	58±4
Aspartic acid + Glutamic acid	403±60	1752±13	1004±11	1349±19	1476±127
Glutamine	601±30	176±7	178±10	396±16	224±13
Proline	394±22	509±7	376±8	437±25	475±8
Lysine	—	—	Traces	35±2	Traces
Arginine	371±19	88±3	536±82	51±2	122±7
Tryptophan	Traces	140±4	164±3	93±6	37±2
Phenylalanine	Traces	55±2	Traces	Traces	34±2
Tyrosine	486±56	115±4	186±7	94±4	153±17
Histidine	511±18	48±1	744±24	86±6	96±6
Totals	6174	5659	5602	4514	5188

experimental error were calculated. Since the error showed rather large variations among the amino acids, it is reported in Tables 1*a* and 1*b*.

The separation of aspartic acid and glutamic acid was done only twice for each sample, therefore in Tables 1*a* and 1*b* the combined values are reported and in Tables 2*a* and 2*b* the proportions of the dicarboxylic amino acids are shown separately.

Glutamine but not asparagine appeared to be present.

RESULTS

The values for 17 amino acids found in the haemolymph of the stages so far studied are reported in Tables 1*a* and 1*b*. Two DNP derivatives, not yet identified, are not reported in the tables; they are present in such small amount that their determination was not possible.

To facilitate a comparison of the relative concentrations of amino acids at different stages of development, the percentages of each amino acid in the total content are given in Tables 2*a* and 2*b*.

Glycine is the most abundant amino acid, representing more than 25% of the total content of amino acids at all stages. Glutamic acid, glutamine, proline and tyrosine also occur in high concentration and show quantitative variations at different stages. Other amino acids, namely α-alanine, serine, threonine, valine and leucine and isoleucine,

Table 2. (*a*) *Amino acids of haemolymph of pigmented* Schistocerca gregaria

Values are expressed as percentages (by wt.) of the total content.

Amino acids	3rd instar	5th instar		Immature adults		Mature adults	
		Males	Females	Males	Females	Males	Females
Glycine	22·48	32·30	40·66	24·38	31·54	25·42	31·73
α-Alanine	5·75	5·76	4·53	6·01	6·04	3·86	2·16
Serine	5·14	4·33	3·59	4·56	3·25	6·32	4·59
Threonine	5·90	4·75	4·04	3·81	3·34	2·83	0·45
Valine	7·01	5·90	6·22	6·33	4·78	6·84	3·12
Leucine and isoleucine	5·36	5·14	4·42	3·99	3·25	3·75	2·56
Methionine	3·28	1·65	2·44	2·48	0·44	0·37	4·21
Aspartic acid	1·68	1·15	2·00	1·57	0·63	1·30	0·63
Glutamic acid	5·64	2·68	7·50	12·97	10·79	9·50	9·26
Glutamine	9·06	11·87	1·46	10·31	11·68	0·37	8·08
Proline	9·33	8·22	7·73	9·66	14·05	21·36	21·39
Lysine	—	0·52	0·74	2·47	0·44	1·03	0·45
Arginine	2·20	0·84	0·99	0·94	6·23	2·54	0·45
Tryptophan	3·69	1·22	1·41	2·04	1·67	0·37	2·42
Phenylalanine	2·25	0·41	0·48	1·13	0·44	0·37	0·45
Tyrosine	6·77	11·35	8·62	6·81	0·89	8·09	7·58
Histidine	4·45	1·92	3·22	0·54	0·56	5·68	0·45
Totals	99·99	100·01	100·05	100·00	100·02	100·00	99·98

Table 2. (*b*) *Amino acids of haemolymph of albino* Schistocerca gregaria

Values are expressed as percentages (by wt.) of the total content.

Amino acids	3rd instar	5th instar		Immature adults	
		Males	Females	Males	Females
Glycine	37·30	29·40	24·43	23·77	29·56
α-Alanine	6·42	4·35	3·34	4·16	3·80
Serine	2·14	3·82	4·42	2·93	2·67
Threonine	3·42	4·05	3·49	3·39	3·72
Valine	3·05	4·03	3·73	4·78	5·33
Leucine and isoleucine	2·14	3·20	2·71	3·02	3·13
Methionine	0·40	0·44	0·58	1·41	1·11
Aspartic acid	1·39	3·88	2·76	3·95	7·16
Glutamic acid	5·06	29·94	15·00	25·77	21·15
Glutamine	9·61	3·10	3·15	8·72	4·30
Proline	6·30	8·95	6·65	9·63	9·11
Lysine	—	—	0·44	0·77	0·48
Arginine	5·93	1·55	9·48	1·12	2·34
Tryptophan	0·40	2·46	2·90	2·05	0·71
Phenylalanine	0·40	0·97	0·44	0·55	0·65
Tyrosine	7·78	2·02	3·29	2·07	2·93
Histidine	8·31	0·84	13·16	1·89	1·84
Totals	100·05	100·00	99·97	99·98	99·99

occur almost always in the same proportions (about 5% of the total amino acid content). Methionine, aspartic acid, lysine and arginine are present in smaller quantity, each representing about 1% of the total amino acids. Tryptophan and histidine represent about 2% of the total, with large variation among the stages. Phenylalanine is the compound found in the smallest quantity.

DISCUSSION

The number of amino acids identified in *S. gregaria* haemolymph by us is the highest, so far as we know, found in Orthoptera. In the blood of nymphs of *Locusta migratoria migratorioides* 16 amino acids were determined (Duchateau & Florkin, 1958), in *Anacridium aegyptium* L. 14 (Benassi *et al.* 1959), and in *S. gregaria* 11 (Treherne, 1959). These differences might be due to different analytical methods and to the small concentration of some amino acids. However, it may also depend on species differences and on variations connected with the physiological and environmental conditions of the insects.

The concentrations of almost all the free amino acids found by others in Orthoptera are of the same order as those reported here, though Treherne (1959) found a larger amount of serine in *S. gregaria*.

Normal pigmented and albino locusts of different sexes were analysed separately at different stages of development. Rather large variations of the content of total free amino acids were found at different stages of the normal pigmented insects. These changes are possibly related to difference of water content of the blood, due to different environmental conditions, such as humidity and food succulence, since samples of blood were taken in different periods of the year, from January to June. Comparable variations were not found at different stages of development in the albino strain, which was analysed in a shorter period. Although there is evidence of a decrease of most amino acids in the mature old animals, mainly in the females, differences between sexes are probably not significant. Moreover, the proportions of each amino acid do not show a parallel change.

Analyses of free amino acids in the haemolymph provide no evidence for the possible role of the amino acids in the physiological changes that accompany development and maturation. Nevertheless, it is of interest to consider the relative concentration of certain amino acids in relation to the metabolic activity in the fat bodies of *S. gregaria*. In insects, fat bodies are spread in the general cavity where haemolymph freely diffuses, and extensive exchanges between fat bodies and haemolymph are very likely.

Glycine, always present in the largest quantity, in the haemolymph is involved in many metabolic processes (Clements, 1959), but its high concentration is possibly related to its osmotic function in insect blood (Buck, 1953). The presence of glutamic acid and glutamine in a high concentration can be related to transaminase and glutaminase activities in fat bodies (Kilby & Neville, 1957; Clements, 1959). Proline also occurs in relatively high quantity; its relations to the metabolic processes are not yet clarified. In *S. gregaria* fat bodies, Kilby & Neville (1957) observed no synthesis of glutamate from proline.

Tyrosine is an important compound for cuticle synthesis and melanogenesis (cf. Hackman, 1958); its concentration in haemolymph is relatively large and higher in normal pigmented animals than in albino strain. A similar difference is observed with phenylalanine and tryptophan. The analysis of these three amino acids of the cuticle may indicate more important differences between pigmented and albino strains, since insectorubin, a pigment formed from tryptophan (cf. Cromartie, 1959), decreases by about 85% in albino *S. gregaria* (Bellamy, 1958).

Data on the exchanges of free amino acids between blood and tissues are still scanty; some free amino acids appear to be present only in the tissues, e.g. β-alanine, cysteine and cystine were found in ovaries and in embryos of *S. gregaria* but not in the blood of hopper and adult locust (G. Colombo, C. A. Benassi, G. Allegri & E. Longo, in preparation).

SUMMARY

1. Seventeen free amino acids were identified in the whole blood of normal pigmented and of albino locust *Schistocerca gregaria*.

2. No significant differences in the content of free amino acids were found during nymphal development and sexual maturation except a decrease in total free amino acids in the oldest animals.

3. Glycine, proline, glutamic acid and glutamine represent the largest part of the free amino acids in all the stages.

4. In normal pigmented hoppers and locusts, tyrosine, phenylalanine and tryptophan are present in larger concentration than in albino insects.

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Paper-Chromatographic Separation of Chlorophylls and Carotenoids from Marine Algae

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Although many methods have been described for the partial separation of chloroplast pigments by paper chromatography (see review by Šesták, 1958) the species examined have been mainly higher plants and algae of the class Chlorophyceae, in which the main chloroplast pigments are chlorophylls *a* and *b*, with β-carotene and lutein as the major carotenoids. In the present work, a method was required for studying the pigment composition of planktonic algae which occur in the oceanic waters off Sydney. Representatives are found not only of the Chlorophyceae but also of the Bacillariophyceae, Dinophyceae and Chrysophyceae, and one would expect to find chlorophylls *a*, *b* and *c* together with a wide range of carotenoids (Strain, 1958). The method described below, which is a modification of the two-dimensional method of Lind, Lane & Gleason (1953), has enabled studies of the pigment composition of such diverse algal groups to be made, since it provides a complete separation of mixtures of chlorophylls *a*, *b* and *c*, carotenes and the xanthophylls lutein, violaxanthin, neoxanthin, fucoxanthin, peridinin and astaxanthin, as well as a number of xanthophyll pigments which occur in relatively small quantities.

EXPERIMENTAL

Materials

Representatives from four classes of marine algae were studied: green flagellates from the Chlorophyceae, diatoms from the Bacillariophyceae, naked dinoflagellates from the Dinophyceae and golden-brown flagellates from the Chrysophyceae (Fritsch, 1948). The material used was obtained either from uni-algal, but not bacteria-free, cultures of marine algae, or from mixed natural plankton in sea-water samples. The uni-algal cultures studied included four green flagellates [*Dunaliella tertiolecta* Butcher, *Nannochloris atomus* Butcher, *Chlorella stigmatophora* Butcher and *Tetraselmis suecica* (Kylin) Butcher], three diatoms [*Phaeodactylum tricornutum* Bohlen, *Nitzschia closterium* (Ehr.) and *Skeletonema costatum* (Grev.)], one dinoflagellate (*Gymnodinium* sp.) and two golden-brown flagellates (*Isochrysis galbana* Parke and *Sphaleromantis* sp.). *Phaeodactylum*, *Dunaliella* and *Isochrysis* were obtained from Dr Mary Parke of the Marine Laboratory, Plymouth. The other organisms were isolated from mixed plankton samples taken from the coastal waters off Sydney.

The organisms were grown in an Erdschreiber medium, consisting of sea water enriched with nitrates, phosphates and soil extract. The soil extract was prepared by autoclaving 1 kg. of soil with 1 l. of tap water at 15 lb./in.^{2} for 2–3 hr., and filtering through an Eaton–Dikeman Paper 541. After diluting 1 vol. of the resulting extract with 2 vol. of tap water, concentrated stock nutrient media were prepared by adding 0·2 g. of $NaNO_3$ and 0·03 g. of $Na_2HPO_4,12H_2O$ to every 50 ml. of soil extract, and autoclaving at 5 lb./in.^{2} for 1 hr. The final culture medium was prepared when needed by adding 50 ml. of this enriched extract to 1 l. of filtered sea water, and autoclaving at 5 lb./in.^{2} for 1 hr. Stock cultures of algae were maintained in 200 ml. of culture medium in 250 ml. Erlenmeyer flasks at 18°, and illumination was from 40 w fluorescent tubes giving a light intensity of approx. 400 ft.-candles. Bulk cultures (4 l.) were continuously aerated in 5 l. Erlenmeyer or Haffkine flasks, and were used for analysis after 2–4 weeks' growth. The algae were harvested by continuous centrifuging at 5000*g* and the cells were washed several times by centrifuging at about 2000*g* for 10 min. This process removed most of the bacteria, as judged by microscopic examination under phase contrast.

In addition to these uni-algal cultures, mixed natural plankton from sea-water samples was used for pigment analysis. At least 35 l. of sea water was needed to obtain sufficient pigments for chromatography. The sea water was