CHANGES IN THE RELATIVE AMOUNTS OF SOLUBLE PROTEIN AND AMINO ACID IN THE HAEMOLYMPH OF THE LOCUST, *CHORTOICETES TERMINIFERA* WALKER (ORTHOPTERA : ACRIDIDAE), IN RELATION TO DEHYDRATION AND SUBSEQUENT HYDRATION

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[*Manuscript received May* 19, 1966]

Summary

Starvation and dehydration of fifth-instar female larvae of *C. terminifera* over concentrated sulphuric acid for 24 hr causes a loss in volume of the haemolymph of over 25% but little change in osmotic pressure. When dehydrated insects imbibe distilled water in the absence of food, the volume of the haemolymph shows an increase after 7 hr which is lost over the succeeding 17 hr. Compared with controls, the decreases in volume are associated with a loss of free amino acids and gain of soluble protein in the haemolymph as a whole; and the increase with a loss of soluble protein and gain of amino acids. Although amino acids contribute no more than 15% of the osmotically active constituents of the haemolymph, it is possible that they interchange with soluble protein and play a limited role in maintaining osmotic pressure, especially during rapid increases in volume of the haemolymph in the absence of dietary salts.

I. Introduction

During investigations of the composition of the haemolymph of the Australian black-tipped locust, *Chortoicetes terminifera* Walker, it was found that the concentrations of free amino acids varied individually and collectively in locusts fed on different diets (T.D., unpublished data). In particular, the omission of bran from a diet that consisted otherwise of fresh grasses resulted in a threefold rise in the total concentration of amino acids in the haemolymph. This result is consistent with the suggestion that free amino acids in the haemolymph of insects are involved in osmoregulation (Beadle and Shaw 1950; Schoffeniels 1960); and the following is an account of experiments designed to cause fluctuations in the water content of the haemolymph and to measure their effects on the osmotic pressure and nitrogenous constituents of the haemolymph–plasma. Chemical desiccants were used to dehydrate the live insects, which were then given access to distilled water for the next 24 hr; this means of altering the water content of *C. terminifera* does no more than accentuate types of stress to which the species is normally subjected in its natural environment, which is often hot and arid (Swan 1956).

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II. Materials and Methods

(*a*) *Design of Experiments*

Three batches of insects were taken at random from a uniformly reared population. One (the control) was kept over, but not in contact with, distilled water for 24 hr; the others were kept meanwhile over sulphuric acid and phosphorus pentoxide respectively. All three batches were then given access to distilled water for the next 24 hr. No food or salts were available to the insects for the entire experimental period of 48 hr. The following estimations were made on samples from each batch: fresh body weight, volume of the haemolymph, the osmotic pressure of the haemolymph, the concentration of protein nitrogen in the plasma, the concentration of non-protein nitrogen in the plasma, the total concentration of free amino acids (as nitrogen) in the plasma, and the concentration of 20 individual amino acids in the plasma. Samples were taken at the following times: immediately before the "dehydration" period (time = 0); immediately after the dehydration period (time = 24); after 7 hr of subsequent "hydration", i.e. access to distilled water (time = 31); and after 24 hr of hydration (time = 48).

Determinations of fresh body weight and volume of the haemolymph were done separately on five or more individuals per sample; these values are quoted $\pm$ their standard errors. Other properties of the haemolymph were determined on plasma taken and pooled from a sample of 17 or more (average 21) insects. Pooled samples of haemolymph taken at time = 0 and time = 24 were replicated six times, but all subsequent pooled samples were unreplicated, except that values for insects dehydrated over the two desiccants can be checked against one another. Once insects had been sampled for properties of the haemolymph they were discarded from the experiment.

The composition of the haemolymph may vary considerably with development (Wyatt, Loughheed, and Wyatt 1956). Thus all observations were made on females midway through their fifth (and final) larval stadium.

(*b*) *Rearing*

The insects were reared *en masse* by a method essentially similar to that described by Hunter-Jones (1956). Their standard diet was blades of Kikuyu grass (*Pennisetum clandestinum* Hochst. ex Chior.) renewed daily, wheat bran, and water.

(*c*) *Experimental Batches*

Fourth-instar females that had a distended abdomen and raised wing pads were placed, 30 at a time, in well-ventilated, 10-litre "preparation cages". These cages were kept at $32{\cdot}0 \pm 0{\cdot}5$°C, and exposed to light for 15 hr per day; they were cleaned daily and provisioned with fresh grass, water, and wheat bran. Larvae that had moulted within 24 hr of removal from the mass-rearing cages were marked on the pronotum with Dulon touch-up paint; they were allowed to feed for a further 48 hr in the preparation cages before removal for experiments. The greatest possible range

in their ages since the fourth moult was thus 48–72 hr, with an average of 2·5 days. The average length of the fifth stadium was 5 days.

(*d*) *Dehydration and Hydration*

Fifth-instar females, 2·5 days old, were weighed and placed individually in 4-dram clear plastic vials. These were capped with Terylene gauze and placed, 10 at a time, on their sides over 100 ml conc. sulphuric acid (analytical grade) or 100 g phosphorus pentoxide (laboratory grade) in a 1 l. air-tight container. The controls were placed over water in place of desiccant. The acid was replaced after two uses, the phosphorus pentoxide was used fresh every time. The insects were reweighed 24 hr later and transferred individually to 8-dram plastic vials that had a direct connection with a water reservoir.

(*e*) *Volume of Haemolymph*

Determination of haemolymph volume was by the dye-dilution method using amaranth (Yeager and Munson 1950; Lee 1961).

(*f*) *Collection of Pooled Plasma*

Haemolymph was taken up into a Pyrex capillary tube from a dorsal puncture in the neck membrane. The haemolymph from several insects was collected in a Pyrex centrifuge tube which had been cooled in an ice-bath. When about 0·3 ml had been amassed, the pooled haemolymph was centrifuged for 15 min at 1500 *g* and 2°C. A few microlitres were stored at -20 ± 2°C for later determination of osmotic pressure, and the rest was analysed immediately for its nitrogenous constituents.

(*g*) *Osmotic Pressure*

Determination of osmotic pressure was by the freezing-point method (Ramsay 1949).

(*h*) *Nitrogenous Constituents*

Protein was precipitated from the pooled plasma with 95% ethanol (Dent 1946), washed three times with 95% ethanol, freeze-dried, and resuspended in 1N NaOH to four times the original volume of plasma. Two 5-μl aliquots were taken for estimates of protein nitrogen by the method of Weed and Courtenay (1954). From the collected supernatant and washings of the precipitate (about 20 times the original volume of plasma), one-tenth was set aside to provide two aliquots for the determination of non-protein nitrogen, and the remainder was run through a column of Amberlite CG-120 (100–200 mesh), 1 cm in diameter and 1 cm deep, and prepared according to Brimley and Barrett (1953). The loaded column was washed with distilled water until the effluent no longer smelled of alcohol. The amino acids were then eluted with 25 ml of 0·15N ammonium hydroxide, freeze-dried, and redissolved in borate buffer, pH 10 (Hackman and Lazarus 1956), to make nine-tenths the original volume of plasma. Two 10-μl aliquots were taken for estimation of "total amino acids" and the rest was analysed qualitatively and quantitatively by one-dimensional paper chromatography, as described by Hackman and Lazarus (1956).

IV. Experimental Results

(a) *Volume of Haemolymph*

The initial weights of the insects used in the experiments (302±4 mg) varied little, and volumes of haemolymph are depicted in graphs as microlitres per individual.

Lee (1961) observed that amaranth did not appear in the malpighian tubes of *Schistocerca gregaria* within 15 min of injection; thereafter, estimates of volumes based on dilution of the dye increased, presumably due to its excretion. In the experiments with *Chortoicetes*, however, amaranth appeared in the malpighian tubes after periods in excess of 5 min, which proved to be near the minimum time required for adequate circulation of the dye. Thus, although the haemolymph was sampled

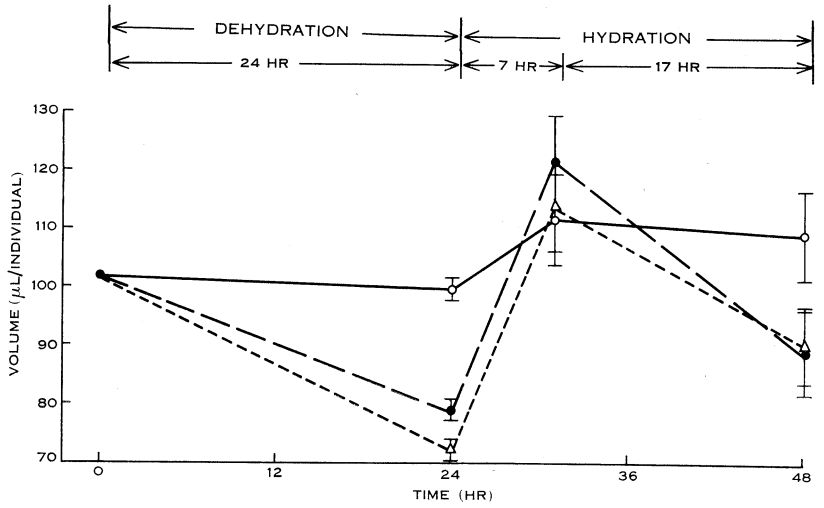

Fig. 1.—Effect of dehydration and subsequent hydration on the volume of the haemolymph of fifth-instar females of *C. terminifera*. ○ Water (control); ● phosphorus pentoxide; △ concentrated sulphuric acid.

at this time, there is a possibility that the values obtained for the volume [334·6± 8·9 μl/g (= 101·6±3·3 μl/individual for females midway through the fifth stadium)] are too high. Moreover, if estimates were affected by excretion, it is possible that they would be affected to different extents in different treatments. Nevertheless, it must be assumed that the rate of excretion of the dye would tend to vary with its concentration in the haemolymph, and hence that the lower the actual volume of the haemolymph, the greater the bias to overestimation. Thus any inaccuracies in estimates can be expected to mask differences rather than exaggerate them.

Highly significant differences in volume of haemolymph between the dehydrated insects and their controls were found (Fig. 1), and it is clear that the dehydrated insects did indeed lose water from the haemolymph. Of the desiccants used, concentrated sulphuric acid proved unexpectedly superior, but the phosphorus pentoxide tended to crust over rapidly and this may have caused some loss of its effectiveness.

When given access to water, the dehydrated insects drank readily and, during the next 7 hr, the volume of their haemolymph was more than restored. During the following 17 hr, however, the volume of the haemolymph dropped again.

Changes in estimated volume of haemolymph were paralleled by changes in fresh body weight—closely over the first 24 hr, and more approximately thereafter (Fig. 2). The fresh weights of dehydrated insects became greater during the subsequent hydration period than might have been expected solely from the volume of their haemolymph, and this may indicate the uptake of water into body tissues.

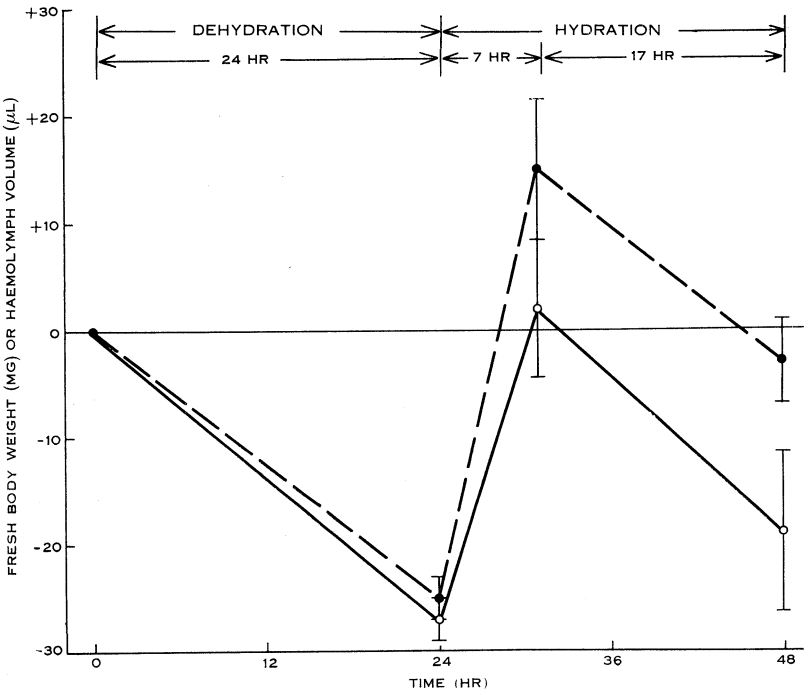

Fig. 2.—Changes in haemolymph volume (○) and in fresh body weight (●) of fifth-instar females of *C. terminifera* due to dehydration over concentrated sulphuric acid (i.e. observed values minus control values obtained from insects kept over distilled water during the "dehydration" period).

(*b*) *Osmotic Pressure*

Immediately after dehydration of the insects over sulphuric acid, which caused the loss of approximately a quarter of the volume of the haemolymph, the osmotic pressure of the haemolymph was $250 \cdot 7 \pm 4 \cdot 7$ mM NaCl compared with $229 \cdot 3 \pm 4 \cdot 6$ mM in the controls. No other changes in osmotic pressure observed during the experiments were statistically significant. The average values obtained are compared in Figure 3 with "expected" values, i.e. those that would have been obtained had changes in volume of the haemolymph reflected changes in water content alone, unaccompanied by withdrawal or addition of osmotically active materials. Clearly, some degree of

osmoregulation occurred, and hence osmotically active solutes must have been removed from the haemolymph whenever water was lost from it, and must have been replaced when water was regained.

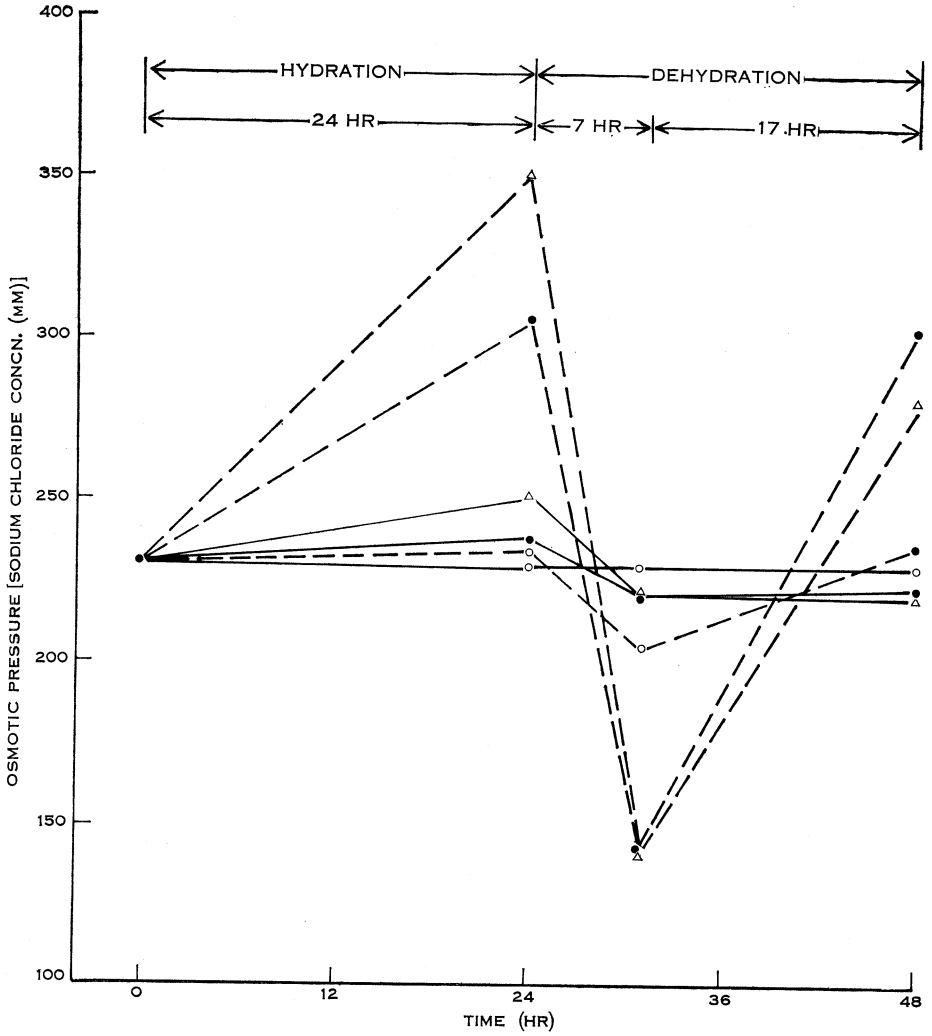

Fig. 3.—Effect of dehydration and subsequent hydration on the osmotic pressure of the haemolymph of fifth-instar females of *C. terminifera*. ——— Observed values; – – – – expected values (calculated from changes of haemolymph volume). ○ Water (control); ● phosphorus pentoxide; △ concentrated sulphuric acid.

(c) *Nitrogenous Constituents*

Two estimates are available for the content of free amino acids in the haemolymph, viz. the "Nessler" value, and the sum of 20 "common" amino acids estimated after separation on paper chromatograms. The latter values were between 80 and 92% of the former, which is reasonable agreement considering that some amino

acids may not have been included in the sum of individual compounds, and that some nitrogenous compounds other than amino acids may have been included in the Nessler value. Nevertheless, it is unlikely that the Nessler values overestimated amino acids, for some amino acid was lost, prior to determination, during the process of adsorption and elution in the resin column. The average recovery from standard solutions was 88% for all but the more basic compounds lysine and arginine, only 70% of which were recovered. It is probably safe to conclude that the true values for the total amino acid are not far from the Nessler estimates.

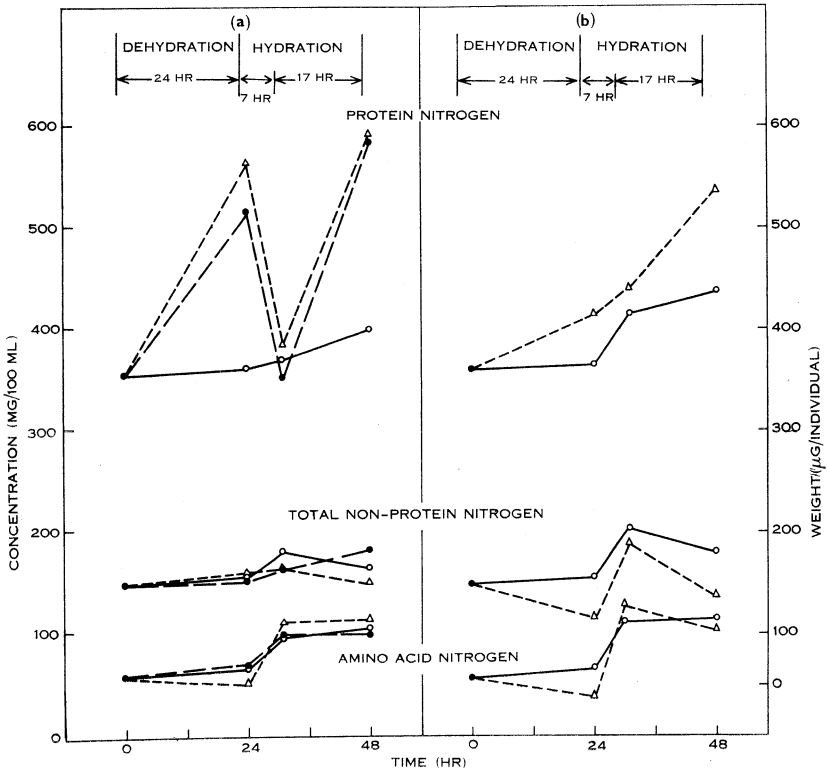

Fig. 4.—Effect of dehydration and subsequent hydration on (*a*) the concentration of nitrogenous constituents in the haemolymph plasma, and (*b*) the weight of nitrogenous constituents in the total volume of haemolymph plasma of fifth-instar females of *C. terminifera*. ○ Water (control); ● phosphorus pentoxide; △ concentrated sulphuric acid.

Protein nitrogen concentration in the haemolymph of the dehydrated insects increased greatly whenever the volume fell [Fig. 4(*a*)]. Such changes would merely be passive, however, if the actual weight of the solute in the total volume of haemolymph remained much the same while the water content varied; and in Figure 4(*b*) are shown the *total weights* of protein and non-protein nitrogen in the haemolymph of the controls and the insects dehydrated over sulphuric acid (values for the insects kept over phosphorus pentoxide are either intermediate, in keeping with the lower

degree of dehydration achieved with this desiccant, or close to those for sulphuric acid, and have been omitted for the sake of clarity). In the controls, the volume of the haemolymph remained constant during the first 24 hr (when they had no access to water) and there was no significant change in the total nitrogen content of the haemolymph, except perhaps a slight rise in amino acids from 57 to 66 ± 2 μg nitrogen per individual. Imbibition of water, however, apparently triggered a rise in the amounts of both protein and total non-protein nitrogen in the whole volume of haemolymph, perhaps because of a release of reserves from storage tissues and an increase in metabolism. Later, the non-protein nitrogen fell slightly, perhaps indicating the excretion of nitrogenous waste products. In the dehydrated insects, on the other hand, the weight of protein nitrogen in the haemolymph rose during the initial period of water loss and continued to rise throughout the experiment; whereas the weight of non-protein nitrogen showed a marked tendency to fluctuate with changes in the volume of the haemolymph.

When differences between dehydrated insects and their controls, with respect to the weights of nitrogenous constituents in the haemolymph, are plotted in relation to the corresponding differences in volume of the haemolymph (Fig. 5), it is found that increases in protein due to dehydration were accompanied by decreases in free amino acids and *vice versa*. Statistically, differences at the end of the dehydration period between the haemolymph of the dehydrated insects and their controls were significant with respect to weight of protein, and highly significant with respect to weights of amino acid and total non-protein nitrogen.

The concentrations of individual amino acids separated by paper chromatography (Table 1) followed the same trends as the total amino acids, as estimated using Nessler's reagent. Despite inaccuracies inherent in the method of chromatographic determination, glycine, proline, and serine, in particular, tended to increase both in concentration and in weight in the total volume of haemolymph during the experimental period, and fluctuations in the amount of these amino acids in the haemolymph contributed greatly to the overall fluctuations of the amino acids as a group.

V. Discussion

The Possible Role of Amino Acids in Osmoregulation

In aquatic insects, amino acids have been shown to provide an increased share of the osmotically active molecules in the haemolymph when the insects were starved in hypotonic solutions (Beadle and Shaw 1950; Schoffeniels 1960). In terrestrial insects, the corresponding situation would be either the loss of salts from, or an increase in the water content of, the haemolymph in the absence of dietary salts, and this has been achieved in the experiments reported here. When dehydrated insects were given access to distilled water, the volume of the haemolymph was temporarily restored and osmotically active molecules apparently reappeared. Salts retained in the malpighian tubes and rectum could conceivably have been returned to the haemolymph at this time, and possibly the contents of the gut, even after 24 hr starvation, may have provided some osmotically active molecules; but otherwise

these must have been furnished from body cells or by the transformation of osmotically inactive or weakly active substances already in the haemolymph.

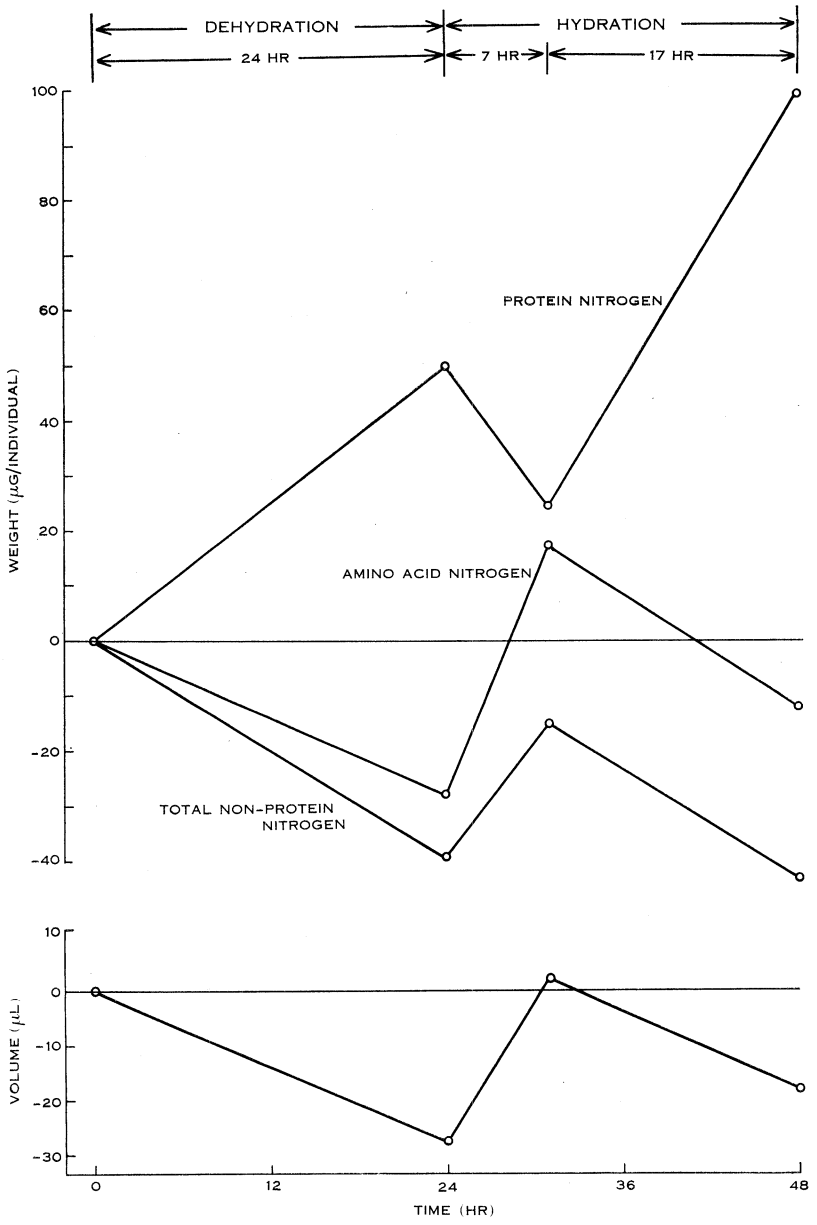

Fig. 5.—Changes in the weights of nitrogenous constituents and volume of the haemolymph plasma of fifth-instar females of *C. terminifera* due to dehydration over concentrated sulphuric acid (i.e. observed values minus control values obtained from insects kept over distilled water during the "dehydration" period).

The inverse relation, during fluctuations in volume of the haemolymph, between total weight of protein in the haemolymph on the one hand and of amino acids on the other might well be interpreted as a reversible conversion related to osmoregulation; but calculations of the osmotic contributions of amino acids in the haemolymph

TABLE 1

EFFECT OF DEHYDRATION AND SUBSEQUENT HYDRATION ON THE CONCENTRATIONS OF AMINO ACIDS IN THE HAEMOLYMPH OF FIFTH INSTAR FEMALES OF *C. TERMINIFERA*

\+ = Trace; — = not found

Amino Acid*	Amino Acid Concentration (mM) for Times †									
	0‡	24‖	31	48	24	31	48	24§	31	48
		Control			*Desiccant* P_2O_5			*Desiccant* H_2SO_4		
Glycine	5·5	12·2	15·6	20·5	13·0	17·6	20·4	8·5	18·7	22·2
Alanine	3·7	3·0	4·2	3·5	3·4	4·5	3·4	2·8	5·1	3·0
Glutamine	3·0	2·2	2·6	2·2	2·3	3·9	2·4	2·3	3·0	2·3
Proline	2·6	5·5	6·6	4·4	4·0	7·2	3·6	3·2	5·8	3·9
Serine	2·2	2·9	3·7	3·5	3·1	4·6	4·3	2·3	4·8	4·0
Histidine	1·6	1·0	0·9	0·7	0·7	0·9	0·7	0·8	0·9	0·7
Lysine	1·6	2·5	5·8	8·4	2·9	5·3	7·9	1·8	5·6	7·4
Leucine	1·2	0·9	1·0	1·8	0·7	0·9	1·9	0·7	1·1	1·8
Valine	1·2	0·9	1·2	3·1	0·8	1·2	3·3	0·8	1·5	3·6
Phenylalanine	1·0	0·7	0·9	0·6	0·5	0·8	0·6	0·9	0·7	0·5
Threonine	0·8	0·8	1·4	1·9	0·9	1·6	1·9	0·6	1·4	2·0
Tyrosine	0·6	0·6	0·8	1·6	0·7	1·0	1·7	0·6	0·9	1·5
Methionine	0·6									
Arginine	0·5	0·5	0·5	+	0·5	0·8	+	0·5	0·6	+
Asparagine	0·5	0·3	0·1	0·2	0·3	0·3	0·4	0·3	0·4	0·3
Glutamic acid	0·3	0·4	0·6	0·6	0·3	0·4	0·5	0·3	0·6	0·5
Aspartic acid	—	0·1	0·3	—	—	0·2	—	—	—	—
Tryptophan	+	+	+	+	+	+	+	+	+	+
Total (mM)	27	35	46	53	34	51	53	26	52	54
Total (mg nitrogen/100 ml)	51	61	82	92	60	91	92	47	89	92
Total (as % of Nessler estimate)	90	92	83	88	88	92	92	90	80	81

* Cysteine or cystine and hydroxyproline were not found in any of these samples. Methionine values are suspect because of interference from other compounds. The reproducibility of results using standards were similar to those given by Hackman and Lazarus (1956).

† Time = 0, before dehydration; 24, after 24 hr dehydration; 31, after 7 hr subsequent access to water; 48, after 24 hr access to water. Control insects were kept over distilled water during the "dehydration" period.

‡ Average of 2 samples. ‖ Average of 4 samples. § Average of 3 samples.

show that, in our experiments, this never became very great. The values shown in Figure 6 for the osmotic contribution of amino acids are based on the sum of molar concentrations of individually separated amino acids, adjusted to agree with the Nessler estimates of total amino acid acid given in Table 1. It has been assumed that a molecule of amino acid in solution produces only half the osmotic effect of a

molecule of sodium chloride, and although this is a slight underestimate, for amino acids are weak electrolytes, amino acids contributed at most in the region of 15% of the total osmoles present. Such an evaluation may underestimate the role of amino acids in osmoregulation, however, for, as Figure 7 illustrates, amino acids contributed

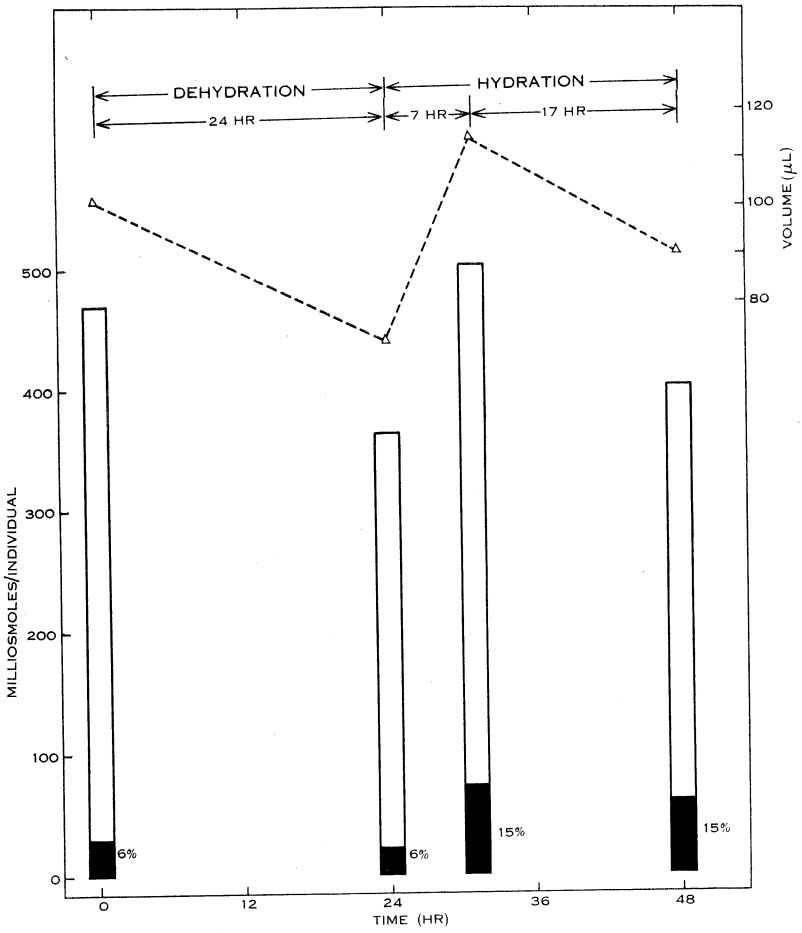

Fig. 6.—Osmoles present in the total volume of haemolymph of fifth-instar females of *C. terminifera*, showing the contribution of amino acids (■) in relation to fluctuations in the volume of the haemolymph brought about by dehydration over concentrated sulphuric acid followed by access to distilled water.

over a third of the total osmoles *added back* to the haemolymph when dehydrated insects imbibed water and thus increased the volume of their haemolymph in the absence of dietary salts.

The temporary nature of the restoration of volume of the haemolymph of starved and dehydrated insects when they drank water was an unexpected result in our experiments. The osmotic pressure of the haemolymph remained nearly

constant and, during dehydration, is presumably regulated mainly by the excretion of osmotically active molecules. [The tendency to increased tonicity at this time may possibly promote the synthesis of protein by cells that regulate the protein content of the haemolymph (Shigematzu 1960) at the expense of its amino acid

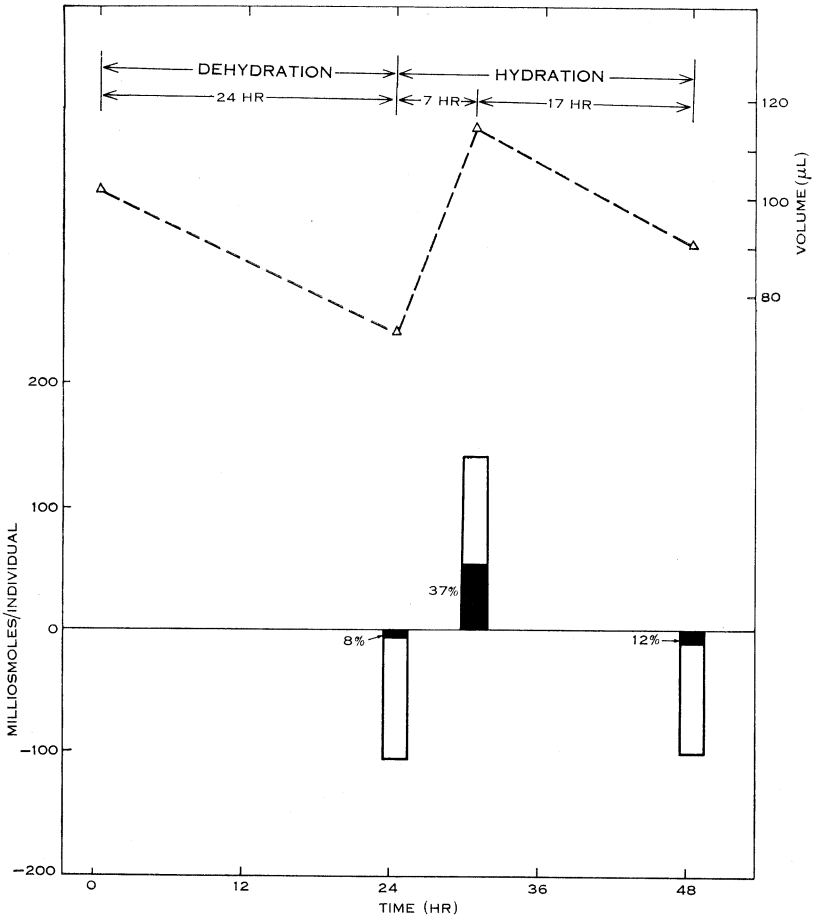

Fig. 7.—Contribution of amino acids (■) to losses and gains of osmoles present in the total volume of haemolymph of fifth-instar females of *C. terminifera* in relation to fluctuations in the volume of the haemolymph brought about by dehydration over concentrated sulphuric, followed by access to distilled water.

content.] Subsequent imbibition of water causes an immediate increase in the water content of the haemolymph and it must be assumed that either salts pass from the tissues to the haemolymph or that osmotically active organic molecules replace the salts previously excreted (e.g. by the hydrolysis of protein to amino acids). But the insects' ability to provide osmotically active molecules for the haemolymph during starvation must surely reach a limit when the supply of salts is exhausted and when osmotically

active organic substances reach toxic concentrations. Thus, in our experiments, total amino acids appeared to reach a limiting concentration in the haemolymph of *C. terminifera* of about 1·1 mg nitrogen per millilitre, coincident with a limit in their contribution to osmotic pressure of about 15% of the total osmoles present. In the continued absence of dietary salts, once reserves of osmotically active substances have been utilized to the maximum extent possible, the only remaining counter to hypotonicity of the haemolymph is the excretion of water; and even without continued imbibition, hypotonicity of the haemolymph can be expected to result from the utilization of osmotically active metabolites and the excretion of osmotically active waste products.

Blackith (1961) presents a different view of the interchange of protein and amino acid with respect to dehydrated hatchlings of the locust *Nomadacris septemfasciata*. He found an increase in water-soluble solids in the body, and suggested that this was brought about by depolymerization of protein, with the result that the osmotic pressure of the haemolymph would increase even further, and thus such water as remained would be held even more tenaciously. Nevertheless, the results quoted by Blackith refer to the water-soluble solids of the entire body, as well as to a different stage of development. Thus there is not necessarily any conflict between Blackith's results and our own.

VI. Acknowledgments

One of us (T.D.) wishes to acknowledge gratefully a Colombo Plan Fellowship from the Commonwealth of Australia and a Research Grant from the University of Adelaide. These made possible her contribution, which represents part of a thesis presented to the University of Adelaide for her Ph.D.

We wish to thank Miss C. M. Hill for the preparation of diagrams.

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