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Some Aspects of Amino Acid Metabolism in the Adult Housefly, *Musca domestica**

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The concentration of free amino acids in insect haemolymph is in general much greater (often 10–50 times) than it is in vertebrate blood (Auclair & Dubreuil, 1953; Bishop, Briggs & Ronzoni, 1925; Buck, 1953; Duchateau & Florkin, 1955; Duval, Portier & Courtois, 1928; Florkin, 1954; Pratt, 1950). Other invertebrates, such as the lobster (*Homarus vulgaris*) and the crayfish (*Astacus fluviatilis*) possess high concentrations of amino acids in their tissues but the concentrations in their blood are of the magnitude found in vertebrate blood (Florkin, 1954). The significance of the high concentration of free amino acids in insect haemolymph has intrigued many workers and it has been wondered whether some of the roles played by amino acids in insects are particular to that class. Winteringham (1958) has suggested that in insects some of the amino acids may provide a soluble and readily available substrate reserve for the Krebs cycle. Winteringham & Harrison (1956) showed that α -alanine, aspartic acid, glutamic acid, glutamine and proline became labelled in adult houseflies (*Musca domestica*) after these insects had been injected with [2-¹⁴C]acetate as labelled precursor. Kasting & McGinnis (1958) found that certain amino acids became labelled in *Phormia regina* after it was fed with [¹⁴C]glucose. Clements (1959) found that when preparations of the fat-body of the desert locust (*Schistocerca gregaria*) were incubated with [2-¹⁴C]acetate, aspartic acid, glutamic acid and proline became labelled. In the present work the distribution in the fly and the metabolic relationships of some of the amino acids are investigated.

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METHODS

The L- or DL-forms of amino acids were purchased from L. Light and Co. Ltd, Colnbrook, Bucks. Each amino acid gave only one ninhydrin-positive spot after two-dimensional chromatography (see below) and they were therefore used without further purification. Anhydrous sodium [2-¹⁴C]acetate, with a specific activity of 5.16 $\mu\text{C}/\mu\text{mole}$, was purchased from The Radiochemical Centre, Amersham, Bucks. A pyridine-acetate buffer, pH 6.0, was prepared by mixing 10 ml. of pyridine, 1 ml. of acetic acid and 89 ml. of water. Methyl Cellosolve (ethylene glycol monomethyl ether) and lutidine were redistilled before use. Phenol was redistilled according to the method of Draper & Pollard (1949). Ninhydrin and isatin (indoline-2:3-dione) were used as 0.2% solutions in acetone.

Sarcosine-isolation medium. The final concentrations were: 0.32 M-sucrose; 0.01 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), pH 7.2; 0.01 M-ethylenediamine-tetra-acetate, pH 7.2; 1% of bovine plasma albumin.

Albumin-free isolation medium. The same as above but without albumin.

Breeding of houseflies (*Musca domestica*). Adult houseflies were kept at 27° and 60% relative humidity and were fed on glucose, milk and water. Only females, 5–6 days old, were used in the experiments.

Injection with [2-¹⁴C]acetate. Each fly was anaesthetized with cyclopropane for 1 min. and then injected from an Agla micrometer syringe with 2 $\mu\text{l.}$ of a solution containing 63.6 $\mu\text{g.}$ of anhydrous sodium acetate (4 $\mu\text{C.}$). The hypodermic needle was inserted 1–2 mm. into the dorsal posterior end of the thorax just under the left-wing base. After being injected, each fly was put into a 150 mm. $\times$ 15 mm. test tube which was sealed with a rubber bung. Soda lime was present in the base of each tube to absorb the CO₂ expired, and the fly was prevented from coming into contact with the alkali by a loosely packed plug of cotton wool placed about 30–40 mm. from the open end. The fly was left in the tube at room temperature (approx. 22°) for the required period, after which it was treated in one of three

ways: (1) it was killed by plunging it into aq. 95% ethanol at -70° ; (2) it was cooled at 0° to immobilize it, and its head was cut off and placed in aq. 95% ethanol at -15° ; (3) a sample of haemolymph was obtained, as described below.

Supply of haemolymph. Withdrawal of a sample of haemolymph was carried out under a binocular microscope (mag. $\times 8$). The left wing of the fly was cut off near to its base and the fly was held between the thumb and forefinger of each hand and gently squeezed. The gentle pressure forced a globule of haemolymph out of the fly through the cut wing-base. A capillary tube (held in a clamp) was applied to the globule which rose up the tube. After as much haemolymph as possible had been obtained, the capillary was weighed, the haemolymph was blown into aq. 95% ethanol at 0° , and the tube weighed again.

Extraction of whole flies, of heads and of haemolymph. Ten flies were homogenized in 2 ml. of aq. 95% ethanol in a Potter-Elvehjem homogenizer for 5 min. at -15° , and the homogenate was centrifuged at 2100g for 15 min. The residue obtained was extracted twice by homogenizing in 1 ml. of aq. 95% ethanol and centrifuging as before. The three supernatants were combined and evaporated to dryness at room temperature. The residue was extracted twice at room temperature by homogenizing in 0.4 ml. of water and centrifuging at 22 000g for 15 min. The two supernatants were combined and filtered to remove particles of fat, and the filtrate was evaporated to dryness. The residue was resuspended in 0.33 ml. of aq. 95% ethanol to give the whole fly extract.

The heads from 30 flies were homogenized in 1 ml. of aq. 95% ethanol at -15° and the homogenate was centrifuged at 2100g for 10 min. The residue was extracted twice at room temperature by homogenizing in 0.5 ml. of aq. 95% ethanol and centrifuging as before. The three supernatants were combined, evaporated to dryness at room temperature and the residue was resuspended in 0.3 ml. of water to give the head extract.

Haemolymph from 25 flies was collected in 1.5 ml. of aq. 95% ethanol at 0° and centrifuged at 2100g for 10 min. The residue was washed twice by dispersion in 0.5 ml. of aq. 95% ethanol and centrifuged as before. The three supernatants were combined to give the haemolymph extract. All the extracts were stored at -15° .

Paper chromatography. One- and two-dimensional paper chromatography was carried out on strips (approx. 3 cm. wide) and on sheets (28 cm. $\times$ 28 cm.) of Whatman no. 1 paper. Solvents were: 1, water-saturated phenol (Pratt & Auclair, 1948); 2, butan-1-ol-acetic acid-water (50:10:40) (Phillips, 1949); 3, 98% formic acid-acetone-water (14:60:26) (Burrows, Grylls & Harrison, 1952); 4, lutidine-collidine-water (1:1:2) (Dent, 1948); 5, water-saturated isobutyric acid; 6, water-saturated butan-1-ol; 7, phenol-0.1% NH_3 ; solvents 5, 6 and 7 were according to Consden, Gordon & Martin (1944). One-dimensional chromatography was carried out in a descending manner with solvents 1, 2, 4, 5 and 6, the aqueous phase being placed in the bottom of the cylinder. With solvent 3, ascending chromatography was carried out as described by Winteringham, Bridges & Hellyer (1955). Chromatograms were developed at 25° for a period of 24-40 hr. in solvents 1, 2, 4, 5 and 6 and at 5° for the same period in solvent 3.

Elution of amino acids. After a [^{14}C]amino acid had been located, the section of chromatogram containing it was cut

out, and the compound was eluted onto a second strip as described by Winteringham (1953) and chromatographed in a second solvent. By repetition of this procedure it was possible to chromatograph a [^{14}C]amino acid in a series of solvent systems. For all the amino acids tried, elution seemed to be complete after 8 hr. since no radioactivity could be detected on the strip. The elution solvent used was 98% formic acid-acetone-water (1:500:500).

Two-dimensional chromatography. Ascending two-dimensional chromatography was carried out in a conventional manner. Chromatograms were developed for approximately 12 hr. and dried in a stream of air for 4-5 hr. at room temperature.

Chromatograms were developed in the first direction with solvent 2 and in the second direction with solvent 7. When solvent 7 was used aq. NH_3 soln. (sp.gr., 0.88) was added to the aqueous phase to a final concentration of 0.1%.

When the solvents were used in the order cited above, 'tailing' was considerably less and the spots were much more compact than when they were used in the reverse order.

Paper electrophoresis. Paper electrophoresis of 30 μl . of the head extract was carried out at 300 v with Whatman no. 1 paper and the pyridine-acetate buffer, pH 6.0. During electrophoresis, which lasted for approx. $1\frac{1}{4}$ hr., most of the strip, apart from the two ends which dipped into the buffer, was immersed in CCl_4 .

Location of amino acids. (a) Chemical methods. Amino acids were located by dipping the developed chromatogram in a 0.2% solution of ninhydrin in acetone, and then heating the chromatogram at $80-90^{\circ}$ for 5 min. (Toennies & Kolb, 1951). For the detection of proline the appropriate part of the chromatogram was dipped in a solution of isatin (0.2%) in acetone and heated at 105° for 3 min. (Smith, 1953).

(b) Radiometric method. The ^{14}C -labelled compounds were located and assayed on the unidimensional chromatograms by a method in which the paper chromatogram is passed automatically beneath a thin end-window Geiger-Müller tube and the radioactivity is automatically plotted (Winteringham, Harrison & Bridges, 1952). Each zone of radioactivity on the strip is represented by a peak on the graph and the area enclosed by the peak is proportional to the amount of ^{14}C in the zone which it represents.

(c) Radioautographic method. ^{14}C -Labelled compounds were located on a two-dimensional chromatogram by leaving it in contact with an X-ray film for 4 days. The film (radioautograph) was then developed and radioactive compounds showed up as dark spots the intensities of which were proportional to the amount of radioactivity.

Identification of amino acids. [^{14}C]Amino acids were identified on unidimensional chromatograms by their coincidence with known 'marker' amino acids in a series of solvent systems. It is assumed that no isotopic exchange takes place between the labelled compounds and the added 'marker' amino acids. On two-dimensional chromatograms amino acids were identified by comparing chromatograms of the extracts with those of known amino acids.

Chemical estimation of amino acids. After chromatography of a labelled amino acid in solvents 1 and 2, the section of chromatogram containing it was cut out and treated with a 1% solution of KOH in methanol (approx. 20 μl ./cm.² of paper). The paper was dried in a stream of

warm, but not hot, air and left in an evacuated desiccator over conc. H_2SO_4 overnight. This procedure removed traces of NH_3 which give rise to high and variable blank readings (Fowden, 1951). The amino acid was then eluted with water into a graduated tube and estimated essentially in the manner described by Yemm & Cocking (1955).

Estimation of proline. The section of chromatogram containing proline was treated with isatin (as already described) and the amount of blue colour produced was measured on an EEL photoelectric densitometer with a yellow filter. A standard curve for proline was constructed with this method of estimation.

Measurement of haemolymph volume. Evans blue dye (100 mg.) (Prosser, 1952) was dissolved in 1.0 ml. of water and 1 μl . of this solution was injected into the thorax of the fly just under the left wing-base. The fly was left for 15 min. in order to give the dye time to diffuse throughout the haemolymph, a sample of which was weighed and transferred to 2.5 ml. of water in a 1 cm. spectrophotometer cell. The solution was thoroughly stirred and its absorption was measured at 600 $m\mu$ against that of water. The absorption of 1 μl . of dye solution in 2.5 ml. of water was also measured. From these values the volume of haemolymph was calculated.

Isolation of flight-muscle sarcosomes. Sarcosomes were isolated by the method of S. E. Lewis & K. S. Fowler (personal communication). Fifty 6–7-day-old female houseflies were collected in a conical flask and immobilized by cooling at 3°. They were transferred to an ice-cold dish and their heads and abdomens were removed, the thoraces being transferred to an ice-cold mortar where they were gently crushed in 1.5 ml. of isolation medium. The resulting brei was squeezed through six layers of muslin (which had previously been washed in water and wrung out), and the filtrate was collected in a tube surrounded by crushed ice. A further 0.75 ml. of isolation medium was added to the residue, which was again squeezed and filtered. The combined filtrates were centrifuged at 134g for 4 min. at 0°, to remove particles of chitin. The supernatant was centrifuged at 2100g for 10 min. and the sedimented sarcosomes were resuspended by adding 1.9 ml. of albumin-free isolation medium and centrifuged at 2100g for 10 min. The resulting pellet of washed sarcosomes was resuspended by adding 0.3 ml. of albumin-free isolation medium; 0.1 ml. of this preparation was used in each transaminase determination.

Transaminase activity of sarcosomes. Four tubes were set up, one pair containing: potassium phosphate buffer (KH_2PO_4 – K_2HPO_4 , 0.33M, pH 7.3, 0.3 ml.); L- α -alanine (0.045M, 0.2 ml.); α -oxoglutaric acid (0.12M, pH 7.3, 0.1 ml.); sucrose (1.66M, 0.1 ml.); bovine plasma albumin (10%, 0.1 ml.); sarcosomes (0.1 ml.); water (0.1 ml.). The second pair contained the same medium except that L- α -alanine was replaced by aspartic acid (0.045M, 0.2 ml.) The tubes were incubated at 25°, and to stop enzyme activity 0.1 ml. of 50% trichloroacetic acid was added to one tube of each pair at zero time, and to the other tubes after 45 min. The tubes were centrifuged at 2100g for 10 min. and 20 μl . of each supernatant was chromatographed in solvent 7 with glutamic acid, aspartic acid and alanine as markers. Before chromatography the extract on the chromatogram was exposed to the vapour from aq. 4N- NH_3 soln. for 5 min. to neutralize the trichloroacetic acid present. After development of the chromatograms the amino acids were located with ninhydrin as already described.

Transaminase activity of haemolymph. This was determined as described above by using the haemolymph from 10 flies in place of 0.1 ml. of sarcosomes.

Production of $^{14}\text{CO}_2$. Five flies were injected with [2- ^{14}C]acetate and the $^{14}\text{CO}_2$ they expired was trapped in 1 ml. of N-NaOH. The flies were placed in a glass chamber and air was sucked through the chamber and then over activated charcoal to absorb any volatile ^{14}C -labelled compounds (other than CO_2). At intervals 10 μl . amounts of the NaOH solution were removed, applied to filter-paper disks and evaporated to dryness in a current of hot air. The ^{14}C -activity, which was then measured with a conventional thin end-window Geiger-Müller counter, was over a range of 200–45 000 counts/5 min.

Estimation of [2- ^{14}C]acetate in flies. Flies were injected with [2- ^{14}C]acetate and left for different periods. Individual flies were killed by immersion in 0.9 ml. of boiling water in a Potter-Elvehjem homogenizing tube. Inactive sodium acetate (0.1M, 0.1 ml.) was added as carrier, and the whole homogenized for 2 min. at room temperature and then centrifuged at 2100g for 10 min. The supernatant (10 μl .) was applied to a filter-paper disk and evaporated to dryness in a stream of warm air. The ^{14}C -activity was then measured as described for the $^{14}\text{CO}_2$. N-HCl was sprayed on the disk very carefully, so as not to alter the geometry of the sample unduly, and the disk was heated to dryness in a stream of hot air. After two such treatments the ^{14}C -activity was measured as before. The difference in activity between the two measurements is taken as the activity due to the [2- ^{14}C]acetate which is volatilized by the HCl treatment. When known amounts of [2- ^{14}C]acetate were applied to filter-paper disks, the above treatment removed 96% of the ^{14}C -activity.

RESULTS

Identity of [^{14}C]amino acids

One-dimensional chromatography. When extracts of the whole fly (10 μl .) and of the haemolymph (100 μl .) were chromatographed together with 'marker' amino acids, in solvent 1, the ^{14}C -labelled compounds were separated into four fractions. These fractions, R_f 0.14 (aspartic acid), 0.23 (glutamic acid), 0.59 (α -alanine and glutamine) and 0.89 (proline), were eluted and rechromatographed in a selection of solvents 2, 3, 4, 5 or 6 so that each fraction, or part of it, coincided with a 'marker' amino acid in at least three solvents. The R_f values of the ^{14}C -labelled fractions and of the coincident 'marker' amino acids are shown in Table 1. The amino acids that became labelled, namely, α -alanine, aspartic acid, glutamic acid, glutamine and proline, were present in both the whole fly and in the haemolymph. It was calculated that the labelled amino acids accounted for 70% of the ^{14}C -activity in the whole fly extract but for only 30% of that in the haemolymph extract.

Two-dimensional chromatography. Two-dimensional chromatography of a number of known amino acids and of extracts of the whole fly (20 μl .), head (50 μl .) and haemolymph (500 μl .) was

carried out as described in the Methods section. A comparison of the chromatogram of the known amino acids with those of the extracts showed that the latter contained α -alanine, β -alanine, aspartic acid, glutamic acid, glutamine, glycine, leucine or isoleucine, lysine, methionine and/or valine, methionine sulphoxide, phenylalanine, proline, serine, taurine, threonine and tyrosine. γ -Aminobutyric acid was discernible only in the head extract, whereas aspartic acid and glutamic acid were present in relatively large amounts in the whole fly and head extracts but were hardly discernible in the haemolymph extract.

Radioautography. Radioautography of a chromatogram of the whole fly extract showed the presence of labelled α -alanine, aspartic acid, glutamic acid, glutamine and proline. This result is in complete agreement with that obtained by uni-dimensional chromatography.

After electrophoresis of the head extract, one of the radioactive, ninhydrin-positive spots obtained coincided with γ -aminobutyric acid. It was eluted from the strip and chromatographed in solvent 2 where it again coincided with γ -aminobutyric acid.

Amino acid content of tissues and haemolymph

Reference to standard curves for α -alanine, aspartic acid, glutamic acid, glutamine and proline showed that after chromatography in solvent 1 and then in solvent 2 the recoveries of these amino acids were 72, 70, 74, 50 and 89% respectively. These values were taken into account when the amounts of [^{14}C]amino acids in the fly were calculated.

To ascertain the distribution of amino acids between the tissues and the haemolymph of the fly it was necessary to determine the volume of the haemolymph and this was carried out as described in the Methods section. The haemolymph volume varied from fly to fly over a range of 3–9 $\mu\text{l.}$ for 10 flies, the average value per fly being 6 $\mu\text{l.}$ (S.D. $\pm$ 1.92 $\mu\text{l.}$).

The amounts of amino acids in the whole fly and in the haemolymph are shown in Table 2. The volume of the haemolymph extract from 25 flies was reduced to 0.5 ml. and 0.2 ml. of this concentrated extract was chromatographed together with 5 $\mu\text{l.}$ of the whole fly extract as marker. The concentrations of aspartic acid and glutamic acid are much less in the haemolymph than they are in the whole fly, whereas those of α -alanine, glutamine and proline in the haemolymph are of the same order as in the whole fly. These quantitative results confirm the results obtained by two-dimensional chromatography. The amounts of amino acids present in the tissues are shown in columns (5) and (6). The concentrations of aspartic acid and glutamic acid in the tissues are three and six times respectively those in the haemolymph. The concentrations of α -alanine, glutamine and proline in the tissues are similar to those in the haemolymph.

Values of 14.7 mg./100 g. and 131.4 mg./100 g. respectively, have been reported (Bellamy, 1958) for the concentrations of glutamic acid and glutamine in the blood of the desert locust (*S. gregaria*). Thus the glutamic acid concentration is very similar to that found in housefly blood, whereas that of glutamine is between two and three times that in the housefly.

Table 1. R_f values of ^{14}C -labelled compounds present in the whole fly and haemolymph extracts and of the amino acids with which they coincided after chromatography in various solvents

Amino acid	R_f in solvent					
	1	2	3	4	5	6
α -Alanine	0.59	0.14	0.73	0.14	0.53	0.02
Aspartic acid	0.14	0.14	0.58	0.18	—	—
Glutamic acid	0.24	0.12	0.65	0.20	0.50	—
Glutamine	0.59	0.08	0.48	0.17	—	0.01
Proline	0.90	0.17	—	0.29	0.59	0.10

Table 2. Amounts of amino acids in the whole fly, in the haemolymph and in the tissues

The amino acids were estimated as described in the text. The average weight of a housefly was 21.4 mg., the average value for the volume of haemolymph in a fly was 6 $\mu\text{l.}$, and the average weight of tissue in each fly was taken as 15.4 mg.

Amino acid	(1)	(2)	(3)	(4)	(5)	(6)
	$\mu\text{g./fly}$	mg./100 g. of fly	$\mu\text{g. in}$ haemolymph of 1 fly	mg./100 ml. of haemolymph	$\mu\text{g. in tissues}$ of fly	mg./100 g. of tissues
α -Alanine	6.30	29.5	1.49	24.8	4.81	31.4
Aspartic acid	2.62	12.2	0.28	4.6	2.34	15.2
Glutamic acid	11.80	55.2	0.74	12.3	11.06	72.0
Glutamine	12.26	57.4	3.51	58.6	8.75	56.8
Proline	20.20	94.5	4.70	78.3	15.50	100.0

Specific activity of [^{14}C]amino acids

In the following experiments the chromatograms were scanned with a 4π scanner and the activity was recorded as counts/min. by a printing-out scaler. By using a known amount of standard [^{14}C]acetate solution, counts/min. could be converted to μC and the values expressed as $\mu\text{C}/\mu\text{mole}$ of amino acid.

Flies were injected with [$2\text{-}^{14}\text{C}$]acetate and left for periods of 5, 10, 30, 60, 90, 120 and 180 min. (10 flies for each period). The subsequent stages of extraction, unidimensional chromatography and amino acid estimation were carried out as described in the Methods section and the results are shown in Fig. 1. The specific activities of α -alanine and glutamic acid were maximal 60 min. after injection of the flies with [$2\text{-}^{14}\text{C}$]acetate, whereas that of proline was maximal at 90 min. The peak of the specific activity-time curve of glutamine is not as sharp as those of α -alanine and glutamic acid, its specific activity remaining near its maximum over the period 60–90 min. The specific activities of α -alanine, glutamic acid, glutamine and proline were also measured in a haemolymph extract obtained from flies killed 30 min. after injection

Fig. 1. Specific activity of α -alanine (▽), glutamic acid (□), glutamine (○) and proline (△) in the whole fly at various periods after injection with [$2\text{-}^{14}\text{C}$]acetate.

with [$2\text{-}^{14}\text{C}$]acetate. The results, together with those of a whole fly extract obtained from flies killed after the same period, are shown in Table 3. The specific activities of α -alanine and proline in the whole fly and haemolymph extracts were very similar. The specific activities of glutamic acid and glutamine in the whole fly extract were approximately double their respective values in the haemolymph extract.

Incorporation of [^{14}C]amino acids into protein

The residue obtained from the third ethanol extraction of whole flies treated for 180 min. with [$2\text{-}^{14}\text{C}$]acetate was homogenized twice with 1 ml. of N-HCl (to remove labelled water-soluble compounds), twice with 1 ml. of ethanol-ether (3:1) (to remove labelled phospholipids), twice with 1 ml. of acetone and once with 1 ml. of CHCl_3 (to remove labelled lipids). Each homogenate was centrifuged at $2100g$ for 10 min. and the supernatants were discarded. The final residue was dispersed in 1 ml. of 6N-HCl and heated in a stoppered tube at 108° for 8 hr. in order to hydrolyse the protein present (Roberts, Abelson, Cowie, Bolton & Britten, 1957). Aq. 95 % ethanol (1 ml.) was added to the hydrolysate, which was then centrifuged at $2100g$ for 15 min. The supernatant was decanted and the residue washed with 1 ml. of ethanol and centrifuged, as before. The combined supernatants were repeatedly (5 times) evaporated to dryness to remove the HCl . The final residue was resuspended in 0.5 ml. of aq. 95 % ethanol. One- and two-dimensional chromatography of this extract (10 $\mu\text{l.}$) showed that it contained labelled α -alanine, aspartic acid, glutamic acid and proline. There were also some other labelled compounds present but these have not been identified. Glycine, methionine and/or valine, serine, threonine and tyrosine were some of the unlabelled amino acids present in the hydrolysate. β -Alanine and methionine sulphoxide were absent from the hydrolysate.

Transaminase activity

Barron & Tahmisian (1948) reported the presence of glutamic-alanine transaminase in cockroach-muscle preparations. Kilby & Neville (1956)

Table 3. *Specific activities of amino acids in whole fly and haemolymph extracts obtained from flies killed 30 min. after injection with [$2\text{-}^{14}\text{C}$]acetate*

Amino acid	Whole fly extract		Haemolymph extract	
	$10^2 \times \text{Amount estimated}$ (μmoles)	Specific activity ($\mu\text{C}/\mu\text{mole}$)	$10^2 \times \text{Amount estimated}$ (μmoles)	Specific activity ($\mu\text{C}/\mu\text{mole}$)
α -Alanine	15.3	0.77	7.5	0.86
Glutamic acid	17.8	1.74	2.3	0.77
Glutamine	12.6	1.15	7.5	0.62
Proline	47.0	0.28	22.8	0.28

demonstrated transaminase activity in the fat-body, malpighian tubules and gut wall of the desert locust (*S. gregaria*), and McAllan (1958) demonstrated glutamic-aspartic acid-transaminase activity in the fat-body, malpighian tubules and flight-muscle of the cockroach (*Periplaneta americana*), and in the housefly (*M. domestica*) throughout its life-cycle. In the present work glutamic-alanine transaminase and glutamic-aspartic acid transaminase enzyme systems have been investigated in flight-muscle sarcosomes and in haemolymph as described in the Methods section. The criterion for their presence was the production of glutamic acid. With the sarcosomes an amino acid was produced which behaved chromatographically in a similar way to glutamic acid, whether α -alanine or aspartic acid was added as the donor of the amino group. These results showed the presence of glutamic-alanine and glutamic-aspartic acid-transaminase activity in the sarcosomes. However, these two enzyme systems were not detectable in the haemolymph.

Production of $^{14}\text{CO}_2$ and metabolism of $[2-^{14}\text{C}]\text{acetate}$

The production of $^{14}\text{CO}_2$ and the disappearance of $[2-^{14}\text{C}]\text{acetate}$ were measured as described in the Methods section and the results are shown in Fig. 2. Curves A and B show that the production of $^{14}\text{CO}_2$ by the 'labelled' insects ceased about $5\frac{1}{2}$ hr. after they were injected with $[2-^{14}\text{C}]\text{acetate}$. Curve B, which is derived from curve A, also shows that the rate of $^{14}\text{CO}_2$ production was maximal during the period 50–70 min. after the flies were injected with $[^{14}\text{C}]\text{acetate}$. Curve C shows that at the end of this period 94 % of the acetate originally present in the flies had been metabolized.

Fig. 2. $^{14}\text{CO}_2$ production and $[2-^{14}\text{C}]\text{acetate}$ utilization by houseflies injected with $[2-^{14}\text{C}]\text{acetate}$. A, Evolution of $^{14}\text{CO}_2$ by five flies as % of that evolved after 18 hr. (100 % = 900 000 counts/min.); B, rate of evolution of $^{14}\text{CO}_2$ as % of rate between 50 and 70 min.; C, utilization of $[2-^{14}\text{C}]\text{acetate}$ (by individual flies) as % of that present at zero time (100 % = 3000 counts/min.).

DISCUSSION

In the present work the amino acids identified in the whole fly and in the haemolymph are, in general, the same as those reported present in the haemolymph of the housefly by Pratt (1950). The one exception is arginine, which he reported to be absent from the haemolymph. Arginine was identified in the whole fly extract by comparative chromatography and by the Sakaguchi test, and in the haemolymph extract by comparative chromatography. Winteringham *et al.* (1955) have also reported the presence of free arginine, as well as the insect phosphagen, phosphoarginine, in thoracic tissues of the adult housefly. Five of the amino acids (α -alanine, β -alanine, glutamic acid, glutamine and methionine sulphoxide) are present in relatively high concentrations in the whole fly, and three of these (α -alanine, glutamine and methionine sulphoxide) are also present in high concentrations in the haemolymph. The relatively high concentration of methionine sulphoxide found is not thought to be due to its formation from methionine during chromatography, for under the same conditions added methionine is not oxidized to its sulphoxide. High concentrations of methionine sulphoxide have also been found in female adult mosquitoes (Chen, 1958). The fact that this acid was absent from a hydrolysate of 'housefly' protein indicated that it is not a constituent of the protein of the fly. A similar observation was made with regard to β -alanine. The isatin test showed that proline is present in high concentrations both in the tissues and in the haemolymph, a fact which was not obvious from the chromatograms treated with ninhydrin because of the pale-yellow colour that proline gives with this reagent.

In bacteria β -alanine can be obtained by the decarboxylation of aspartic acid (Virtanen, Rintola & Laine, 1938). However, it appears very unlikely that it is obtained in this manner in the fly, because aspartic acid is labelled, but there is no evidence of β -alanine's being labelled.

It is well known that in vertebrates γ -aminobutyric acid is specially concerned in the metabolism of nervous tissue, such as the brain (Elliott, 1958; Roberts & Baxter, 1958). The fact that this amino acid was discernible only in the housefly head extracts shows that it is more concentrated in the head than in the rest of the fly and suggests that in insects, too, it may be specially concerned in the metabolism of nervous tissue. Further, since both glutamic acid and γ -aminobutyric acid were labelled in the fly, it is quite likely that the latter amino acid was derived from the former as a result of glutamic acid-decarboxylase activity. This suggestion is supported by the fact that glutamic acid-decarboxylase activity has

been demonstrated in bee brain *in vitro* (Toschi, 1961).

One of the most striking differences between the amino acid composition of the tissues and that of the haemolymph is the high concentration of aspartic acid and glutamic acid in the former and their low concentration in the latter. Kermack & Stein (1959) have observed a similar distribution in the locust (*Locusta migratoria migratoroides*), where these amino acids are present in muscle extracts but are absent from haemolymph. Bellamy (1958) has shown that glutamic acid is more concentrated in the head than in other tissues of the desert locust (*S. gregaria*). Turner, Eggleston & Krebs (1950) have shown that glutamic acid is concentrated in rat-brain cells, and there it is thought to play a highly specific and important role in the transport of potassium ions across cell membranes. It may be that it is playing a similar role in the fly.

Winteringham & Harrison (1956) showed that when houseflies are injected with [2-¹⁴C]acetate, α -alanine, aspartic acid, glutamic acid, glutamine, and proline become labelled. A similar result was obtained by Clements (1959), who showed that when the fat-body of the desert locust (*S. gregaria*) is incubated with [2-¹⁴C]acetate, glutamic acid and proline become labelled. The present work with the housefly confirms the observations of Winteringham & Harrison and has been extended to investigate the nature of the specific activity-time curves of the labelled amino acids. The steep slope of the specific activity-time curve of glutamic acid and of glutamine (Fig. 1) indicates that these two

amino acids are rapidly 'turned over' in the tissues of the fly.

That glutamic acid is playing an important role as a 'carbohydrate-amino acid' link in the fly is indicated by the following observations: (1) glutamic acid is concentrated in the tissues rather than in the haemolymph; (2) after injection of the flies with [2-¹⁴C]acetate, glutamic acid is rapidly labelled, presumably by its interconversion with α -oxoglutaric acid; (3) Glutamic-alanine transaminase and glutamic-aspartic acid transaminase enzyme systems are present in the sarcosomes. These observations indicate that glutamic acid plays a very similar role in the fly and in vertebrate tissues.

The presence of transaminase activity in the sarcosomes and its absence from the haemolymph is not surprising, because aspartic acid and glutamic acid, which are major participants in the transaminase reactions, are concentrated in the tissues and not in the haemolymph. This result is in keeping with that of McAllan (1958), who found no transaminase activity in the haemolymph of the cockroach (*P. americana*).

The results show that α -alanine and aspartic acid are labelled in the fly. Since glutamic acid is not a precursor of either of these acids they must become labelled from another source. It is well known that oxaloacetic acid can be decarboxylated, resulting in the production of pyruvic acid (Ochoa & Weisz-Tabori, 1948; Lewis & Price, 1956) and, when this transaminates with glutamic acid, alanine is one of the products. Similarly, when oxaloacetic acid transaminates with glutamic acid, aspartic acid is

Table 4. Concentrations (mg./100 ml.) of some amino acids present in the blood of insects and of vertebrates

	Insecta				
	<i>Musca domestica</i> (1)	<i>Locusta migratoria</i> (2)	<i>Bombyx mori</i> (2)	<i>Sphinx ligustri</i> (2)	<i>Schistocerca gregaria</i> (3)
Alanine	24.7	34.1	37.2	52.8	33.0
Aspartic acid	4.6	4.7	0.0	7.9	—
Glutamic acid	12.2	0.0	0.0	25.6	75.0
Glutamine	58.6	165.0*	209.0*	121.0*	139.0
Proline	78.3	62.9	18.0	34.1	46.0
	Vertebrata				
	<i>Cyprinus carpio</i> (2)	<i>Gallus bankiva</i> (2)	<i>Oryctolagus cuniculus</i> (2)		
Alanine	5.6	4.6	—		
Aspartic acid	0.0	3.4	0.0		
Glutamic acid	2.0	16.5	3.4		
Glutamine	6.7*		14.8*		
Proline	0.0	3.5	3.1		

(1) Data obtained in present work. (2) Florkin (1954). (3) Treherne (1959).

* Calculated from the difference between total and free glutamic acid. Total glutamic acid = free glutamic acid + glutamine.

produced. Thus labelled oxaloacetate can act as the source of both labelled alanine and aspartic acid.

If one of the pathways of acetate metabolism in the fly leads to the Krebs cycle, then after injection of [2-¹⁴C]acetate one would expect ¹⁴CO₂ to be expired by the fly. That this is so is shown by curve *A* in Fig. 2. It might also be expected that the evolution of ¹⁴CO₂ would continue for a period after the time when there is no [¹⁴C]acetate left. This would be due to the fact that the precursors of the carbon dioxide, namely the Krebs cycle intermediates and other labelled intermediates which are in equilibrium with this cycle, would still be labelled for a period after all the [¹⁴C]acetate had been metabolized (curves *B* and *C*, Fig. 2). If it is assumed that the rate of evolution of carbon dioxide is constant over the period 0–5 hr. (Fig. 2) then curve *B* represents the change in specific activity of the ¹⁴CO₂ over this period. If a decrease in the specific activity of the ¹⁴CO₂ is indicative of an immediately previous decrease in the specific activities of the Krebs cycle intermediates, then one would expect the specific activity–time curves of the amino acids, which are in rapid equilibrium with these intermediates, to correspond closely in time to the specific activity curve of the ¹⁴CO₂. That they do so can be seen by comparing the curve for glutamic acid (Fig. 1) with that of the ¹⁴CO₂ (Fig. 2). That the equilibrium between proline and the Krebs cycle intermediates is not so rapid as that between glutamic acid or glutamine and these intermediates is shown by the fact that proline attains its maximum specific activity later than do either of these acids (Fig. 1) and later than the ¹⁴CO₂.

Finally, the housefly is typical of the Insecta in that the concentrations of amino acids in its haemolymph are similar to those in the haemolymph of other insects and are greater than those in vertebrate blood (Table 4).

SUMMARY

1. The amino acids α -alanine, β -alanine, arginine, aspartic acid, γ -amino butyric acid, glutamic acid, glutamine, glycine, leucine, lysine, methionine sulphoxide, phenylalanine, proline, serine, taurine, tyrosine and valine have been identified in extracts of adult houseflies.

2. Aspartic acid and glutamic acid are concentrated in the tissues of the fly and were hardly detectable in the haemolymph. γ -Aminobutyric acid was detectable only in the head extracts.

3. After injection of the fly with [2-¹⁴C]acetate, α -alanine, aspartic acid, γ -aminobutyric acid, glutamic acid, glutamine and proline became labelled.

4. Labelled α -alanine, aspartic acid, glutamic acid and proline were identified in a protein hydrolysate from 'labelled' flies.

5. Glutamic–alanine- and glutamic–aspartic acid-transaminase activity was demonstrated in sarcosomes isolated from flight-muscle, but these two enzyme systems were not detectable in haemolymph.

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The Metabolism of Isolated Rat-Liver Nuclei during Chemical Carcinogenesis

THIOACETAMIDE AND *p*-DIMETHYLAMINOAZOBENZENE (BUTTER YELLOW)

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The feeding of thioacetamide to rats for 6 months or more results in the appearance of liver tumours (cholangiocarcinoma) (Fitzhugh & Nelson, 1948). Histological changes are, however, apparent in the liver after a few days of feeding (Gupta, 1956). The most striking of these early changes is the enlargement of the parenchymal cell nucleus and nucleolus. Preliminary studies on the nuclear fraction of liver homogenates after several weeks of thioacetamide feeding showed an altered chemical composition and changes in metabolism (Grant & Rees, 1957). With the development of improved methods for isolating liver nuclei free from contaminants, a more detailed study has now been undertaken.

During the 6 months of feeding, liver nuclei were isolated at various intervals from 1 to 22 weeks. Chemical analysis of these preparations showed quantitative changes in the nucleic acids, proteins and phospholipids. In view of these findings the incorporation of labelled metabolites into protein and ribonucleic acid *in vitro* was studied.

Histological studies of livers of rats fed on a range of hepatocarcinogens have been well documented (Firminger, 1955). The histological changes during the induction period vary according to the compound used. Thus it was of interest to know whether the biochemical changes observed in the nuclei after the feeding of thioacetamide are due to the carcinogenic process or to the specific substance fed. For this reason similar studies were undertaken by using the hepatocarcinogen *p*-dimethylaminoazobenzene (butter yellow).

MATERIALS AND METHODS

Nuclear preparations. Nuclei were isolated in 0.25 M-sucrose as described by Rees & Rowland (1961).

Radioactive substances. [2-¹⁴C]Glycine, [6-¹⁴C]orotic acid and [8-¹⁴C]adenine hemisulphate were obtained from The Radiochemical Centre, Amersham, Bucks.

For incorporation of [6-¹⁴C]orotic acid and [8-¹⁴C]adenine into ribonucleic acid (RNA) the system used was: sodium phosphate buffer, pH 7.4, 20 mM; glucose, 12 mM; NaCl, 10 mM; 1 μ C of [¹⁴C]adenine or [¹⁴C]orotic acid; 1.5 ml. of nuclear suspension in 0.25 M-sucrose; water to 2.5 ml. The temperature was 30°. The reaction was stopped by the addition of 2.0 ml. of ice-cold 10% (w/v) HClO₄. The precipitate was washed four times with 2.0 ml. of ice-cold 2% (w/v) HClO₄, twice with ethanol and twice with ether.

For incorporation of [2-¹⁴C]glycine into nuclear protein the system used was: sodium phosphate buffer, pH 7.4, 20 mM; NaCl, 20 mM; [¹⁴C]glycine, 1 μ C; 1.5 ml. of nuclear suspension in 0.25 M-sucrose; water to 2.5 ml. The temperature was 38°. The reaction was stopped by the addition of 2.0 ml. of 20% trichloroacetic acid. The precipitate was extracted with 5 ml. of 5% (w/v) trichloroacetic acid for 20 min. at 90°. The protein residue was then washed successively with 5 ml. of 5% trichloroacetic acid containing 1% (w/v) of glycine and 5 ml. of 5% trichloroacetic acid, then twice with ethanol and twice with ether. The protein residue was dried at room temperature.

The methods for determining radioactivity are as described by Rees & Rowland (1961).

Chemical determinations. Nuclear counts, nitrogen, RNA, deoxyribonucleic acid (DNA) and phospholipids were determined as described by Rees & Rowland (1961).

Animals. Male Norwegian hooded rats bred from the same colony were used. Rats were put on the diets when their body weights reached 140–170 g. The basic diet was