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EFFECT OF INSECTICIDES, ESPECIALLY DIAZINON, ON THE AMINO ACIDS OF ADULT HOUSEFLIES *MUSCA DOMESTICA*

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Abstract—1. The amounts of individual free amino acids in different strains of housefly can differ. These differences are not related to selection by or resistance of the flies to Diazinon.

2. The amounts of free amino acids in houseflies change with changing age and nutritional status.

3. Poisoning caused changes in the amino acids of houseflies. Each poison produced characteristic effects, but the changes caused by diazinon resembled those from oyxgen excess and poisoning by dinitrophenol. Changes caused by the pyrethrins resemble those of oxygen lack and poisoning by cyanide and fluoracetate.

4. Changes in the amino acids of houseflies caused by diazinon suggest that organophosphorus poisons may affect the metabolism of fats or carbohydrates in addition to inhibiting cholinesterase of the nervous system.

INTRODUCTION

LORD and SOLLY (1961) reported that the amounts of some free amino acids in houseflies were changed by poisoning with organophosphorus insecticides The changes appear to be related to the toxic action of organophosphorus insecticides and differ from those caused by chlorinated hydrocarbons.[1]

This investigation is an attempt to determine the effects more precisely than previously and to seek amino acids other than α-alanine affected by organophosphorus poisons. In an attempt to obtain evidence to decide whether the changes observed reflect general disturbance of metabolism or are specific to the poisons used, the effects of short periods of starvation, modified diet, and other poisons, mostly of known toxic action, were examined.

EXPERIMENTAL

Houseflies

To obtain frequent supplies of houseflies, two susceptible strains A and B and five strains C, D, E, F, G, of differing resistance to organophosphorus compounds were used, and differences in the amino acid contents of these strains were noted. These differences seem not to be simply related to degree of resistance and are not considered in this investigation.

All strains were reared by the standard method used in this laboratory.[2] Resistant strains were selected in each generation by exposing adults to Diazinon-treated filter papers.[3]

Methods of Poisoning

Before treatment the flies were immobilised by cooling in a refrigerator, and afterwards were kept at room temperature (18–20°) for 24 hr in 4-in. Petri dishes containing a 9-cm disc of Whatman No. 1 filter paper in the lower half. The flies were fed with dried milk and sucrose solution unless otherwise stated.

(a) *Topical application.* Most poisons were applied as solutions in acetone. An Agla micrometer syringe was used to apply a one μl drop of an appropriate concentration of poison in acetone to the ventral surface of the thorax. Control flies were treated with 1 μl of acetone.

(b) *Feeding.* The flies were offered an unrestricted amount of an appropriate concentration of poison (0·1% sodium fluoracetate or 0·4% dinitrophenol) in a 1% solution of sucrose until they were extracted. Control flies were offered sucrose solution.

(c) *Gases and vapour.* Batches of 30 flies were treated in glass stoppered jars, $4\frac{1}{2}$ in. high × $3\frac{1}{2}$ in. dia., at 18–20°. They were fed on dried whole milk and sucrose solution in these containers until they were examined 24 hr after treatment began.

Control flies were kept in a jar of air. Similar tests were made in jars containing air + $1\frac{1}{2}$% carbon dioxide, pure oxygen and oxygen + $1\frac{1}{2}$% carbon dioxide.

Flies were poisoned with cyanide vapour by introducing into the jar a 2 in. × $\frac{1}{2}$ in. test tube lightly stoppered with cotton wool which contained a few crystals of sodium cyanide.

Extraction and assay of amino-acids

The free amino acids were extracted in 50% aq. ethanol, their 2,4-dinitrophenyl derivatives prepared and separated by paper chromatography and assayed spectrophotometrically.

(a) *Extraction procedure.* Fifteen flies were macerated with 1·0 ml 50% aq. ethanol in an all glass homogeniser. The extract was transferred to a centrifuge tube, and the homogeniser washed with 0·5 ml more aq. ethanol. The combined washing and extract were then centrifuged and the supernatant fluid removed. A further 1·5 ml ethanol was then used to wash out the homogeniser and to extract the solids from the first extract. After centrifuging, the superatant fluids were combined. This procedure completely and rapidly extracted amino acids but not proteins, which interfered with subsequent operations.

(b) *Preparation of dinitrophenyl derivatives of amino acids.* The dinitrophenyl derivatives of amino acids were prepared by the method of Sanger[4] modified to give maximum yields with minimum dinitrophenol. One ml of fly extract was mixed with 0·1 ml M $NaHCO_3$ and 0·1 ml 5% v/v fluoro-2,4-dinitrobenzene in absolute alcohol in a 10-ml graduated stoppered tube, and heated at 40° for 1 hr, with occasional gentle shaking. After cooling, the mixture was extracted with 5 ml diethyl ether and centrifuged, the ether layer was removed and the extraction was repeated with another 5 ml diethyl ether. After removing the bulk of ether layer, the last traces of ether were evaporated in a water bath at 40°.

The aqueous layer was then acidfied with 0·2 ml 5 N HCl and extracted with 2 ml methyl isobutyl ketone, according to the procedure of Axelrod *et al.*[5] Centrifugation completed the separation. Both layers were kept for chromatography and examination of the dinitrophenyl derivatives. Compounds found in the aqueous layers are not

reported on, because poisoning did not change significantly the amounts of the four major constituents. These amino-compounds were not identified.

(c) *Chromatography.* The dinitrophenyl derivatives of the amino acids were separated by paper chromatography using the method of Pairent and Williamson.[6] 150 μl of dinitrophenyl derivatives of amino acids, as prepared from the extracts of flies, were applied to sheets of Whatman No. 1 filter paper impregnated with 0·05 M potassium hydrogen phthalate pH 6·0. The material was then chromatographed at 25° in two directions at right angles. The solvent used in the first direction was *n*-pentanol equilibrated with aq. 0·05 M potassium hydrogen phthalate buffer pH 6·0 and 1·5 M Na_2SO_4. After drying in air the material was chromatographed in the second direction using an aqueous solution of 0·05 M potassium hydrogen phthalate pH 6·0 and 1·5 M Na_2SO_4 equilibrated with *n*-pentanol.

The chromatograms were then dried in air, viewed under blue light, which increased the contrast between the yellow of the spots and the paper background, and the spots marked. The spots were then cut out and placed in 3 ml of water. Extraction was completed by heating to 55–60° for 15 min and allowing 15 min to cool to room temperature.

The optical density of the extract of each spot was read at 360 mμ in a 1-cm cell. In addition, the proline derivative was read at 385 mμ[7] and the β-alanine derivative, which is not completely separated from dinitrophenol, was acidified with 0·1 ml 5 N HCl to decolourise the dinitrophenol and the colour remaining read at 360 mμ. These optical densities were used as a measure of the amounts of free amino acids present.

TABLE 1. RELATIVE AMOUNTS OF FREE AMINO ACIDS IN 3-DAY-OLD MALE HOUSEFLIES OF TWO STRAINS

	Mean Value — Optical density × 1000		
	Strain A (8 assays)	Strain C (11 assays)	S.D. of single determination
α-alanine	78 ± 12·0	102 ± 10·2	34·0
β-alanine	337 ± 21·8	302 ± 18·6	61·8
Aspartic acid	32 ± 5·5	36 ± 4·7	15·7
Glutamic acid	129 ± 8·1	128 ± 6·9	23·0
Glycine	30 ± 3·0	48 ± 2·6	8·6
Leucine	0 ± —	30 ± 4·7	15·6
Proline	191 ± 15·2	206 ± 13·0	43·0
Serine	142 ± 20·3	158 ± 17·6	58·4
Taurine + Glutamine	428 ± 23·8	382 ± 20·4	67·4
Valine	3 ± 5·7	36 ± 2·6	16·3
Unknown 1	25 ± 5·8	29 ± 5·0	16·6
Unknown 2	45 ± 8·8	87 ± 7·6	25·1
Unknown 3	68 ± 9·1	61 ± 4·9	25·8
Unknown 4	2 ± 3·0	16 ± 2·6	8·6

RESULTS

Amino acids found in several strains of housefly

Table 1 shows the amino acids present and indicates their relative abundance in 3-day-old male houseflies from strains A and C. The amounts of amino acids are expressed in arbitrary units which are 1000 times the light absorption determined for each substance as described in the experimental section. The values are the means of

8 independent assays for strain A and 11 assays for strain C. They were collected as control analyses on fully fed untreated flies used as controls in experiments to determine changes caused by various treatments. The means, standard errors and deviations of single determinations are given to indicate the reliability of the test and assay procedures.

The amino acid contents of strains A and C differ, with strain C containing significantly more glycine, valine, leucine and unknown substances 2 and 4. Strain A contains little or no valine, leucine or unknown substance 4. Strain A probably also contains more β-alanine and less α-alanine than strain C. Strain A also appears to contain more of a material, probably a mixture of taurine and glutamine (not separated from each other by our procedure) than strain C.

Measurements of amino acids of strains B, D, E, F, and G (not reported here) indicate differences in the contents of amino acid. These differences are neither statistically significant, nor can they be related to differences in resistance to diazinon. This shows a lack of a general correlation between amino acid content and resistance.

The dinitrophenyl derivatives of four unidentified substances were detected. The movement on paper chromatograms of each material relative to dinitrophenol with *n*-pentanol and with the aqueous buffer, sodium sulphate solution were: substance 1: 0·1, 0·5; substance 2: 0·6, 1·8; substance 3: 0·8, 0·2; substance 4: 1·3, 0·2.

Effects of various treatments on amino acid contents of houseflies

For the sake of brevity, the effects of various treatments on the amino acids of houseflies are given as the ratio of the amino acid in treated flies to that in untreated flies. The treated and untreated flies were always from the same batch, kept under the same conditions and extracted at the same time.

Nutritional status and age seemed to be two possible factors, other than poisoning, causing changes in amino acid status, and these were examined to avoid confusing their effects with those produced by poisons.

TABLE 2. CHANGES IN AMINO-ACIDS WITH AGE OF HOUSEFLIES

	Relative amounts of amino acids (day 3 = 1·0)									
Age (days) Amino acid	0	1	2	3	4	5	6	8	10	11
β-alanine	0·60	0·52	0·48	1·00	0·93	1·06	1·50	1·89	1·78	1·60
Leucine	1·20	1·22	1·51	1·00	0	0	0	0	0	0
Proline	1·22	1·15	0·84	1·00	0·74	0·76	1·07	0·86	0·79	0·94
Serine	1·18	1·06	0·89	1·00	0·94	0·65	0·80	0·64	0·52	0·94
Valine	2·05	1·51	1·49	1·00	0·12	0	0	0	0	0

Changes of other amino compounds were too small to be considered significant. Values for glutamine were erratic.

Values are means from 2 independent determinations.

(a) *Effect of age.* Flies from strain C fed on a normal diet of dried milk, sugar and water were extracted and assayed for free amino acids daily from time of emergence from the pupae to eleven days old. Table 2 shows there was a progressive fall in the amounts of valine, leucine, serine and proline while the amount of β-alanine increased.

(b) *Nutrition effects.* Flies from strain C were divided into three batches, one given water only, another sucrose solution and the third (control) sucrose, dried milk and water. All were kept at 20°. Flies were extracted 3, 6, 24 and 48 hr after the start of treatment. Table 3 shows that flies fed with sucrose and milk for 24 hr had more serine than those fed on sucrose. No other amino acid was significantly affected during the 48-hr period.

TABLE 3. EFFECTS OF NUTRITION ON AMINO ACIDS OF HOUSEFLIES

		Relative amounts of amino acids (test/control)			
Time (hr)		3	6	24	48
α-alanine					
	Sugar solution	0·97	1·30	1·25	1·10
	Water	0·90	0·99	0·95	0·50
β-alanine					
	Sugar solution	0·83	0·74	0·91	1·21
	Water		0·95	1·03	0·97
Proline					
	Sugar solution	0·91	1·14	1·08	1·03
	Water	1·15	1·35	1·31	0·58
Serine					
	Sugar solution	0·84	1·07	0·52	0·75
	Water	0·93	1·10	0·85	0·59

Values are means from 2 independent assays.

Supplying only water diminished α-alanine, proline and serine. The effects only showed after 48 hr.

(c) *Diazinon* (O,O-Diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate. In the first experiments all the strains, A, B, C, D, E, F, G, some susceptible some resistant to diazinon were used. Individuals were treated with 1-μl drops of 1% diazinon in acetone; a dose sufficient to kill at least 99% of the most resistant strains. After 24 hr the poison had decreased proline and α-alanine in all strains. In addition, poisoning probably also altered the amounts of aspartic acid, glutamic acid, β-alanine and unknown substance 1. Table 4 shows the results obtained with strains A and C, the most susceptible and most resistant.

In later experiments doses were used that killed 99% or 50% of the flies. Twenty-four hours after poisoning the amino acids were extracted and assayed. The flies were treated with 1-μl drops of an acetone solution of diazinon. Two strains were used, the concentrations to kill 50% and 90% of strain A were 0·004% and 0·011%, of strain C 0·12% and 0·35%. In both strains, flies killed by the poison showed a decrease in α- and β-alanine and proline. These amino acids were not affected in flies not killed by the poison (Table 5).

(d) *Pyrethrum.* Flies from strains A and E were poisoned with a 0·133% solution of pyrethrins in acetone, applied topically. This dose gave 100% kill with strain A and 88% kill with strain E. Twenty-four hours after treatment the amino acids were extracted from the dead flies and assayed.

Most amino acids were not significantly affected, but in both strains α-alanine increased and in strain E there was a large increase in proline. (Table 6).

(e) *Sodium fluoroacetate.* All flies of strain D fed for 24 hr at 27–28° on a 0·1% solution of sodium fluoracetate in sucrose were killed. Poisoned flies contained more α-alanine and less glutamic acid, serine and proline than untreated flies (Table 6).

TABLE 4. RELATIVE AMOUNTS OF AMINO ACIDS (POISONED/UNTREATED) IN HOUSEFLIES AFTER POISONING WITH DIAZINON

Test	Strain A				Strain C				
	1	2	3	4	1	2	3	4	5
α-alanine	0·98		0·41	0·21		0·57	0·91	0·66	0·66
β-alanine	0·65	0·69	0·81	0·82	0·58	0·85	1·09	1·00	0·85
Aspartic acid	1·24		1·33	1·44			1·20	0·62	0·90
Glutamic acid	0·87	0·67	0·76	0·47			1·06	0·93	0·98
Glycine	0·37	0·87	1·03	0·63		0·98	1·74	0·95	1·08
Leucine					0	0	1·30	1·90	
Proline	0·43	0·22	0·25	0·42	0·72	0·34	0·68	0·83	0·88
Serine	0·98	0·99	0·93		1·06	1·46	1·59	1·41	1·33
Taurine + Glutamine	1·14	0·84	0·81	0·86	1·16	0·78	1·39	1·04	1·40
Valine		0·80	0	2·38	0	0	0·93	3·20	1·00
Unknown 1		0	0·76	0·47			2·08	1·10	3·80
Unknown 2	0·17	1·57	0·47	0·54			1·41	0·86	0·95
Unknown 3	0·86	1·12	1·04	1·00	1·20	0·48	0·60	0·59	
Unknown 4				0·87	0·10	0		0·17	0

TABLE 5. EFFECT OF DOSAGE LEVEL ON CHANGES OF AMINO ACIDS OF HOUSEFLIES POISONED WITH DIAZINON

	Relative amounts of amino acids (Poisoned/control) LD50		LD99
	Live	Dead	
α-alanine	1·18	0·77	0·93
β-alanine	0·97	0·81	0·71
Glutamic acid	1·00	0·80	0·77
Proline	1·02	0·30	0·30

Values are means from 4 independent assays.

(f) 2,4-*Dinitrophenol.* A solution of 0·4% dinitrophenol in sucrose fed to flies of strain C for 48 hr at 24° killed about half the flies. Flies that survived poisoning contained more β-alanine and taurine + glutamine but less glutamic acid, α-alanine and proline than control flies fed on sucrose. Killed flies contained less taurine + glutamine, glutamic acid, α- and β-alanine, proline and serine than untreated flies. There was less α-alanine and proline in killed flies than in those that survived poisoning (Table 6).

(g) *Cyanide.* Flies of strain C exposed to cyanide vapour at 20° for 24 hr all died; they contained more α-alanine and proline, but less taurine + glutamine and glutamic acid than control flies (Table 6).

(h) *Oxygen and carbon dioxide.* Exposure to a mixture of oxygen and 1½% carbon dioxide instead of air decreased proline and β-alanine and tauramine + glutamine. Oxygen alone and air + 1½% carbon dioxide did not cause any change in amino acids (Table 6).

TABLE 6. EFFECTS OF VARIOUS TREATMENTS ON AMINO ACIDS OF HOUSEFLIES

		Relative amounts of amino acids (Poisoned/untreated)					
	Strain	α-alanine	β-alanine	Glutamic acid	Proline	Serine	Taurine and glutamine
Pyrethrins	A*	1·83	0·97	1·11	1·07	0·95	0·86
	E†	1·54	1·06	1·07	2·03	0·90	1·20
Sodium fluoracetate	D	1·13	0·99	0·38	0·45	0·78	0·95
Dinitrophenol 'Live'	C	0·39	0·97	0·60	0·32	1·01	1·28
'Dead'		0·14	0·64	0·56	0·14	0·67	0·82
Cyanide	C	3·82	0·91	0·61	1·17	0·98	0·77
O_2/CO_2	C	0·84	0·64	1·18	0·69	0·84	0·78

Air/CO_2 mixture and pure O_2 had no effect,
No other amino acids were affected.
Values are from means of 2 independent assays except for * —4 assays and † —3 assays.

DISCUSSION

Because the object of the experiments was to compare the effects of poisoning on strains susceptible or resistant to diazinon, considerable information was collected about the kind and quantities of free amino acids in different strains. Table 1 summarises analyses on 3-day-old male houseflies of two strains A and C and shows they are significantly different in their content of glycine, valine, leucine and two unidentified substances.

The larger scatter in results is partly caused by difficulties in standardising exactly the age and nutritional status of houseflies. Both were expected to affect the free amino acids in houseflies, because amino acids are actively metabolised,[8, 9] and Tables 2 and 3 confirm the expectation. However these effects do not increase the difficulties of interpreting the experiments on the effects of poisons, because these are characteristic and cannot easily be ascribed to the inactivation and inability of insects to feed when poisoned. (Compare Tables 2 and 3 with 4).

We confirmed that poisoning by diazinon decreases the amount of α-alanine.[10] In addition, proline was always decreased. The effects on both amino acids were irrespective of the strain of houseflies used. Other amino acids also appear to be affected by organophosphorus poisoning, although less and not so consistently (Table 4 shows results obtained with strains A and C). Reviewing all the results available (some not reported here), it seems likely that poisoning by diazinon not only decreases α-alanine and proline but also aspartic, glutamic acids and β-alanine, and the unidentified substance 1. The apparent increase in aspartic acid for one strain (Table 4) seems to be exceptional and no explanation for it is offered. It may be significant that these amino acids are closely related to some of the acids in the tricarboxylic acid cycle.

The decrease in α-alanine and proline cannot be related to oxygen deficiency because Price[11] showed that anoxia in houseflies increases the amount of α-alanine. The

effects of poisons interfering with respiration and the tricarboxylic acid cycle (Table 6) confirm this opinion. Cyanide and fluoracetate, which block the oxidative processes of the tricarboxylic acid cycle, increase the levels of α-alanine. In contrast, dinitrophenol, which uncouples oxidation and phosphorylation in the tricarboxylic acid cycle, accelerates oxidative metabolism and oxygen uptake and decreases α-alanine in houseflies. Sodium fluoracetate, caused a slight increase in the amount of alanine and a decrease in the amount of glutamine. These amino acids are closely linked to pyruvic acid and α-ketoglutaric acid by transaminases and changes in the amounts of the keto-acids may be readily accounted for by the known block in the tricarboxylic acid cycle by inhibition of aconitase following fluoracetate poisoning. The falls in serine and proline are less easy to explain. Block of respiration by cyanide, a cytochrome poison, gave a fourfold increase of alanine and a smaller increase of proline. This is presumably because of an initial increase in tricarboxylic acids, resulting from arrest of the tricarboxylic acid cycle, but by a different mechanism from that caused by sodium fluoracetate.

The effects of fluoracetate and cyanide are opposite from those observed with organophosphorus poisons, which affect amino acids in a way (i.e. loss of alanine and other amino acids related to the tricarboxylic acid cycle) suggesting a depletion of tricarboxylic acids. This could arise from a too rapid (uncontrolled) oxidation such as occurs with dinitrophenol, a suggestion supported by the fall in α-alanine, glutamic acid, and proline in houseflies poisoned by dinitrophenol. Similar but smaller effects were observed when flies were exposed to high oxygen tensions, especially when in conjunction with CO_2 to prevent the closing of spiracles which may occur in low CO_2 tensions.

These effects lead to the conclusion that houseflies when poisoned with organophosphorus compounds, unlike mammals, do not die from oxygen lack caused by a failure of respiratory movement. On the contrary, the changes in amino acid content suggest rather an oxygen excess (see Tables 1 and 3), although similar effects might be expected from a shortage of metabolites arising from some block in the use of either carbohydrate or fat. Evidence for such a block in mammals has been deduced from the effects of cold on rats treated with malathion.[12] The lack of metabolites is unlikely to arise from metabolism because respiration studies[13] indicate that insects can sustain respiration rates much greater than those caused by organophosphorus insecticides. Dinitrophenol, which greatly increases respiration, causes a fall in α-alanine and proline, thus having at least a superficial resemblance to the action of diazinon, but toxic doses of dinitrophenol deplete amino acids more.

The fall in α-alanine and proline caused by diazinon poisoning is similar to that on starvation, but the effect with poisoning is so much more rapid, occurring in less than half the time, that inability to feed is unlikely to be the sole cause of the observed changes.

In addition, the pyrethrins, whose rapid knockdown action quickly stops feeding increased α-alanine and sometimes proline, effects opposite to those of starvation or oxygen excess (Tables 3 and 6). The effects resemble those of oxygen lack or cyanide poisoning, although the effects of the pyrethrins on the oxygen uptake of *T. castaneum* are not unlike those of organophosphorus compounds.[13]

It must be concluded that the changes in amino acids of poisoned flies are not from starvation but from more specific actions of poisons. Of particular interest are the

opposite effects of the pyrethrins and diazinon, both reputedly nerve poisons, which suggests that both substances may have widespread direct effects on insect metabolism not in themselves lethal.

CONCLUSIONS

1. The amounts of some individual free amino acids differ from one strain of houseflies to another, one strain may contain an amino acid absent from another. The differences do not appear to be related to selection by or resistance to diazinon.

2. The action of diazinon on the amino acid status of houseflies resembles that of oxygen excess or lack of metabolites, so that organophosphorus compounds may have effects on insect metabolism in addition to inhibition of cholinesterase of the nervous system. Organophosphorus poisons may interfere with the metabolism of fats or carbohydrates.

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