cisternally administered [^{3}H]norepinephrine into a superficially situated intraneuronal pool of newly synthesized norepinephrine (*17*) and its subsequent rapid release from this compartment into the extraneuronal space. Thus, the techniques of the present study may provide a means for studying, in vivo, the kinetics and metabolism of the labile pool of newly synthesized norepinephrine in the brain under various physiological and pharmacological conditions.

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References and Notes

1. I. J. Kopin, G. Hertting, E. K. Gordon, *J. Pharmacol. Exp. Ther.* **138**, 34 (1962).
2. J. Axelrod, G. Hertting, R. W. Patrick, *ibid.* **134**, 325 (1961); W. R. Burack and P. R. Draskoczy, *ibid.* **144**, 66 (1964); R. Montanari, E. Costa, M. A. Beaven, B. B. Brodie, *Life Sci.* **2**, 232 (1963); J. Glowinski, I. J. Kopin, J. Axelrod, *J. Neurochem.* **12**, 25 (1965); L. L. Iversen and J. Glowinski, *ibid.* **13**, 671 (1966); G. C. Sedvall, V. K. Weise, I. J. Kopin, *J. Pharmacol. Exp. Ther.* **159**, 274 (1968); J. M. Stolk and J. D. Barchas, *Pharmacologist* **12**, 268 (1970).
3. S. M. Schanberg, J. J. Schildkraut, I. J. Kopin, *J. Pharmacol. Exp. Ther.* **157**, 311 (1967).
4. N. H. Neff, T. N. Tozer, W. Hammer, E. Costa, B. B. Brodie, *ibid.* **160**, 48 (1968).
5. I. J. Kopin, G. R. Breese, K. R. Krauss, V. K. Weise, *ibid.* **161**, 271 (1968).
6. A. M. Thierry, G. Blanc, J. Glowinski, *Eur. J. Pharmacol.* **10**, 139 (1970).
7. J. J. Schildkraut and S. S. Kety, *Science* **156**, 21 (1967); N. Weiner, *Annu. Rev. Pharmacol.* **10**, 273 (1970).
8. These findings were reported, in part, at the meeting of the Psychiatric Research Society, New Haven, Conn., December 1968, and at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 1970; J. J. Schildkraut and P. R. Draskoczy, *Fed. Proc.* **29**, 679 (1970).
9. J. Glowinski and J. Axelrod, *Pharmacol. Rev.* **18**, 775 (1966).
10. A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.* **138**, 360 (1962); L. G. Whitby, J. Axelrod, H. Weil-Malherbe, *ibid.* **132**, 193 (1961); I. J. Kopin, J. Axelrod, E. Gordon, *J. Biol. Chem.* **236**, 2109 (1961); S. M. Schanberg, J. J. Schildkraut, G. R. Breese, I. J. Kopin, *Biochem. Pharmacol.* **17**, 247 (1968); J. J. Schildkraut, *Amer. J. Psychiat.* **126**, 925 (1970).
11. I. J. Kopin and E. K. Gordon, *J. Pharmacol. Exp. Ther.* **138**, 351 (1962); A. Carlsson and N.-A. Hillarp, *Acta Physiol. Scand.* **55**, 95 (1962); J. Glowinski, L. L. Iversen, J. Axelrod, *J. Pharmacol. Exp. Ther.* **151**, 385 (1966); J. Axelrod, *Pharmacol. Rev.* **18**, 95 (1966); I. J. Kopin, *ibid.*, p. 513.
12. J. J. Schildkraut, S. M. Schanberg, G. R. Breese, I. J. Kopin, *Amer. J. Psychiat.* **124**, 600 (1967).
13. J. M. Stolk and J. D. Barchas, in preparation.
14. These experiments were done in collaboration with S. R. Tarlov.
15. J. Glowinski and J. Axelrod, *Nature* **204**, 1318 (1964).
16. That is, Δ[^{3}H]NE/control. See Table 1.
17. That is, a pool situated in, or adjacent to, the axonal membrane.
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Activity of an NADPH-Dependent Nitroreductase in Houseflies

Abstract. *A nitroreductase, reducing parathion to aminoparathion, was found in the soluble fraction that was obtained from abdomens of female houseflies. The reaction required reduced nicotinamide adenine dinucleotide phosphate (NADPH), but was not affected by the presence or absence of oxygen. Further degradation of aminoparathion into water-soluble compounds occurred in NADPH-fortified incubation mixtures over prolonged incubation periods. The effect of sesamex or SKF 525-A on these reactions is described.*

Oxidative enzymes in houseflies, *Musca domestica* L., were reported to "metabolize a wide variety of insecticide chemicals when the enzyme preparations were fortified with reduced nicotinamide adenine dinucleotide phosphate (NADPH)" (*1*). Considerable strain differences existed in the activity of these enzymes in vitro. Nakatsugawa and Dahm demonstrated that "rat liver microsomes degraded parathion by splitting at the aryl phosphate bond," that the system was specific for parathion, and that it did not degrade paraoxon (*2*). With the use of microsomes from houseflies and from rat and rabbit livers, it was also shown that parathion and other "P = S compounds were metabolized via two oxidative pathways, activation to P = O analogs and cleavage at the aryl phosphate bond," reactions that required oxygen and NADPH (*3*). A reductive degradation of parathion to aminoparathion has been demonstrated, after yeast had been added to parathion-treated soils or water (*4*) and also with two species of bacteria (*5*).

Degradation in vitro of parathion to aminoparathion, which is due to the presence of a nitroreductase in houseflies, is described here. Enzyme preparations were obtained from a DDT susceptible strain (CSMA-1948) of houseflies, a strain resistant to DDT and naphthalene vapors (P_2/sel), and another one resistant to DDT only (F58-W). The CSMA strain was used in all tests, while the other two were occasionally used for comparison. Female houseflies were frozen with Dry Ice, and the abdomens were removed and homogenized in grinding medium at 0° to 4°C at a ratio of ten abdomens per milliliter. The medium consisted of 0.15*M* phosphate buffer, *p*H 7.4, and 0.25*M* sucrose. Various cell fractions were obtained by differential centrifugation as described (*6*), except that the soluble fraction was obtained at 150,000*g* for 60 minutes. Pellets were resuspended in 5 to 7 ml of grinding medium. Reaction mixtures were kept in open, 10-ml Erlenmeyer flasks and consisted of 1 to 3 ml of the various cell fractions to which NADPH at $1.3 \times 10^{-4}M$ and 0.22 μc of ^{14}C–ring-labeled ethylparathion (100 μg) in 10 μl of ethanol were added. These mixtures were incubated for 60 minutes in a water bath of a gyratory shaker at 30°C and 120 rev/min. In some tests, shorter or longer incubation periods were employed. After that, the reaction was terminated by adding 3 ml of acetone, followed by extraction with 3×10 ml of a 2 : 1 mixture of hexane and acetone and separation into a hexane and water-acetone phase as described (*6*).

Portions of the hexane phase were analyzed by gas-liquid chromatography (GLC) with a Packard model 7834 gas chromatograph, the conditions being as follows: two different column packings (5 percent Dow 11 on 60-80 chromosorb W and 5 percent QF-1 on 80-90 Anakrom AS) at 175° and 190°C, respectively, helium as the carrier gas, and a hydrogen flame detector coated with potassium chloride, thus rendering the detector especially sensitive to phosphorus (*7*). Labeled metabolites in the hexane fraction were resolved by thin-layer chromatography (TLC) on silica gel, with hexane-chloroform-methanol (7 : 2 : 1) as developing solvents. The metabolites were then detected by radioautography. In addition, color reactions were utilized by spraying the thin-layer plates successively with the reagents of the Averell-Norris method (*4, 8*), except that the reduction step was eliminated. In this way, the presence of previously reduced compounds was indicated by a positive color test. Additional spraying of the plates with a palladium chloride solution followed by spraying with 5*N* NaOH resulted in spots of different colors for parathion, paraoxon, aminoparathion, *p*-nitrophenol, and *p*-aminophenol. To determine the radiocarbon content of isolated compounds, silica-gel areas which had the same R_F value as reference grade chemicals were scraped from the plates into scintillation vials containing 12 ml of dioxane scintillation solvent and 4 percent Cab-O-sil

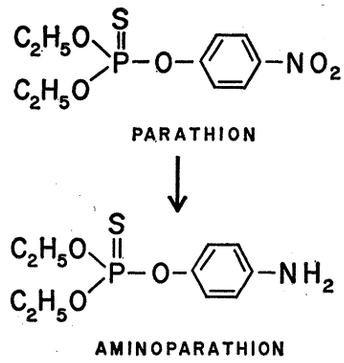

Fig. 1. Reduction of parathion to aminoparathion.

thixotropic gel powder. Radioactivity was determined with a Packard liquid scintillation spectrometer, model 3320. In other tests, isolated silica gel areas were extracted with acetone and analyzed by GLC as described. For additional verification of the presence of metabolites, compounds were isolated by GLC and subjected to mass spectrometry. The radiocarbon content in the water-acetone phase was in some tests determined by liquid scintillation counting (LSC).

Results obtained from these enzyme assays indicated that aminoparathion had been formed from parathion (Fig. 1). Analyses by GLC of the hexane fractions from the extraction of incubation mixtures resulted in peaks with retention times identical to aminoparathion. In addition, analyses of the hexane fraction by TLC and autoradiography resulted in a spot with the same R_F value as reference grade aminoparathion. Extraction of this area and analyses by GLC with two different columns yielded peaks with the same retention times as that of aminoparathion. Verification of the identity of this compound by mass spectrometry was accomplished after its isolation by TLC and GLC. The aminoparathion area, as detected by radioautography, also gave a positive color reaction with the Averell-Norris reagents, although the reduction step had been omitted. This then indicated the presence of an amino compound. Additional spraying with palladium chloride and sodium hydroxide yielded the same color as did reference grade aminoparathion.

Evidence of enzyme activity was demonstrated by heating the enzyme source for 10 minutes in boiling water, thus achieving a protein denaturation. Even with several analytical methods, no aminoparathion could be detected in these reaction mixtures, while with nonheated cell fractions aminoparathion was produced. In the absence of fly material, no aminoparathion could be detected.

Localization of the enzyme activity in subcellular fractions was determined by assaying each fraction as described, with the exception of the 1200g pellet of "nuclei and debris." Based on LSC of "aminoparathion areas" that had been isolated by TLC, the supernatant of the 10,000g centrifugation was most active. The 10,000g "mitochondria" pellet did not reveal any nitroreductase activity. Further centrifugation at 150,000g for 60 minutes yielded the "microsome" pellet. This fraction, tested in four different experiments, did not indicate nitroreductase activity. However, utilization of the 150,000g supernatant as the enzyme source, resulted in the formation of aminoparathion. Liquid scintillation counting of the "aminoparathion areas" obtained in the "microsome" fraction (3 ml out of 7 ml suspended pellet) after TLC and radioautography resulted in 650 ± 120 disintegrations per minute (dpm), but was 7720 ± 160 dpm when the soluble fraction had served as the enzyme source (3 ml out of 24 ml soluble fraction). The "aminoparathion area" obtained by the same thin layer procedure with 0.22 μc of [^{14}C]parathion reference standard yielded 624 dpm (Table 1).

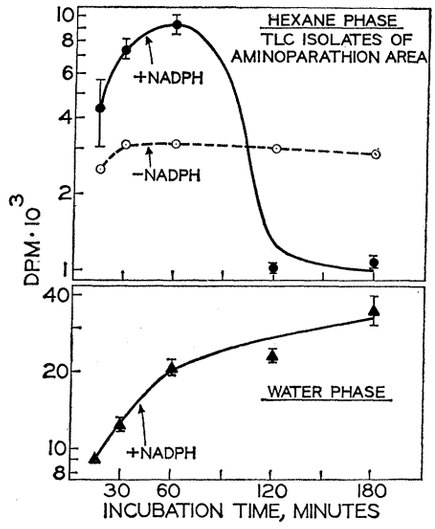

Fig. 2. Radioactivity in the "aminoparathion area" isolated by thin-layer chromatography of hexane phases or in the total water phases, obtained after extraction of duplicate housefly incubation mixtures (10,000g supernatant) that had been treated with 0.22 μc of [^{14}C]parathion.

The dependency of the reductase activity on NADPH was demonstrated in assays that were conducted with and without this coenzyme. Using the 10,000g supernatant as the enzyme source (P_2/sel strain) and later analyses by GLC of the hexane fraction, we showed that the amount of aminoparathion produced within 1 hour from 100 μg of parathion in the presence of NADPH was 7.9 ± 1.4 μg and only 2.75 μg in its absence. In another test (CSMA strain), these figures were 12.1 ± 0.3 μg and 7.2 ± 0.3 μg, respectively. LSC of "aminoparathion areas," isolated by TLC and autoradiography, showed similar differences.

Enzyme assays with and without NADPH were also conducted for periods of 15, 30, 60, 120, and 180 minutes (Fig. 2). In the absence of

Table 1. Distribution of radioactivity in hexane fractions of extracts from duplicate, housefly (CSMA) incubation mixtures (10,000g supernatant of abdominal homogenate in phosphate buffer plus NADPH) after treatment with [^{14}C]parathion (0.22 μc) and incubation at 30°C; dpm, disintegrations per minute.

TLC area*	Standard 0.22 μc ^{14}C		Radioactivity after incubation for:									
			15 minutes		30 minutes		60 minutes		120 minutes		180 minutes	
	dpm	%T†	dpm	%T	dpm	%T	dpm	%T	dpm	%T	dpm	%T
I	500,498	98.9	407,894	98.1	382,299	97.1	409,873	96.8	405,612	99.0	414,220	98.9
II	3,618	0.7	2,141	0.5	2,256	0.6	2,107	0.5	1,647	0.4	1,784	0.4
III	*624*	0.1	*4,400*	1.1	*7,480*	1.9	*9,396*	2.2	*1,024*	0.2	*1,108*	0.3
IV	1,224	0.3	1,385	0.3	1,703	0.4	2,214	0.5	1,593	0.4	1,605	0.4
Total	505,964	100	415,820	100	393,738	100	423,590	100	409,876	100	418,717	100

* Solvent system: hexane, $CHCl_3$, methanol (7 : 2 : 1). Designated areas had R_F values identical with parathion (area I); paraoxon (area II); aminoparathion (area III), and p-nitrophenol, p-aminophenol (area IV). † Radioactivity in percent of total recovered.

NADPH (− NADPH) the amount of aminoparathion, determined by LSC of TLC isolates of "aminoparathion areas," increased up to 30 minutes, and remained the same thereafter. In experiments with NADPH-fortified incubation mixtures (Table 1 and +NADPH in Fig. 2) addition of the cofactor resulted in a marked increase in aminoparathion formation, which reached its peak after 60 minutes of incubation. At that time, three times more aminoparathion had been produced with NADPH as compared to those assays, to which no coenzyme had been added. After the first 60 minutes of incubation, the amount of aminoparathion recovered from the hexane fraction dropped sharply, while the radioactivity in the total water-acetone fraction increased constantly (Fig. 2). Although the radioactivity in this fraction was not only due to the degradation of aminoparathion, there was indication that the aminoparathion had been metabolized into water-soluble products. The water fraction—obtained from the 120- and 180-minute assays—were combined and reextracted with chloroform. This removed over 50 percent of the radioactivity from the water. The extracted radiocarbon content in the chloroform was then resolved by TLC and autoradiography. LSC of isolated areas indicated that only 7 percent of the totally recovered radioactivity was located in the area that corresponded to aminoparathion, but 23 and 22 percent were located in those areas that corresponded to *p*-nitrophenol and *p*-aminophenol, respectively. Of the totally recovered radioactivity, 40 percent was located in the parathion area, and 8 percent in the paraoxon area.

In separate tests NADPH-fortified incubation mixtures were extracted after different incubation times. Analyses by GLC of the hexane fraction showed the presence of 1.35 μg of aminoparathion per incubation mixture after 15 minutes of incubation, 5.75 μg after 30 minutes, 7.1 μg after 60 minutes, 5.6 μg after 120 minutes, and 0.9 μg after 180 minutes.

Since oxygen is required for the functioning of oxidative enzymes, the activity of the nitroreductase should not be dependent on the presence of oxygen. To prove this point, triplicate assays were conducted with and without atmospheric oxygen (or a very low partial pressure of oxygen) in the 10-ml Erlenmeyer flasks. This was accomplished by bubbling nitrogen for 2 minutes through the assay mixture (10,000*g* supernatant as the enzyme source) and immediate sealing of the flasks with four layers of Saran wrap. Controls were conducted with incubation mixtures that had not been purged with nitrogen and were kept in open Erlenmeyer flasks. After incubation and extraction, the formation of aminoparathion was confirmed by the described test procedures. Results obtained by GLC of the hexane phase showed that oxygen was not required for the nitroreductase activity; the amount of aminoparathion produced in the absence of atmospheric oxygen was 11.17 ± 0.52 μg per incubation mixture and 9.80 ± 0.96 μg in its presence. Differences observed under the two experimental conditions are not significant.

To investigate the effects of some enzyme inhibitors, duplicate tests were conducted in which sesamex or SKF 525-A—a known inhibitor of microsomal enzymes—were added to the regular incubation mixture at $5 \times 10^{-4}M$ as described (*6*). Results showed that, in the presence of these chemicals, the amount of aminoparathion produced per incubation mixture was only 45 ± 1 percent (with sesamex) and 63 ± 0 percent (with SKF 525-A) of the amount that was produced in control tests.

No protein measurements of the various subcellular fractions were performed to determine specific enzyme activity. However, based on the amounts of aminoparathion produced, differences in enzyme activity were evident in assays conducted at different dates with one fly strain, and also in assays conducted simultaneously with all three strains. Both the CSMA and P_2/sel strain were a good source for the nitroreductase, whereas the F58-W strain was a poor source.

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References and Notes

1. I. Yamamato, E. C. Kimmel, J. E. Casida, *J. Agr. Food Chem.* **17**, 1227 (1969).
2. T. Nakatsugawa and P. A. Dahm, *Biochem. Pharmacol.* **16**, 25 (1967).
3. T. Nakatsugawa, N. M. Tolman, P. A. Dahm, *ibid.* **17**, 1517 (1968).
4. E. P. Lichtenstein and K. R. Schulz, *J. Econ. Entomol.* **57**, 618 (1964).
5. D. L. Mick and P. A. Dahm, *ibid.* **63**, 1155 (1970).
6. E. P. Lichtenstein and J. R. Corbett, *J. Agr. Food Chem.* **17**, 589 (1969).
7. L. Giuffrida, *J. Ass. Off. Agr. Chem.* **47**, 293 (1964).
8. P. R. Averell and M. V. Norris, *Anal. Chem.* **20**, 753 (1948).
9. Special thanks are expressed to Dr. H. K. Schnces, for assistance in the mass spectrometric analyses, and T. T. Liang for his technical help. Published with the approval of the Director of the Research Station, College of Agricultural and Life Sciences. Research supported in part by a grant from the PHS (FD-00258). Contribution by project 1387 from the Wisconsin Agricultural Experiment Station as a collaborator under North Central Regional Cooperative Research Project 96, entitled "Environmental Implications of Pesticide Usage."

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Neural Regeneration: Delayed Formation of Central Contacts by Insect Sensory Cells

Abstract. *Correlated anatomical and electrophysiological results demonstrate that sensory neurons, which differentiate de novo within the epidermis of regenerate abdominal cerci of crickets, enter the terminal ganglion and form functional central connections even when regeneration of the cerci is delayed through the greater part of postembryonic development. Stimulation of regenerate cerci evokes activity in giant interneurons which is normal by several physiological criteria.*

Paired cerci, which form sensory appendages on the 11th abdominal segment of orthopteroid insects, are exceptionally well developed in crickets (Fig. 1A). They are clothed with numerous sensory hairs (Fig. 1C) and other mechanoreceptors. Cell bodies of sensory neurons situated in the cercal epidermis send dendrites to the sensilla, and axons which travel via the purely sensory cercal nerve to the terminal abdominal ganglion of the central nervous system (Fig. 1D) (*1*). There they contact collaterals of giant interneurons which send axons anteriorly through the paired connectives of the ventral nerve cord (*2*) (Fig. 1G).

As with insect appendages in general, new abdominal cerci regenerate after amputation in immature stages (instars), growing vigorously and developing all components of normal cerci within several instars. They are repeatedly formed when regenerates are amputated following each molt. Regeneration of functional cerci can thus be prevented during any desired sequence of instars, after which regeneration may be allowed to proceed.

Their cell bodies having been re-