Tyrosine hydroxylase activity and tyrosine titers during cockroach metamorphosis

T. L. Hopkins[1]

Department of Entomology, Kansas State University, Manhattan (KS 66506, USA), 3 June 1981

Summary. Tyrosine hydroxylation (TH) to dopa in vivo in nymphs and pharate adults of *Periplaneta americana* was very low, but increased over 30-fold beginning at ecdysis. Free tyrosine increased in pharate adults and peaked at ecdysis, whereas TH activity peaked 6 h later. Both TH activity and tyrosine titers declined the next 24 h as the cuticle tanned. TH appears to regulate tanning substrate biosynthesis in cockroaches.

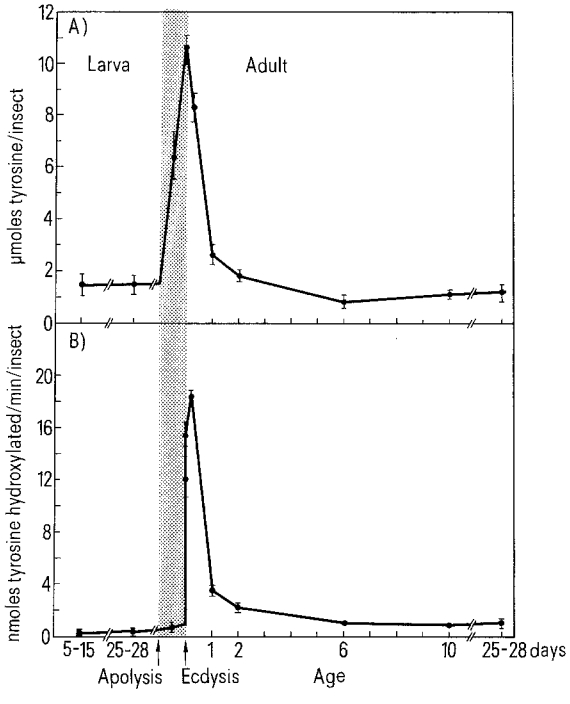
Catecholamine biosynthesis for tanning of insect cuticle is regulated by tyrosine hydroxylase and dopa decarboxylase systems, which in turn are under neurohormonal and/or molting hormone control[2-4]. We have shown that, in cockroaches, dopa decarboxylase levels begin to increase in the hemolymph of pharate adults a few hours before ecdysis[5,6]. In vivo studies show, however, that rates of decarboxylation remain very low until after ecdysis, suggesting that the tyrosine hydroxylase system necessary for dopa production is not activated or synthesized until ecdysis occurs[7,8]. In the present paper, a method for assaying tyrosine hydroxylation rates in vivo has been developed and used to correlate the activity of TH with its substrate titers and other events during cockroach development and metamorphosis.

Materials and methods. Tyrosine hydroxylase activity in vivo was determined by methods adapted from in vitro assays for tyrosine hydroxylase[9] and tyrosinase[10]. When hydroxylation of L-(3,5 ^{3}H) tyrosine is catalyzed by either enzyme, tritium displacement is equivalent to dopa formation and can be determined by separating and counting the tritiated water. Hydroxylation of tyrosine to dopa in insects appears to be catalyzed by a tyrosinase or phenoloxidase (EC 1.10.3.1), which also oxidizes diphenols to quinones[11]. One of the problems of correlating tyrosine hydroxylase activity with cuticle tanning is that tyrosinases are distributed in several tissues and are activated for wound healing, parasite encapsulation, and pigmentation, in addition to their role in tanning. To date a specific tyrosine-3-monooxygenase (EC 1.14.3.a) has not been reported for insects. This method has the advantage of measuring total TH rates in the live insect at specific times during development regardless of enzyme characteristics.

Aliquots of L-(3,5 ^{3}H) tyrosine (54 Ci/mmole, Amersham-Searle, Arlington Heights, IL) were pipetted into small conical vials and evaporated under a stream of dry nitrogen to remove any tritiated water formed by exchange during storage. The tyrosine was redissolved in aqueous 2% ethanol and stored at 2–4 °C. Radiochemical purity checks were made periodically by paper chromatography[7]. Cockroaches, *P. americana,* of known ages and stages of development were anesthesized with carbon dioxide (to prevent bleeding) and were injected with 10 µl of tritiated tyrosine solution (about 1 µCi) in the abdominal hemocoele. The injected animals were held in metabolism chambers for 100 min at 26 °C and then were rapidly frozen by immersion in liquid nitrogen. To recover tritiated water each insect was homogenized in 10 ml of ice-cold distilled water and 1 ml aliquots were rapidly lyophilized[12]. Keeping the homogenates ice-cold until lyophilization was completed was sufficient to prevent in vitro hydroxylation by released enzymes in the homogenates.

The water recovered from each sample was mixed in 10 ml of liquid scintillation fluid for aqueous samples (3a70B, Res. Prod. Internat. Corp., Elk Grove Village, IL) and counted to obtain a SE of 2%. Injection standards containing 10 µl of the ^{3}H-tyrosine solution, 1 ml water and 10 ml of scintillation fluid, were prepared with each group of animals injected. Recovery of tritiated water carried through the lyophilization step was essentially 100%. Only one-half of the total dpm injected was used in the calculation of TH activity because only about half of the label would be displaced if all of the tritium were in the 3 and 5 positions on the ring[9]. To calculate total tyrosine hydroxylated in each animal, it was necessary to determine total endogenous substrate. Total free tyrosine was determined by extraction of individual animals followed by GLC analysis of the extracts[13,14]. During the 100-min incubation period some loss of tritium may occur by respiration of tritiated water and by incorporation of tritium into molecules other than water by exchange with labile hydrogen atoms. To determine the extent of this loss, respired water vapor form injected animals held in metabolism chambers was collected in cold traps and counted. The losses were found to be less than 1.0% of the injected radioactivity during the 100 min, so no correction was made. Tritium loss by incorporation of tritiated water into molecules other than water was assumed to be constant for all samples and very small due to the fact that the tritiated water released by hydroxylation is an extremely small fraction of the total body water of the animal.

Results and discussion. The free tyrosine concentration was consistently low (1–2 µmoles/insect) during the feeding period of the last instar, but increased significantly in the pharate adult a few hours before ecdysis (figure, A). The tyrosine pool increased more than 5-fold to maximal size



Free tyrosine titers (A) and tyrosine hydroxylase activity (B) in the cockroach, *Periplaneta americana,* during the last larval, pharate adult (shaded area), and adult periods. At each interval 3–5 insects were analyzed for tyrosine and 8–9 insects for tyrosine hydroxylase activity.

(10 µmole/insect) at the time of ecdysis and the start of cuticle tanning as was shown in previous studies[13,14]. During the time of tyrosine accumulation in the pharate adult, tyrosine hydroxylase activity remained very low (< 1 nmole/min/insect) its activity was not significantly greater than during the nymphal period (figure, B). Total tyrosine hydroxylase activity increased over 30-fold at the time of ecdysis suggesting enzyme activation or synthesis perhaps linked to the release of bursicon during ecdysis[3]. Tyrosine hydroxylase activity then continued to rise to a peak at 6 h after ecdysis (18 nmoles/min/insect), whereas tyrosine titers rapidly decreased during the post ecdysial period of intense cuticular tanning. Both enzyme activity and substrate titers decreased rapidly during the next 18 h, then more gradually the next few days.

Previous studies have shown that dopa decarboxylase increases during the pharate adult period concomitant with the rise in free tyrosine[5]. However, little or no metabolism of tyrosine occurs because the amino acid is not a good substrate for that enzyme[5,6]. In vivo studies have also shown very little decarboxylation prior to ecdysis but high levels thereafter[7,8]. Therefore, tyrosine is not metabolized to any appreciable extent prior to the activation or synthesis of tyrosine hydroxylase, a critical factor in the build-up of a large substrate pool[15]. Tyrosine hydroxylase which arises during or shortly after ecdysis appears to be the controlling enzyme system for initiating tanning substrate biosynthesis in the cockroach, *P. americana.*

1 Contribution No. 81-294-j, Department of Entomology, Kansas Agricultural Experiment Station, Manhattan, KS. Research supported in part by National Science Foundation grant No. PCM-8003859. I wish to thank Dr M.A.J. Al-Izzi for assistance with the enzyme analyses.
2 P. Karlson and C.E. Sekeris, in: The Insect Integument p. 145. Ed. H.R. Hepburn. Elsevier, Amsterdam 1976.
3 M. Seligman, S. Friedman and G. Fraenkel, J. Insect Physiol. *15,* 553 (1969).
4 M. Seligman, A. Blechi, J. Blechi, P. Herman and G. Fraenkel, Proc. natl Acad. Sci. *74,* 4697 (1977).
5 T.L. Hopkins and R.A. Wirtz, J. Insect Physiol. *22,* 1167 (1976).
6 R.A. Wirtz and T.L. Hopkins, Insect Biochem. *7,* 45 (1977).
7 L.L. Murdock, T.L. Hopkins and R.A. Wirtz, J. Insect Physiol. *16,* 555 (1970).
8 T.L. Hopkins, L.L. Murdock and R.A. Wirtz, Insect Biochem. *1,* 97 (1971).
9 T. Nagatsu, M. Levitt and S. Udenfriend, Analyt. Biochem. *9,* 122 (1964).
10 S. Pomerantz, Biochem. biophys. Res. Commun. *16,* 188 (1964).
11 P.C.J. Brunet, Insect Biochem. *10,* 467 (1980).
12 B.E. Vaughan and E.A. Boling, J. Lab. clin. Med. *57,* 159 (1961).
13 R.A. Wirtz and T.L. Hopkins, J. Insect Physiol. *20,* 1143 (1974).
14 R.A. Wirtz and T.L. Hopkins, Comp. Biochem. Physiol. *56A,* 263 (1977).
15 T.L. Hopkins and R.A. Wirtz, Proc. N. cent. Brch ent. Soc. Am. *32,* 44 (1977).

The effect of camphor on mitochondrial respiration

D.F. Guilland-Cumming[1,2] and G.J. Smith[3]

Physics and Engineering Laboratory, Department of Scientific and Industrial Research, Private Bag, Lower Hutt (New Zealand), 15 June 1981

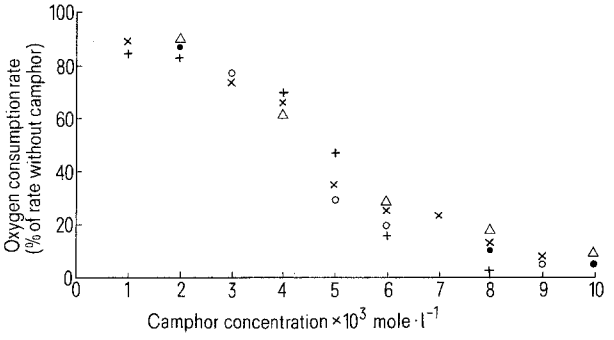
Summary. Camphor at < 8 µmoles/mg protein reduced the rate of oxygen consumption by rat liver mitochondria. The effect occurs only with NAD^+-linked substrates. Succinate linked respiration was inhibited but this appears to be caused by some conversion of succinate to malate. At higher levels, camphor increases oxygen consumption with succinate substrate, by uncoupling at site II.

Camphor has been shown to reduce oxygen consumption in *E. coli*[4,5], and mammalian mitochondria (rat kidney)[6]. The work with *E. coli* suggested that camphor seems to have some effect on the cell membrane. While camphor is not as effective as many other respiration inhibitors, there has been no previous study of the mechanism by which camphor reduces oxygen consumption. This mechanism is the subject of the present study.

A large number of chemicals have been shown to inhibit oxygen consumption of isolated mitochondrial preparations by either blocking electron transport at specific points in the respiratory chain or energy transfer at sites leading to ATP production[7-13]. In the study reported here, inhibitors blocking at different points in the electron transport chain have been used to locate the site of camphor interaction with the respiratory chain.

Materials and methods. Liver mitochondria from 170–220 g male Wistar rats were prepared by a method similar to that of Schneider[14] in a solution containing 0.25 M sucrose, 10 mM HEPES buffer at pH 7.4 and 1 mM ethylenediaminetetraacetic acid.

The mitochondria were washed twice and resuspended in a medium containing only 0.25 M sucrose and 10 mM HEPES. The protein content of each preparation was determined by the biuret reaction. The rate of mitochondrial oxygen consumption was determined by measuring the concentration of oxygen in solution as a function of time with a Clark type electrode. The mitochondria were resuspended in the incubation medium at a concentration of 1 mg protein/ml. Acceptor control ratios at 20 °C were routinely measured for each preparation. The rate of ox-



Rate of oxygen consumption expressed as a percentage of the rate without camphor present. Several determinations were done using different substrates: △ and X malate plus glutamate; + glutamate plus pyruvate; O pyruvate plus malate; ● glutamate.