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Pepsin-like Enzyme in Larvae of Stable Flies

Abstract. A pepsin-like enzyme has been found in larvae of the stable fly. The activity is detectable in homogenates of whole larvae and in homogenates of isolated midguts, the latter of which possess a *p*H environment low enough to support a peptic enzyme. The *p*H optimum of the activity was determined to be 2.4.

Prior to the work of Greenberg and Paretsky (*1*), it was assumed that the enzyme pepsin was absent from insects, and indeed, was restricted to vertebrates. However, these workers showed that a pepsin-like activity is present in all three larval stages of the house fly. Although Champlain and Fisk (*2*) were unable to detect pepsin in homogenates of whole, adult, blood-fed stable flies, we felt that the results of Greenberg and Paretsky warranted a closer investigation in other stages of the stable fly, and also in the expected site of protein digestion, the midgut. The present report (*3*) summarizes the results of a study of a pepsin-like enzyme in the stable fly, *Stomoxys calcitrans* (L.), and the house fly, *Musca domestica* (L.).

Fully mature, third instar larvae, reared on CSMA medium after the method of Champlain *et al.* (*4*) were used throughout. Homogenates of whole larvae (final concentration 10 per milliliter) or dissected midguts (final concentration 50 per milliliter) were prepared with a Teflon-Pyrex tissue grinder in glycine-NaOH buffer (*p*H 2.5) in the cold, and the cuticular debris was removed by centrifugation. Homogenates were stored frozen before use. Estimation of pepsin-like activity was made essentially by the method of Fisk and Shambaugh (*5*) by incubating 0.1 ml of homogenate with 0.9 ml of glycine-NaOH buffer (*p*H 2.5) and 0.25 ml of sulfanilic acid azoalbumin substrate (*6*) at 37°C for 2 hours. Analysis of the diazotized, colored amino acid or peptide fragments, released in direct proportion to enzyme activity (*5*), was accomplished with a Klett-Summerson photoelectric colorimeter with the 420-mμ filter. Controls consisted of reaction mixtures containing homogenate which had been placed in a boiling water bath for 5 minutes prior to assay, and also of reaction mixtures in which the homogenate was replaced by an equal volume of buffer to determine autolysis and residual color of the substrate. Although the investigation was primarily qualitative, the same wet weight of tissue, or number of midguts or whole larvae, was used for comparable assays.

Table 1 is a summary of the results, which confirm the presence of a pepsin-like enzyme in both whole larvae and midguts of *Musca domestica* and *Stomoxys calcitrans*. There is a small but consistent increase in Klett-Summerson colorimeter units in the unaltered mixtures compared with the values obtained for boiled homogenate mixtures or with the residual color of the substrate alone. Variations occurred from preparation to preparation. An additional series of experiments was carried out in which the Folin-Ciocalteau technique employed by Greenberg and Paretsky was followed. By this technique we showed that larvae of both species possessed nearly equal pepsin-like activities. A later comparison of the activities of homogenates from third instar larvae, pupae, and adult stable flies showed that the larvae had the most activity, followed closely by the pupae, but with the adults quite low.

To insure that the enzyme activity was not due to microorganisms, tests were repeated with axenic larvae (*7*). Essentially the same results were obtained (see Table 1). Sterility checks on representative samples of media and larvae showed them to be negative for bacteria, yeasts, and molds.

It was also necessary to determine whether the larval stable fly possessed an alimentary *p*H acid enough to support peptic activity. An experimental procedure was followed (*8*) in which young third instar larvae were reared on enriched artificial medium plus *p*H indicator dyes. The larvae fed for 2 days and were then dissected to determine *p*H. The *p*H of the medium itself was also noted.

The results of the *p*H tests of the digestive tract clearly indicated that the anterior portion of the midgut, and part of the mid portion of the midgut actually could provide a suitable *p*H environment for peptic activity. The following *p*H ranges were recorded: anterior midgut, 2.8 to 3.0; middle midgut, 2.8 to 4.8; posterior midgut, 6.8 to 7.9; hindgut, 7.9 to 9.6. These conditions prevailed although the food itself was above *p*H 6.8 before ingestion.

The *p*H optimum was determined for the pepsin-like activity by measuring both *p*H and increase in Klett reading after 2 hours' incubation of reaction mixtures buffered at 0.3 *p*H intervals between *p*H 1.0 to 3.1. The highest activity was found at *p*H 2.4, but the activity was only slightly less at *p*H 2.8. Activity was lowest below *p*H 1.3. These results are similar to those of Greenberg and Paretsky (*1*) who found the optimum to be *p*H 2.5 in both whole homogenates and dissected guts of the house fly.

At present it is not known whether the enzyme activity is similar to true gastric pepsin of higher animals or is a cathepsin acting at low *p*H. It is also unknown

Table 1. Results of tests confirming presence of pepsin-like activity in *Musca domestica* (L.) and showing similar activity in third instar larvae of *Stomoxys calcitrans* (L). Except as noted, activities are averages of seven replicates, while boiled homogenate or homogenateless mixtures represent two replicates.

Enzyme source	No. of whole insects or guts per replicate	Reading		Net increase due to active homogenate	Reading of homogenateless mixture
		Active mixture*	Boiled homogenate		
		Normal larvae			
Musca					
Whole larvae	1	44.3	41.5	2.8	
Midguts	5	37.2	20.9	16.3	
Stomoxys					
Whole larvae	1	43.9	36.5	7.4	21.0
Midguts	5	32.8	21.0	11.8	19.9
Midguts	5	23.7	13.4	10.3	8.5
Midguts	5	18.4	12.0	6.4	8.5
		Axenic larvae			
Stomoxys					
Whole larvae	4	16.5†	0‡	16.5	
Fore and Midguts	4	18.3†§	0	18.3	

* Klett units (Klett-Summerson photoelectric colorimeter with 420-mμ filter).
† Klett units derived from optical density readings with Spectronic 20 colorimeter at 440 mμ.
‡ Readings made with boiled homogenate tubes arbitrarily set at 0.
§ Activity readings here represent just six replicates.

whether the observed activity has a functional significance in the alimentary tracts of these insects. A series of tests with homogenates made from larvae from which the alimentary tracts had been removed were negative for pepsin-like activity, indicating that the gut tissue is the principal source of the enzyme.

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Biogenesis of Nicotine

Abstract. Radioactive nicotine was synthesized by *Nicotiana rustica L.* which was fed sodium acetate-1-C^{14}, sodium acetate-2-C^{14}, sodium pyruvate-1-C^{14}, or sodium pyruvate-3-C^{14}. Acetate-2-C^{14} and pyruvate-3-C^{14} were converted to nicotine with the least dilution of radioisotope, whereas pyruvate-1-C^{14} was incorporated with a relatively large dilution. When acetate-1-C^{14} was administered to the plants the nicotine contained C^{14} only in the pyrrolidine ring. After acetate-2-C^{14} was fed, C^{14} was located in both the pyrrolidine and pyridine rings.

Biogenetic pathways for the synthesis of the pyrrolidine and N-methyl groups of nicotine in tobacco plants have been firmly established (*1, 2*). Although Dawson *et al.* (*3*) have demonstrated the incorporation of the pyridine ring of nicotinic acid into nicotine, the *de novo* biogenesis of the pyridine ring of nicotine or other pyridine compounds in higher plants has not been elucidated. It has been shown that nicotinic acid is synthesized from tryptophan in several microorganisms and animals (*4*). However, higher plants do not appear to be capable of synthesizing the pyridine ring system of nicotine, trigonelline, or niacin from tryptophan (*5, 6*).

Recently, Il'in showed that acetate-C^{14} is incorporated to a large extent into nicotine in tobacco plants (*7*) but did not ascertain the location of the radioactivity in the nicotine molecule. Leete *et al.* (*8*) also demonstrated incorporation of acetate-2-C^{14} into nicotine and indicated that the radioactivity was randomly distributed in the alkaloid.

This report is a study of the incorporation of acetate-1-C^{14}, acetate-2-C^{14}, pyruvate-1-C^{14}, and pyruvate-3-C^{14} into nicotine in tobacco plants and presents data of a preliminary degradation to discover the location of radioactivity in nicotine following the feeding of radioactive acetate.

Intact *Nicotiana rustica L.* were prepared for hydroponic administration of the C^{14}-labeled substrates as described in an earlier report (*5*). Each plant was fed 2.44×10^{-5} mole of a given compound which had a specific activity of 4.10×10^{8} count/min per millimole. The plants were harvested 7 days after administration of a labeled compound, and the nicotine was purified and isolated as the dipicrate (*9*).

Nicotine was degraded by oxidation with neutral potassium permanganate to yield nicotinic acid, potassium bicarbonate, and methylamine (*10*). In this procedure 3 moles of potassium bicarbonate were formed from the 3-, 4-, and 5-carbons of the pyrrolidine ring. The nicotinic acid was then decarboxylated, and the carboxyl carbon, which originally was the 2-carbon of the pyrrolidine ring of nicotine, was obtained as barium carbonate (*11*). The pyridine resulting from the decarboxylation was recovered as the picrate. The N-methyl group of nicotine was obtained by treatment of the alkaloid with hydriodic acid and conversion of the resulting methyl iodide to methyltriethylammonium iodide (*9*).

The radioactivity of each compound was determined with a windowless flow counter, and all counts were corrected for self-absorption.

The extent of incorporation of labeled substrates into nicotine is shown in Table 1. Acetate-2-C^{14} and pyruvate-3-C^{14} were converted to nicotine with the least dilution, whereas pyruvate-1-C^{14} was incorporated only to a relatively small extent. These results indicated that pyruvate was metabolized to acetate for the most part before it was incorporated into nicotine. However, the extent of conversion of pyruvate-3-C^{14} and the small but significant incorporation of C^{14} from pyruvate-1-C^{14} are an indication that not all pyruvate was converted to acetate before it was utilized for nicotine synthesis.

Nicotine which was synthesized in plants fed acetate-1-C^{14} and acetate-2-C^{14} was partially degraded to determine the location of C^{14} in the molecule in each case. The results of these degradations are presented in Table 2. When acetate-1-C^{14} was the labeled precursor, about one-half the activity was located in the 2-carbon of the pyrrolidine ring. The remaining C^{14} was located in the barium carbonate obtained from the 3-, 4-, and 5-carbons of the pyrrolidine ring. Previous studies, in which nicotine was degraded following administration of ornithine-2-C^{14} (*12*) and glutamate-2-C^{14} (*1*), have shown C^{14} in the alkaloid to be equally distributed between positions 2 and 5 of the pyrrolidine ring. The precursors of the pyrrolidine ring therefore appear to pass through a symmetrical intermediate during the synthesis of nicotine. Since acetate was probably incorporated first into glutamate by way of the tricarboxylic acid cycle, the quantity of the C^{14} in the 2- and 5-carbons of the pyrrolidine ring should be equal. In the present study one-half the C^{14} in nicotine obtained from plants fed acetate-1-C^{14} was found in position 2 of the pyrrolidine ring. It is postulated, therefore, that the remaining C^{14} resides in position 5 of the pyrrolidine ring. Essentially no C^{14} was incorporated into the pyridine ring when plants were fed acetate-1-C^{14}. In contrast, radioactive carbon in the nicotine from plants fed acetate-2-C^{14} was located in both rings. The pyrrolidine ring contained about 60 percent of the total C^{14} in the molecule. If one again assumes an equal distribution of radioactivity between carbons 2 and 5, these positions contained about 20 percent of

Table 1. Incorporation of several labeled precursors into nicotine in tobacco plants.

Substrate	Nicotine dipicrate (10^5 count/min mmole)	Dilution
Acetate-1-C^{14}	4.50	911
Acetate-1-C^{14}	4.18	981
Acetate-2-C^{14}	7.47	550
Acetate-2-C^{14}	12.70	323
Pyruvate-1-C^{14}	0.49	8370
Pyruvate-3-C^{14}	16.90	243

Table 2. Distribution of C^{14} in nicotine from plants fed sodium acetate-1-C^{14} and sodium acetate-2-C^{14}.

Compound	Specific activity (10^3 count/min mmole)	
	Acetate-1-C^{14}	Acetate-2-C^{14}
Nicotine dipicrate	9.3	21.0
Barium carbonate*	3.7	8.6
Nicotinic acid	4.5	9.3
Methyltriethylammonium iodide	0.1	0.6
Barium carbonate†	4.4	1.8
Pyridine picrate	0.4	8.6

* Barium carbonate from the 2-, 3-, and 4-carbons of the pyrrolidine ring. The specific activity was multiplied by 3.
† Barium carbonate resulting from the decarboxylation of nicotinic acid.