

## EFFECT OF NEUTRAL RED ON CARBOHYDRATE LEVELS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.) (LEPIDOPTERA: SPHINGIDAE)\*

KARL J. KRAMER, CYNTHIA N. CHILDS and ROY D. SPEIRS

U.S. Grain Marketing Research Laboratory, Agricultural Research, Science and Education  
 Administration, U.S. Department of Agriculture, Manhattan, Kansas 66502, U.S.A.

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**Abstract**—1. The effect of acute hemocoel injection (100 mg/kg) of neutral red on hemolymph trehalose and fat body glycogen has been examined in the tobacco hornworm, *Manduca sexta* (L.).

2. Neutral red caused a 70% increase in hemolymph sugar 6 hr after injection.
3. Hypertrehalosemia persisted for 18 hr and was followed by hypotrehalosemia.
4. Fat body glycogen was unchanged up to 12 hr.
5. A 60% glycogenolytic response was observed 48 hr after injection.

### INTRODUCTION

Injection of neutral red into rats induces hyperglycemia and hyperglucagonemia (Okuda & Grollman, 1966; Nevis *et al.*, 1968). The vital dye apparently stimulates the alpha cells of the pancreas to secrete glucagon. It is therefore used experimentally to produce chronic glucagon deficiency and also as a tool to elucidate functions of alpha cells and their role in the homeostatic regulation of glucose metabolism (Malaisse *et al.*, 1968; Loubatieres *et al.*, 1974).

The discovery of physiologically active, glucagon-like polypeptides in tissues from invertebrates indicates that the invertebrate and vertebrate hyperglycemic factors are structurally related (Assan *et al.*, 1969; Tager *et al.*, 1975, 1976; Maier *et al.*, 1975, 1978; Norman & Duve, 1978). The results also suggest that agents (such as neutral red) which affect glucagon action in vertebrates might have a similar effect in invertebrates. To test that hypothesis, we studied the action of this dye in the tobacco hornworm, *Manduca sexta* (L.). Specifically the effect on carbohydrate levels of neutral red injected into the hemocoel was determined.

### MATERIALS AND METHODS

#### Chemicals and insects

Neutral red (3-amino-7-dimethylamino-2-methylphenazine HCl, Fig. 1) was obtained from Sigma. It was dissolved in insect saline solution (0.13 M NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>; Schneiderman, 1967) immediately before testing.

*Manduca sexta* eggs were obtained from Dr J. P. Reinecke (Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Fargo, ND, U.S.A.). Larvae were reared at 28°C and 60% relative humidity with a 16-hr light photoperiod using a standard diet (Bell & Joachim, 1976). Neutral red was injected (10–250 mg/kg) into the hemocoel of fifth stadium insects (3 ± 1 g). Then the insects were observed on alternative days until adult eclosion or mortality or until they were used for carbohydrate analysis.

#### Carbohydrate analysis

After injection, insects that were to be used for carbohydrate analysis were allowed to feed *ab libitum* for 0, 3, 6, 12, 24, or 48 hr and then killed. Hemolymph and fat body were collected and subjected to trehalose and glycogen analyses, respectively, by using the anthrone reagent (Roe, 1955) as described previously (Tager *et al.*, 1976; Kramer *et al.*, 1978). Mean values for six insects (±SE of those values) are reported. Hemolymph trehalose ranged from approx 5–15 mM depending on the particular set of control animals; fat body glycogen ranged from 350 to 500 μmol glucose equivalents/g dessicated tissue.

### RESULTS AND DISCUSSION

To determine whether neutral red might adversely affect insects, we first attempted to determine a dose-mortality relationship for the tobacco hornworm. The dye was only slightly toxic when it was injected into the hemocoel of fifth stadium insects. There was little effect except that the animals became reddish and at the highest dose tested (200–250 mg/kg), approximately half of them failed to complete pupation. Larval-pupal intermediates were produced by some in-

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Fig. 1. The structure of neutral red.

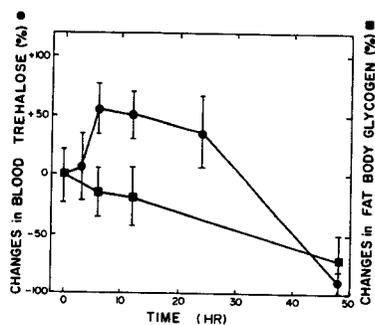


Fig. 2. Changes in blood trehalose (●) and fat body glycogen (■) produced by injection of neutral red (100 mg/kg). Each point represents the mean for 6 insects; the standard error of the mean is indicated. Changes are expressed in percent with reference to control value.

sects, a result reminiscent of the forms obtained when excess juvenile hormone is administered (Slama *et al.*, 1974). Perhaps the dye increased or prolonged juvenile hormone titer, which, in turn prevented morphogenesis.

To determine whether neutral red produced a hyperglycemic response in insects as it does in vertebrates, we injected fifth stadium larvae with a dose of about 100 mg/kg and checked carbohydrate levels in hemolymph and fat body. Neutral red induced a significant rise in blood trehalose that peaked (70% increase) 6 hr after injection (Fig. 2). The hypertrehalosemic response persisted for about 20 hr, then hypotrehalosemia occurred. Fat body glycogen was slightly depressed for the first 12 hr; it was reduced about 60% at 48 hr.

The striking effect of neutral red in carbohydrate metabolism can be best explained by postulating that the dye acted on cells in the neuroendocrine system for about 24 hr post-injection to effect a glucagon-like peptide release, and that the glucagon-like peptide exerted some of its hypertrehalosemic action by inducing glycogenolysis in fat body (Tager *et al.*, 1975, 1976). Over a longer period the neuroendocrine cells might have become depleted of glucagon-like material, and hypotrehalosemia ensued.

The insects responded more slowly than vertebrates to neutral red (30 min versus 6 hr) and the effect persisted for longer periods (24 versus 4 hr; Okuda & Grollman, 1966; Nevis *et al.*, 1968). These differences probably reflect the different experimental animals and conditions, as well as the differences in metabolism between cold-blooded and warm-blooded animals.

We do not know whether the physiological effects of neutral red in the tobacco hornworm are hypertrehalosemic hormone (glucagon)-mediated responses or secondary effects. As with vertebrates, the next logical experiment would be to determine whether hyperglucagonemia also occurred in our invertebrate animal. Small amounts of glucagon-like peptides have been found in rather large quantities of insect tissues (Tager *et al.*, 1976; Maier *et al.*, 1978; Norman & Duve, 1978), but at present there is no assay sensitive enough to measure glucagon titer in individual or

small groups of insects (Kramer, 1979). Additional proof of the pharmacological action of neutral red must await the development of a more sensitive insect glucagon assay and also the results of a morphological analysis for its effects at the cellular level. Attempts so far to detect cellular effects by using microscopic techniques have been unsuccessful (Kramer & Speirs, unpublished).

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