Synthesis of Chitin in Cell-free Extracts of *Prodenia eridania*

Little information has been published on the biosynthesis of chitin in insects despite the fact that this polymer is an important constituent of the exoskeleton[1]. Glaser and Brown[2] demonstrated the presence of a particulate enzyme in *Neurospora crassa* that catalysed the synthesis of chitin from uridine diphospho-*N*-acetylglucosamine (UDPAG) labelled with carbon-14. They demonstrated that chitin formation occurred by the transfer of *N*-acetylglucosamine from UDPAG to a chitodextrin fraction used as a primer. Recently, Candy and Kilby[3] reported investigations on the synthesis of chitin in the desert locust (*Schistocerca gregaria*). Their work indicated a rapid increase in the formation of chitin during and immediately after the moult of the 5th instar larvæ. Similar patterns were found using the thorax, abdomen, and hind leg of the locust. Chitin formation in the wings of the locust was investigated also and dramatic increases in chitin content were found as the locust matured into its adult stage. In the comprehensive work of Candy and Kilby, the presence of enzymes leading to the formation of UDPAG was established unequivocally. They were unable, however, to demonstrate the conversion of ^{14}C-UDPAG into chitin. Developmental investigations at present being made in our laboratories have indicated that chitin synthetase is present in cell-free extracts of *Prodenia eridania*, and the results of the preliminary work are reported here.

The southern army worm (*P. eridania*) was obtained in the 6th instar, pre-pupal, and pupal stages. The procedure followed to obtain the enzyme was that of Glaser and Brown[2]. The animals were rinsed with distilled water and homogenized at 4° C in 3 volumes of 0·05 M *tris*–0·01 M magnesium chloride–0·001 M ethylenediamine tetraacetic acid, *p*H 7·5, containing 0·005 M reduced glutathione. Homogenization was carried out under an atmosphere of nitrogen. The homogenate was centrifuged at 2,000*g* for 10 min, and the supernatant fluid was then centrifuged at 105,000*g* for 1 h. The 105,000*g* pellets were suspended in *tris*-buffer using 1 ml. of buffer for each 2 g of initial weight of the animal, and this material was then used in the assay (Table 1). Following incubation, the enzyme system was treated with 3 N perchloric acid and centrifuged for 30 min at 105,000*g*. The pellet obtained was washed once with 0·3 N perchloric acid, once with distilled water, and then suspended in 1 ml. of distilled water. A 0·5 ml. aliquot was withdrawn and added to 15 ml. of an aqueous counting solution[4] containing 4 per cent 'Cab-O-Sil', a thixotropic gelling agent. The system was counted in a liquid scintillation counter (Packard 'Tri-Carb') to determine the radioactivity of the insoluble particulate.

Table 1. Chitin Synthetase Activity in Cell-free Extracts of *P. eridania**

Stage of development	Radioactivity incorporated into pellet (d.p.m./mg N)	Percentage incorporation of ^{14}C into pellet
Boiled enzyme (6th instar)	9	0·08
6th instar	56	0·52
Pre-pupæ (late 6th instar)	81	0·93
Pupæ	52	0·57

* Total reaction mixture: 1·5 ml. containing 6×10^{-4} M ^{14}C-UDPAG ($3{\cdot}06 \times 10^{4}$ d.p.m.), $1{\cdot}12 \times 10^{-3}$ M *N*-acetylglucosamine, 5 mg chitodextrin and 1 ml. of enzyme preparation. Incubation at 25° C for 2 h.

As may be seen from Table 1, carbon-14 was incorporated into the pellet indicating the presence of chitin synthetase. The level of activity, compared with that found by Glaser and Brown in *Neurospora*, was quite low. The significant result in this investigation is the peaking of enzymatic activity at the pre-pupal (or late 6th instar) stage of development. It is precisely at this stage of metamorphosis that one would expect higher levels of enzyme activity inasmuch as the insect is beginning to form its pupal casing which contains a substantial amount of chitin. These results appear to be consistent with the work of Candy and Kilby on the desert locust.

Work by Carpenter and Jaworski[5] on the presence of isocitrate lyase in *P. eridania* has indicated that the enzyme is present in the pre-pupæ and pupæ but not in the early 6th instar larvæ. It is of interest to speculate, in view of the foregoing findings, that the role of isocitrate lyase may involve the conversion of fat reserves into carbohydrates for chitin biosynthesis. The timing of the appearance of isocitrate lyase and peaking of chitin synthetase activity coincide at a period when the need for carbohydrate for glucosamine synthesis is high, and when exogenous nutrients (carbohydrates) are no longer taken in.

A more extensive examination of the biosynthesis of chitin in *P. eridania* is at present under investigation to establish the relationship between chitin content at various morphological periods in the development of the insect and the level of chitin synthetase at these periods.

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Effects of Cobalt on Mitochondrial Respiration

Dingle, Heath, Webb, and Daniel have suggested that the toxicity of cobalt (II) is due to inhibition of keto-acid oxidation[1]. This hypothesis was supported by the finding that cobalt (II) inhibited oxidation of α-ketoglutarate and pyruvate by rat liver mitochondria during a prolonged incubation without the addition of phosphate acceptor[1]. Since prolonged incubation is deleterious to mitochondria[2], these inhibitory effects of cobalt (II) may have been an artefact. Results obtained in our laboratory show that cobalt (II) does not inhibit oxidation of α-ketoglutarate, β-hydroxybutyrate, or pyruvate plus malate during brief, polarographic experiments. Succinate oxidation was stimulated by cobalt (II) under some conditions and inhibited under others.

Mitochondria were isolated from rat liver in 0·25 M (moles per litre) sucrose using Schneider's method[3]. Oxidative phosphorylation was measured by the procedure of Chance and Williams[4] in an oxygen cathode respirometer[5]. In most experiments cobalt (II) was added to the system as cobalt (II) adenosine diphosphate (Co(II)ADP)[6].

Some effects of cobalt (II) on succinate oxidation are summarized in Table 1. The addition of cobalt (II) ADP stimulated succinate oxidation to a higher level than that obtained with ADP. Succinate was oxidized even more rapidly in the presence of ethylenediamine tetraacetic acid

Table 1. Effects of Co(II) on Succinate Oxidation

Additional reagents in medium	Respiration* (μmoles O_2/sec/g N)
None	1·1
Co(II) (250 μM)	1·1
ADP (250–500 μM, cytochrome *c* (30 μM)	7·0
Co(II)ADP (250 μM), ADP (250 μM), cytochrome *c* (30 μM)	8·5
EDTA (30 μM), ADP (250–500 μM), cytochrome *c* (30 μM)	11
Co(II)ADP (250 μM), EDTA (30 μM), ADP (250 μM), cytochrome *c* (30 μM)	8·5

All reaction media contained 15 mM succinate, 50 mM sucrose, 40 mM KCl, 10 mM $MgCl_2$ and 20 mM potassium phosphate, adjusted to *p*H 7·5. Final concentrations of other reagents are shown in parentheses. Respiration was measured within 1–7 min after adding mitochondria to reaction medium. (25° C)

* Absolute values of respiratory rates had a standard deviation of ± 10 per cent. Even small effects of Co(II), however, were accurately determined because the change in respiration after Co(II) addition was measured.