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Induction of Dopa (3,4-Dihydroxyphenylalanine) Decarboxylase in Blowfly Integument by Ecdysone

A DEMONSTRATION OF SYNTHESIS OF THE ENZYME *DE NOVO*

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The activity of the enzyme dopa (3,4-dihydroxyphenylalanine) decarboxylase, present in the epidermis cells of blowfly larvae, increases during the late third instar under the influence of the steroid hormone, ecdysone. By using the double-labelling technique and immune precipitation with univalent antibody to dopa decarboxylase, we demonstrated that the increase in enzyme activity was due to a stimulation of synthesis of enzyme molecules *de novo*. In this respect, the action of ecdysone is similar to the action of other steroid hormones.

During metamorphosis of dipteran insects, the soft larval cuticle is transformed into the hard darkened puparium by a tanning process involving the sclerotizing agent *N*-acetyldopamine (*N*-acetyl-3,4-dihydroxyphenethylamine) (Karlson & Sekeris, 1966*a*). This metabolite is produced by the decarboxylation of dopa (3,4-dihydroxyphenylalanine) by an enzyme present in the epidermal cells of the integument (Sekeris, 1963) followed by a rapid *N*-acetylation of the amine formed (Sekeris & Karlson, 1962). The activity of the dopa decarboxylase is controlled by the steroid hormone ecdysone (Karlson & Sekeris, 1966*a*). Enzyme activity is low in early third-instar larvae and is high just before pupation, reflecting the ecdysone titre of the larvae (Shaaya & Sekeris, 1965). Further, ecdysone-deprived (ligated) larvae show decreased enzyme activity, whereas injection of ecdysone into the ligated animals restores enzyme activity to normal values (Karlson & Sekeris, 1966*a*). Experiments with metabolic inhibitors of nucleic acid and protein synthesis suggested that the increase in enzyme activity is due to synthesis of enzyme *de novo*, in a process dependent on the presence of newly formed RNA (Sekeris & Karlson, 1964). In the present paper we give direct evidence that the increase in enzyme activity during larval development, or after ecdysone treatment of ligated larvae, is due to an increase in the synthesis of enzyme molecules.

Materials and Methods

Materials

Calliphora vicina (*Calliphora erythrocephala* M.) were reared on bovine meat under conditions described by Congote *et al.* (1969). ^{3}H-labelled L-dopa (sp. radioactivity 22 Ci/mmol), L-[^{14}C]leucine (sp. radioactivity 311 mCi/mmol) and L-[^{3}H]leucine (sp. radioactivity 46 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). L-Dopa and pyridoxal phosphate were purchased from Hofmann–La Roche (Basel, Switzerland), leucine from Fluka A.G. Chemische Fabrik (Buchs, Switzerland), isoleucine from Roth (Karlsruhe, Germany), Triton X-100 from Serva (Heidelberg, Germany), NCS solubilizer from Searle, Nuclear–Chicago Division (Heusenstamm, Germany), chromatography paper 2043 B from Schleicher and Schuell (Dassel, Germany) and DEAE-cellulose from Whatman Biochemicals Ltd. (London, U.K.). All other reagents used were of analytical grade. Agar and anti-(rabbit-γ-globulin) serum were a kind gift from the Behringwerke, Marburg, Germany.

Methods

Injection of precursors. The larvae were first washed gently with tap water and then injected in the posterior part of the body with 0.15 μCi (3 μl) of [^{14}C]leucine/animal. The treated larvae were left on absorbant paper at room temperature for the required length of time. In another series of experiments 7-day-old larvae were ligated at the third to fourth segment. At 24h after pupation of the head part 0.1 μg of β-ecdysone, dissolved in insect Ringer solution (140 mM-NaCl, 2.6 mM-KCl, 2.2 mM-$CaCl_2$, 1 mM-Na_2HPO_4, pH 7.5), was injected as described by Shaaya *et al.* (1971). The control animals received the same amount of insect Ringer solution. At different time-periods after the first injection, the ligated animals were similarly injected once again with [^{14}C]leucine or [^{3}H]leucine and left on filter paper for 1h.

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Assay of enzyme activity. Enzyme activity was assayed by the radiochemical method as described by Sekeris & Karlson (1962) with slight modifications. In general, 0.1ml of enzyme preparation was incubated at 37°C for 30min with 0.18ml of buffer consisting of 10mM-sodium phosphate, 20% (v/v) glycerol, 0.2% phenylthiourea and 1mM-2-mercaptoethanol, pH6.8 (buffer A) containing 0.5μmol of pyridoxal phosphate and with 0.25ml of a 5mM-dopa solution containing 3μCi of ^{3}H-labelled L-dopa. The reaction was terminated by cooling to 0°C. In the blank experiments the enzyme preparation was substituted with buffer or with boiled enzyme. Samples of these incubation mixtures as well as standards of non-radioactive substrate and product were then applied to Schleicher and Schuell 2043 B chromatography paper and submitted to ascending chromatography in butan-1-ol – acetic acid – water (3:1:1, by vol.) at room temperature for 15h. The papers were then dried in air and 0.5cm-wide strips were stained with the Pauly reagent (Smith, 1960), to detect the position of dopa and dopamine. The rest of the paper was then cut into five pieces, one corresponding to dopa (R_F 0.46), another to dopamine (R_F 0.66) and three pieces of equal length corresponding to the intermediate distance between dopa and dopamine. The pieces were placed in toluene scintillator mixture {5g of PPO (2,5-diphenyloxazole) and 0.2g of dimethyl POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene]/l of toluene} for counting radioactivity in a Nuclear–Chicago Mark 1 liquid-scintillation counter. The specific activity of the enzyme was calculated from the percentage transformation of dopa into dopamine.

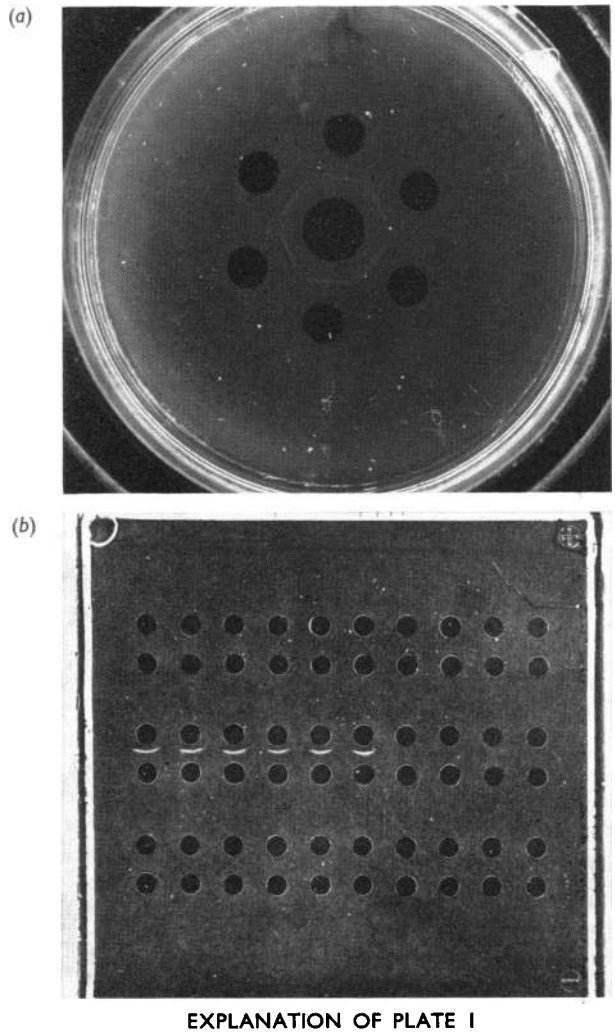
Determination of incorporation of radioactivity into proteins. Portions (100μl) were pipetted on to filter-paper discs (Schleicher and Schuell 2043 B). The paper discs were dropped into ice-cold 5% (w/v) trichloroacetic acid, heated to 90°C for 10min, washed once with 5% trichloroacetic acid, then twice with ethanol and twice with diethyl ether, dried and transferred to scintillation vials. They were counted for radioactivity by using the above conditions, with an efficiency of approx. 10% for ^{3}H and 40% for ^{14}C.

Double-labelling experiments. [^{14}C]Leucine was injected into ligated larvae whereas [^{3}H]leucine was administered to ecdysone-treated ligated animals (after 2 and 6h). The animals were opened with scissors, placed on blotting paper and the integument was separated from the rest of the tissues and the haemolymph. The integuments of control and ecdysone-treated animals were homogenized together in 20ml of buffer A with an Ultraturrax for 1min at 0°C. The homogenate was then centrifuged at 30000*g* in a Sorvall R-C-2B centrifuge for 15min. The supernatant was used for the post-mitochondrial fraction. The post-mitochondrial fractions were chromatographed on a column (8cm×1.5cm) of DEAE-cellulose equilibrated with buffer A. The column was then washed with buffer A and eluted with a linear NaCl gradient made up by mixing 75ml of buffer A with 75ml of buffer A containing 0.3M-NaCl. Fractions (3ml) were collected. The incorporation of the radioactive amino acids into proteins and the ^{3}H/^{14}C ratio in the various fractions were measured.

Immunological titration of dopa decarboxylase. Some 100 integuments from white prepupae were homogenized in 6ml of buffer A with an Ultraturrax for 1min at 0°C. The post-mitochondrial fraction was then dialysed against 10mM-sodium phosphate, pH7.6, containing 0.8% (w/v) NaCl and used as crude enzyme extract. Different portions of this preparation were incubated with equal amounts of antiserum to dopa decarboxylase or with control serum at room temperature for 1h. After incubation, the mixture was centrifuged at 15000*g* for 15min and the supernatant enzyme activity was assayed in duplicate.

Immunochemical experiments. [^{14}C]Leucine was injected for 1h into control and ecdysone-treated larvae as well as into animals of different developmental stages. The integuments were homogenized as described in the section on double-labelling experiments. In the post-mitochondrial fraction immune precipitation of labelled dopa decarboxylase was then done in duplicate as described by Palmiter (1973). To 150μl of post-mitochondrial supernatant was added 50μl of a solution containing 5μg of dopa decarboxylase, 2.5% (w/v) sodium deoxycholate, 2.5% (w/v) Triton X-100, 50mM-leucine, 50mM-isoleucine and 5mM-NaN_3, and the mixture was centrifuged for 10min at 13000*g*. The supernatant was incubated with 20μl of specific anti-(dopa decarboxylase) serum at a concentration 1.2 times that necessary to precipitate the added dopa decarboxylase and 10μl of anti-(rabbit-γ-globulin)serum from goat, at a concentration necessary to precipitate the added anti-(dopa decarboxylase)serum. After incubation for 1h at room temperature each reaction mixture was layered on 100μl of a solution, previously centrifuged at 15000*g*, consisting of 1M-sucrose, 10mM-sodium phosphate buffer, pH7.6, 150mM-NaCl, 10mM-EDTA and 1% (v/v) Triton X-100, and centrifuged for 5min at 15000*g* in the HB-4 Sorvall rotor. The contents of each tube were frozen and the tip containing the antibody was cut off and placed in scintillation vials with 0.7ml of NCS. After incubation for 12h at 37°C, 10ml of scintillator mixture was added and the radioactivity determined by using a Nuclear–Chicago Mark I scintillation counter.

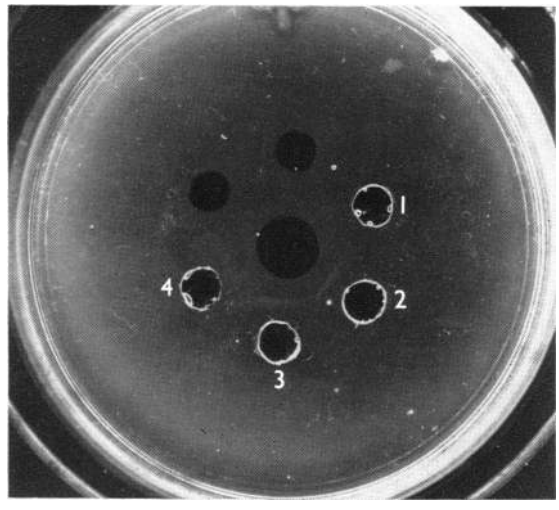
Since unspecific co-precipitation presents a serious problem in these kind of experiments, we introduced the following control. We prepared control serum and repeated the same experiments as above, by



EXPLANATION OF PLATE 1

Interaction of antibody with pure dopa decarboxylase

(*a*) Agar-gel diffusion analysis (Ouchterlony test). The central well contained 50 μg of specific anti-(dopa decarboxylase) serum and the outer wells contained 2 μg of pure enzyme. (*b*) Counter electrophoresis of antibody with dopa decarboxylase. Each of the upper six series of wells contained 30 μg of specific anti-(dopa decarboxylase) serum, each of the lower wells 2 μg of pure enzyme.



EXPLANATION OF PLATE 2

Agar-gel diffusion analysis (Ouchterlony) of antibody interaction with extracts from integuments of animals from different developmental stages

The central well contained 50μg of IgG to dopa decarboxylase. Wells 1–4 contained 300μg of protein of extracts from integuments of: 1, 6-day-old larvae; 2, white prepupae; 3, brown pupae (4h after pupation); 4, black pupae (24h after pupation).

incubating the post-mitochondrial supernatant with control serum, anti-IgG (immunoglobulin G) and dopa decarboxylase as carrier. In all experiments, the values of the precipitates obtained with the control serum were subtracted from the immunoprecipitates obtained with dopa decarboxylase IgG.

IgG was prepared from the rabbit serum as described by Levy & Sober (1960). The double-diffusion technique was done by the method of Ouchterlony (1948), and counter electrophoresis as described by Grabar & Burth (1964). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

In the first series of experiments we determined the activity of the dopa decarboxylase during development of third-instar larvae by using the radiochemical test previously used for such measurements but with more optimum substrate and cofactor concentrations. The general pattern of the curve was similar to that described by Shaaya & Sekeris (1965); however, the enzyme activities were higher. As Fig. 1 shows, activity is low in early-third-instar larvae, increases during the late third instar and maximum values are reached in pupating animals. Ligated larvae show low enzymic activity that can be partially restored to normal values by the injection of β-ecdysone (Fig. 1). To decide whether the increase of enzyme activity reflects the amount of enzyme molecules synthesized or an activation of enzyme, two techniques were used: (*a*) double-labelling with [^{3}H]- and [^{14}C]-leucine; (*b*) immunochemical precipitation of the enzyme.

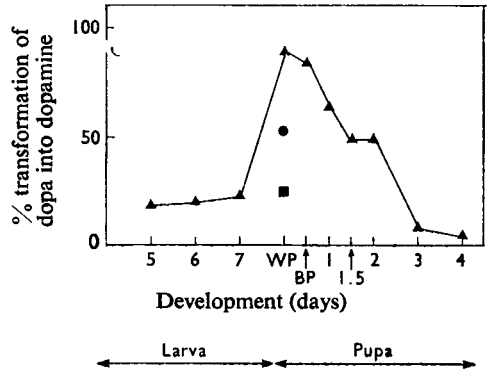


Fig. 1. *Activity of dopa decarboxylation during development of blowfly larvae*

▲, Enzyme activity in normal developing larvae; ■, enzyme activity in ligated 7-day-old larvae, 24h after ligation; ●, enzyme activity in ligated 7-day-old larvae, 24h after ligation, which received 0.1 μg of β-ecdysone, 6h before death. BP, Brown pupae, 4h after white prepupae (WP) stage.

Ligated animals were injected with either insect Ringer or β-ecdysone and 6 or 2h later either [^{3}H]-leucine or [^{14}C]leucine was injected into ecdysone-treated and control larvae respectively 1h before death. The integuments of both groups of animals were homogenized together in buffer as described in the Materials and Methods section and the post-mitochondrial supernatant was submitted to DEAE-cellulose chromatography. In the fractions collected the incorporation of the labelled isotope into protein and the ^{3}H/^{14}C ratio as well as the activity of the dopa decarboxylase were determined. Fig. 2 shows that 3h after injection of β-ecdysone only slight changes in the ratio of ^{3}H/^{14}C incorporated can be detected. At 7h after treatment with the hormone, significant changes appear, especially in those fractions in which the dopa decarboxylase was eluted, as shown by enzymic assays. No detectable enzymic activity can be found in similar fractions of ligated controls or ligated animals 3h after injection of ecdysone. To lend direct proof to the assumption that dopa decarboxylase is a protein whose synthesis is stimulated by ecdysone, we applied immunochemical methods. A prerequisite for our work was the availability of pure dopa decarboxylase which we obtained by standard purification methods (E. G. Fragoulis & C. E. Sekeris, unpublished work). By using the pure enzyme as antigen we obtained from rabbits an antiserum to the enzyme, which on the basis of Ouchterlony tests (see Plate 1*a*) and counter electrophoresis (Plate 1*b*) proved to be univalent. Further, immunoprecipitation of the enzyme results in a loss of the enzymic activity (Fig. 3).

By using the Ouchterlony technique, enzyme extracts from the epidermis of larvae of different developmental stages were assayed with antibody to dopa decarboxylase. Plate 2 shows that extracts from 6-day-old larvae show no or only faintly visible precipitation bands whereas extracts from white prepupae, and brown and black pupae demonstrate very intense bands.

In further experiments the incorporation of radioactive leucine into dopa decarboxylase was measured by precipitating the enzyme from epidermal extracts with its specific antibody. The results of these experiments are depicted in Table 1. The amount of labelled enzyme precipitated by the antibody is low in early third-instar larvae whereas significant amounts are obtained in white prepupae and brown pupae. The ratio of percentage of radioactivity incorporated into the enzyme/percentage incorporated into total protein reaches values of approx. 1.25%, showing that intense enzyme synthesis takes place in pupating animals. As penetration of the labelled leucine into the epidermal

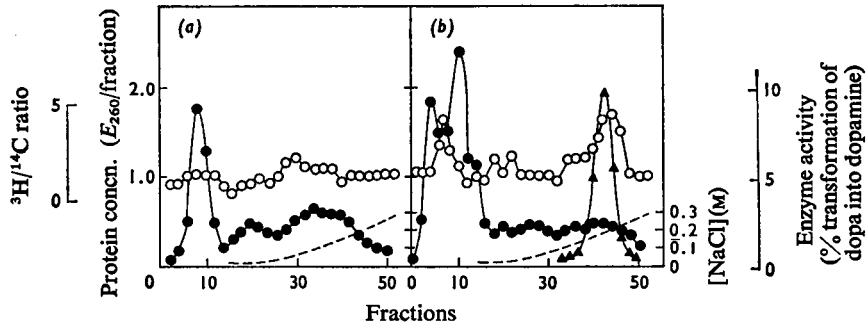


Fig. 2. *DE-32 cellulose chromatography of proteins from the epidermis of ligated blowfly larvae, double-labelled with [^{14}C]leucine (control) and [^3H]leucine (ecdysone treated)*

●, Protein; ○, $^3H/^{14}C$ ratio; ▲, dopa decarboxylase activity; ----, NaCl gradient (M). For experimental details see the Materials and Methods section. (*a*) Ligated larvae, 3h after injection of β-ecdysone; (*b*) ligated larvae, 7h after injection of β-ecdysone.

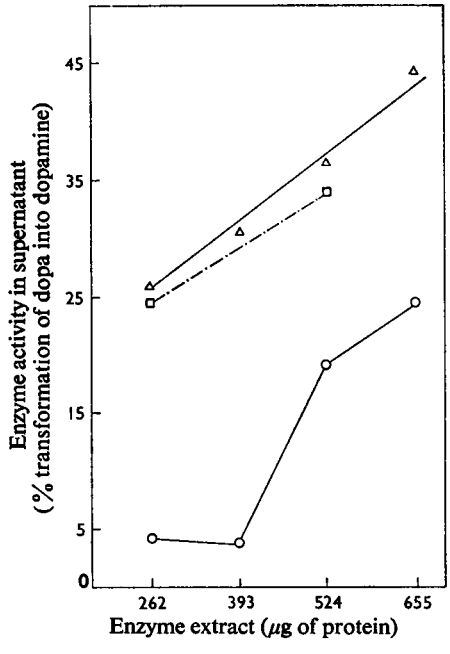


Fig. 3. *Titration of enzyme extracts with antibody to dopa decarboxylase and unspecific antibodies*

Standard amounts of antibody to dopa decarboxylase or of control serum were incubated with portions of crude enzyme extracts (see the Materials and Methods section). After incubation, the mixture was centrifuged at 15000*g* for 15min and the enzyme activity in the supernatant was assayed in duplicate. △, Enzyme activity in the absence of antibody; □, enzyme activity in the presence of unspecific antibody; ○, enzyme activity in the presence of specific antibody.

cells and the endogenous leucine concentration were constant in third-instar larvae, no corrections of these values were necessary.

Lastly, immune titration of labelled enzyme, as described in the experiment of Plate 2, was done with ligated control larvae and ligated ecdysone-treated larvae. From the results in Table 2 it is obvious that a 5h treatment of ligated animals with β-ecdysone leads to a significant increase in the amount of labelled precipitate. It is noteworthy that no general stimulatory effect on protein synthesis is evoked by ecdysone.

Discussion

The experiments described in this paper were aimed at elucidating the nature of the increase of the activity of dopa decarboxylase in epidermis tissue of blowfly larvae during development and after ecdysone action. We had previously described the dependence of enzyme activity on the developmental stage of the larvae (Shaaya & Sekeris, 1965), and had shown that the stimulus leading to the increased activity is the steroid hormone ecdysone (Karlson & Sekeris, 1966*a*). Indirect experiments, i.e. the use of metabolic inhibitors of protein and RNA synthesis, had suggested that the increased enzyme activity could be due to synthesis of enzyme *de novo* and not to activation of pre-existing precursor molecules (Sekeris & Karlson, 1964). By using the double-labelling technique and the immunochemical approach we have clearly demonstrated that our assumptions were correct. The amount of radioactively labelled dopa decarboxylase obtained by precipitation with specific antibody from larvae

Table 1. *Incorporation of [^{14}C]leucine into total protein and enzyme protein of epidermis during development of blowfly larvae*

Groups of 40 animals were injected with [^{14}C]leucine for 1 h. The animals were then homogenized. In the crude homogenate immunoprecipitable material, incorporation of [^{14}C]leucine into total proteins and enzyme proteins was determined as described in the Materials and Methods section.

	Incorporation of [^{14}C]leucine into total epidermal protein (c.p.m.)	Incorporation of [^{14}C]leucine/mg of epidermal protein (c.p.m.)	Incorporation of [^{14}C]-leucine into enzyme protein (c.p.m. in immunoprecipitate)	% radioactivity in enzyme/% radio-activity in total protein
6-day-old larvae	158820	7562	240	0.15
White prepupae	266000	11981	1200	0.40
Brown pupae (2h after pupation)	98000	4734	1240	1.25
Black pupae (24h after pupation)	549400	25793	1900	0.34

Table 2. *Incorporation of [^{14}C]leucine into total protein and enzyme protein of epidermis of ligated control and ecdysone-treated larvae*

[^{14}C]Leucine was injected for 1 h into 40 control ligated animals and into three groups of 40 ligated animals treated for 1, 3 and 5 h with β-ecdysone. The epidermis was then extracted with phosphate buffer and the incorporation of [^{14}C]leucine into proteins (Expt. A) and into immunoprecipitable material (Expt. B) was measured in the extracts as described in the Materials and Methods section.

Expt. A. Incorporation of [^{14}C]leucine into protein (c.p.m./mg of protein)

Treatment ...	Control	1 h with ecdysone	3 h with ecdysone	5 h with ecdysone
	5004	4516	5023	6290

Expt. B. Radioactivity in immunoprecipitate formed against the antibody to dopa decarboxylase (c.p.m.)

Treatment ...	Control	1 h with ecdysone	3 h with ecdysone	5 h with ecdysone
	380	250	350	1860

injected with [^{14}C]leucine increases dramatically in white prepupae. As is well known, during this developmental period the titre of ecdysone also shows a parallel abrupt increase. The effect of ecdysone on enzyme synthesis could be demonstrated more directly by comparing the amount of immune precipitates obtained from ligated ecdysone-depleted larvae and ligated animals subjected to the action of ecdysone. In all present experiments we have used β-ecdysone, which seems to be the intracellular active form of the hormone in most insect species (Moriyama *et al.*, 1970). Injection of ecdysone into ligated larvae results in a significant increase in the amount of labelled precipitate obtained.

Since there is no significant difference in the half-life of the dopa decarboxylase in the non-induced and ecdysone-induced animals (E. G. Fragoulis & C. E. Sekeris, unpublished work) a decreased enzyme breakdown does not seem to play a role in the induction of this protein. Thus the induction of dopa decarboxylase in the integument of blowfly larvae by ecdysone is of the same nature as the induction, among others, of the well-studied rat liver enzymes, tyrosine aminotransferase (Kenney, 1962), tryptophan oxygenase (Feigelson & Greengard, 1962) and alanine aminotransferase (Segal & Kim, 1963) by glucocorticosteroids. As in the case of the liver enzymes, induction of the dopa decarboxylase is dependent on the presence of newly synthesized RNA (Karlson & Sekeris, 1966*a*). We have attributed this to the need of synthesis of the respective mRNA molecules, according to the hormone–gene activation hypothesis (Karlson, 1963; Karlson & Sekeris, 1966*b*). This remains to be demonstrated. RNA fractions have been prepared from epidermal tissue from larvae of different developmental stages and on the basis of their poly(A) content isolated from oligo(dT) cellulose columns (E. G. Fragoulis & C. E. Sekeris, unpublished work). These fractions stimulate protein synthesis in a reconstituted rat liver ribosomal system (Fragoulis & Sekeris, 1975). Analysis of the synthesized products has shown that mRNA for dopa decarboxylase is present in the mRNA fraction isolated and that there are quantitative differences in the mRNA content between the fractions from induced and non-induced animals.

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References

Congote, L. F., Sekeris, C. E. & Karlson, P. (1969) *Exp. Cell Res.* **56**, 338–346

Feigelson, P. & Greengard, O. (1962) *J. Biol. Chem.* **237**, 3714–3717

Fragoulis, E. G. & Sekeris, C. E. (1975) *Eur. J. Biochem.* in the press
Grabar, P. & Burth, P. (eds.) (1964) *Immunoelectrophoretic Analysis*, p. 30, Elsevier Publishing Company, Amsterdam, London and New York
Karlson, P. (1963) *Perspect. Biol. Med.* **6**, 203–214
Karlson, P. & Sekeris, C. E. (1966*a*) *Rec. Progr. Hormone Res.* **22**, 1473–1502
Karlson, P. & Sekeris, C. E. (1966*b*) *Acta Endocrinol.* **53**, 505–518
Kenney, F. T. (1962) *J. Biol. Chem.* **237**, 3495–3498
Levy, H. B. & Sober, H. A. (1960) *Proc. Soc. Exp. Biol. Med.* **103**, 250–252
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Moriyama, H., Nakanishi, K., King, D. S., Okauchi, T., Siddall, J. B. & Hafferl, W. (1970) *Gen. Comp. Endocrinol.* **15**, 80–87
Ouchterlony, O. (1948) *Arkiv. Kemi Mineral. Geol.* **26B**, 1
Palmiter, D. R. (1973) *J. Biol. Chem.* **248**, 2095–2106
Segal, H. L. & Kim, Y. E. (1963) *Proc. Nat. Acad. Sci. U.S.* **50**, 912–916
Sekeris, C. E. (1963) *Z. Phys. Chem.* (*Leipzig*) **332**, 70–78
Sekeris, C. E. & Karlson, P. (1962) *Biochim. Biophys. Acta* **62**, 103–113
Sekeris, C. E. & Karlson, P. (1964) *Arch. Biochem. Biophys.* **105**, 483–487
Shaaya, E. & Sekeris, C. E. (1965) *Gen. Comp. Endocrinol.* **5**, 35–39
Shaaya, E., Schneider, B., Kunz, W. & Sekeris, C. E. (1971) *Insect Biochem.* 113–121
Smith, I. (ed.) (1960) *Chromatographic and Electrophoretic Techniques*, vol. 1, pp. 96–97, William Heinemann Medical Books Ltd. and Interscience Publisher Inc., London and New York