Effect of the Chemosterilant Apholate on the Synthesis of Cellular Components in Developing Housefly Eggs

By WENDELL W. KILGORE AND RUTH R. PAINTER

Agricultural Toxicology and Residue Research Laboratory, University of California, Davis, Calif., U.S.A.

(Received 23 September 1963)

Many antimetabolites and alkylating agents (e.g. 5-fluorouracil, amethopterin, aphoxide and apholate) at concentrations of 0.05–1.0% in food given to adult houseflies (*Musca domestica* L.) induce sterility in one or both sexes (LaBrecque, Adecock & Smith, 1961; LaBrecque, 1961). Some sterilants interrupt or prevent normal oviposition, whereas others cause the deposition of non-viable eggs. Also, depending on the compound used, either permanent or temporary sterility can be induced by many of these chemicals (Painter & Kilgore, 1964).

Kilgore & Painter (1962) reported that the chemosterilant 5-fluoro[¹⁴C]uracil, or a metabolic product, was incorporated into the internal components of housefly eggs after the consumption of a treated diet by the adult insects. There was a correlation between the amount of radioactive material in the eggs and egg viability. Plapp, Bigley, Chapman & Eddy (1962), studying the metabolism of the sterilant methaphoxide, found that the chemical was completely degraded within 48 hr. after injection into the thorax, but that the ³²P from labelled methaphoxide appeared to increase in the ovaries of houseflies for 24 hr. after administration. The methaphoxide content of the ovaries declined rapidly after reaching a peak 1 hr. after treatment. Except for these studies, there have been few reports on the mechanism of action or biological effects of chemosterilants in insects.

Much information has been published on the synthesis of nucleic acids during the development of sea-urchin eggs, frog eggs and chick embryos (Hoff-Jørgensen, 1954), although little is known about the development of these components in viable or non-viable housefly eggs. Therefore, in conjunction with our studies on the mechanism of action of chemosterilants in houseflies, the concentrations of RNA and DNA as well as the concentrations of two enzymes, glucose 6-phosphate dehydrogenase and lactate dehydrogenase, were determined in both viable and non-viable eggs during the normal incubation period immediately after deposition. The non-viable eggs used were deposited by adult insects given diets treated with the chemosterilant apholate. The results of these experiments are reported below.

MATERIALS AND METHODS

Treatment of diets

Control or untreated houseflies (*Musca domestica* L.) were given a normal diet of canned milk (100 ml. of milk, 400 ml. of water and 0.3 ml. of 37% formaldehyde; Kilgore & Painter, 1962) from the time of emergence. The treated insects, a mixed population of 800 houseflies, were given dry diets (6 parts of dried skim milk, 6 parts of finely powdered sucrose and 1 part of powdered eggs; LaBrecque *et al.* 1961) containing either 0.08% or 0.25% of apholate [2,2,4,4,6,6-hexakis(aziridin-1-yl)-1,3,5,2,4,6-triazatriphosphorine] for 48 hr. after emergence followed by normal diets. To ensure complete mixing of the contents, the dry diets were mixed in a high-speed electric mortar (Wig-L-Bug; Crescent Dental Manufacturing Co., Chicago 23, Ill., U.S.A.). Water was given *ad lib.* to the insects receiving the treated dry diets.

Egg collection and incubation

Eggs were collected immediately after deposition from the surface of the oviposition medium as described by Kilgore & Painter (1962). In the present study, however, the oviposition medium was soaked in a 0.5M-sucrose solution when the eggs were to be collected for enzymic studies.

Egg samples weighing 200 mg. each were placed on aluminium foil in separate Petri dishes containing moist filter paper and incubated at 37° for 0–6 hr. At this temperature (37°) normal hatching usually occurs in about 8 hr. (West, 1951). The samples for nucleic acid determinations were frozen immediately after the desired incubation period for subsequent analyses, but the samples for enzymic studies were homogenized and analysed immediately.

Egg extracts

Acid-soluble fractions. Eggs to be analysed for total nucleic acid content were placed in homogenizers specially adapted in this Laboratory for grinding small samples. These were made by grinding the inner tips (1.5 cm. from base to top of ground area) of 12 ml. heavy-walled conical centrifuge tubes and fitting them with tapered ground-glass pestles. After the addition of 0.1 ml. of cold 0.17N-HClO₄, the eggs were homogenized for 2 min. in an ice bath at 5°. After homogenization 0.9 ml. of cold (5°) 0.17N-HClO₄ was added to each tube to wash down the sides of the tubes and pestles, and to dilute the samples. The preparations were then centrifuged at 6000g for 20 min. at 5°. The supernatant solution obtained from each sample was removed with a Pasteur pipette and retained. The residue was

thoroughly mixed with 1.0 ml. of cold (5°) 0.17N-HClO₄ and again centrifuged. The two supernatant solutions from each sample were combined and retained for subsequent analyses.

Acid-insoluble fractions. The residue remaining after the cold-HClO₄ extraction was extracted twice with 5.0 ml. portions of 95% (v/v) ethanol to remove lipids and waxy substances. After each extraction the samples were centrifuged (at 6000 *g* for 20 min.) and the supernatant solutions discarded. Each residue was further extracted with 2.0 ml. of 0.5N-HClO₄ for 20 min. in a water bath at 90° and again centrifuged at 6000 *g* for 20 min. The supernatant solutions were then analysed for nucleic acid content.

Homogenates for enzyme assays. The egg samples weighing 200 mg. each were homogenized for 2 min. at 5° with 0.2 ml. of 0.05M-tris-HCl buffer, pH 8.0, in the special centrifuge-tube homogenizers described above. The pestle and walls of each tube were rinsed with 1.8 ml. of buffer, and the contents were centrifuged at 6000 *g* for 20 min. at 5° . Any solution still turbid after this centrifugation was transferred to a clean tube and recentrifuged. The supernatant solutions obtained after this centrifugation were used for the protein determinations and enzyme assays.

Chemical analyses

The RNA content in the HClO₄ extracts was determined by the orcinol procedure (Mejbaum, 1939), and the DNA content was measured with diphenylamine (Burton, 1956). Reagent-grade RNA (Nutritional Biochemicals Corp.) and the sodium salt of calf-thymus DNA (Sigma Chemical Co.) were used as standards.

Protein was determined by the method of Folin & Ciocalteu (1927) and by measuring the extinction of preparations at 280 *mμ* in a Beckman model DU spectrophotometer. Egg albumin (5 × crystallized; Nutritional Biochemicals Corp.) was used as a standard.

Enzyme assays

Dehydrogenase activities in the homogenates were determined by measuring the extinction of the appropriate coenzyme at 340 *mμ* in a Beckman model DU spectrophotometer. For glucose 6-phosphate dehydrogenase assays, the spectrophotometer cells contained: 5.0 *μ*moles of sodium glucose 6-phosphate (no substrate in endogenous), 0.6 *μ*mole of NADP⁺, 0.2 ml. of egg homogenate and 420 *μ*moles of tris-HCl buffer, pH 7.7; the final volume was 3.0 ml. For the lactate-dehydrogenase assays, the spectrophotometer cells contained: 4.0 *μ*moles of sodium pyruvate (no substrate in the endogenous), 0.5 *μ*mole of NADH, 0.2 ml. of egg homogenate and 420 *μ*moles of tris-HCl buffer, pH 8.0; the final volume was 3.0 ml. For each assay the blank cells of the spectrophotometer contained comparable amounts of buffer and egg homogenate.

RESULTS

Synthesis of nucleic acids in normal eggs

Deoxyribonucleic acid. The DNA content in the acid-insoluble fractions of the normal eggs was very low during the early stages of larval development (Fig. 1*a*) but, after an initial lag period of

about 1 hr., the DNA content increased rapidly until the fourth to fifth hour of incubation at 37° . No further net synthesis of DNA occurred after this period or during the time of larval emergence. The deoxyribosidic compounds in the acid-soluble fractions decreased only slightly during the entire incubation period (Fig. 1*a*).

Ribonucleic acid. The RNA content of the eggs (acid-insoluble fractions) remained almost constant during the normal development period (Fig. 1*b*). In addition, the concentration of ribosidic compounds in the acid-soluble fractions was also constant during this period and was almost as high as the RNA content in the acid-insoluble fractions.

Fig. 1. Synthesis of nucleic acids during the development of normal housefly eggs: (a) DNA; (b) RNA. Δ, Acid-soluble fractions; ●, acid-insoluble fractions. Experimental details are given in the text. Larval emergence usually occurred after an incubation period of 7–8 hr. at 37° .

*Synthesis of nucleic acids in eggs
deposited by flies given apholate*

Deoxyribonucleic acid. Fig. 2(a) shows that on incubation some net synthesis of DNA occurred in eggs from flies given a diet containing 0.08 % of apholate. On the other hand, no net synthesis occurred when the diet contained 0.25 % of apholate. Although the eggs were collected immediately after deposition, some development may have occurred before incubation, as zero-time measurements of egg lots from both treated and untreated insects showed considerable variation. The small amount of DNA synthesized by the eggs deposited by flies given 0.08 % of apholate may be attributed, at least in part, to those few eggs that hatched during the experiments (17 % compared

with 90 % for normal eggs). Even after prolonged incubation none of the eggs deposited by flies given 0.25 % of apholate hatched or showed any change in total DNA content.

Both types of eggs deposited by flies given apholate, when analysed without incubation, contained higher concentrations of acid-soluble deoxyribosidic compounds (Fig. 2b) than did normal eggs of the same age. On incubation these concentrations increased considerably in the eggs deposited by flies given apholate but not in the normal eggs.

Ribonucleic acid. The concentrations of acid-insoluble RNA and acid-soluble ribosidic compounds present in the eggs deposited by flies given apholate at the time of deposition were identical with the concentrations present in the normal eggs. Also, the concentrations of these components did not change appreciably in the eggs deposited by flies given apholate or differ from the normal concentrations (Fig. 1b) during the subsequent incubation period.

*Enzymic activity in normal eggs and
eggs deposited by flies given apholate*

Lactate-dehydrogenase activity. Preliminary experiments indicated that lactate-dehydrogenase activity was present in both normal eggs and eggs deposited by flies given apholate after incubation for 4 hr. at 37° (Fig. 3). The activity was, however, much greater in the normal eggs than in the eggs deposited by flies given apholate.

Further studies showed that the lactate-dehydrogenase activity in normal eggs, incubated at 37° for various periods after deposition, was constant for about 2 hr. and then increased considerably during the subsequent incubation period (Fig. 4). Under similar conditions, only a slight increase occurred in the eggs from flies given 0.08 % of apholate and little change took place in the eggs from flies given 0.25 % of apholate (Fig. 4).

Glucose 6-phosphate-dehydrogenase activity. Fig. 5 shows that the enzyme glucose 6-phosphate dehydrogenase was present in both normal eggs and eggs deposited by flies given apholate after incubation for 4 hr. at 37°. Reaction-rate studies also suggested that about the same concentration of enzyme was present in both kinds of eggs at the 4 hr. stage of development.

To determine whether the concentration of enzyme varied or remained unchanged in the eggs during incubation, normal eggs and eggs deposited by flies given apholate were incubated at 37° for various periods, homogenized and assayed for glucose 6-phosphate-dehydrogenase activity. The results of these experiments showed that the activity remained unchanged during the entire incubation period.

Fig. 2. Synthesis of DNA in normal and non-viable housefly eggs: (a) acid-insoluble fraction [the sampling interval used in these experiments was not short enough to show the initial lag of DNA synthesis characteristic of normal eggs (shown in Fig. 1a)]; (b) acid-soluble fraction. ●, DNA content of normal eggs deposited by flies given an untreated diet; □, DNA content of non-viable eggs deposited by flies given a diet containing 0.08 % of apholate; ○, DNA content of non-viable eggs deposited by flies given a diet containing 0.25 % of apholate. Experimental details are given in the text.

Fig. 3. Lactate-dehydrogenase activity in normal and non-viable housefly eggs. After an incubation period of 4 hr., homogenates were prepared from both normal and non-viable eggs. Δ , Endogenous activity; $\bullet$, lactate-dehydrogenase activity of normal eggs deposited by flies given an untreated diet; $\square$, lactate-dehydrogenase activity of non-viable eggs deposited by flies given a diet containing 0.08% of apholate; $\circ$, lactate-dehydrogenase activity of non-viable eggs deposited by flies given a diet containing 0.25% of apholate. The cuvette contents were: 4μ moles of sodium pyruvate (no substrate in endogenous), 0.5μ mole of NADH, 0.2 ml. of egg homogenate and 420μ moles of tris-HCl buffer, pH 8.0; the final volume was 3.0 ml. Lactate-dehydrogenase activity was determined by measuring the extinction at $340\text{ m}\mu$.

DISCUSSION

The results of the present study show that normal housefly eggs contain very low concentrations of DNA during the early stages of larval development. After an initial lag period of about 1 hr., however, the amount of DNA increases until the time of larval emergence. On the other hand, the amount of RNA in the eggs remains relatively constant. These results are similar to those found in sea-urchin eggs, frog eggs and chick embryos by Hoff-Jørgensen (1954), and in sea-urchin embryos by Elson, Gustafson & Chargaff (1954).

The activity of glucose 6-phosphate dehydrogenase did not vary significantly in the normal eggs from the time of deposition to the period of larval emergence. In contrast, however, the activity of acetate dehydrogenase increased considerably during this period. Also, the formation of the enzyme paralleled the synthesis of DNA. These results suggest that glucose 6-phosphate dehydrogenase could have been incorporated into the eggs by the ovaries and was not synthesized to any appreciable extent during the incubation period, whereas the formation of lactate dehydrogenase

Fig. 4. Formation of lactate dehydrogenase in normal and non-viable housefly eggs. $\bullet$, Lactate-dehydrogenase activity of normal eggs deposited by flies given an untreated diet; $\square$, lactate-dehydrogenase activity of non-viable eggs deposited by flies given a diet containing 0.08% of apholate; $\circ$, lactate-dehydrogenase activity of non-viable eggs deposited by flies given a diet containing 0.25% of apholate. Experimental details are given in the text.

Fig. 5. Glucose 6-phosphate-dehydrogenase activity in normal and non-viable housefly eggs. After an incubation period of 4 hr., homogenates were prepared from both normal and non-viable eggs. Δ , Endogenous activity; $\circ$, glucose 6-phosphate-dehydrogenase activity of normal eggs deposited by flies given an untreated diet; $\bullet$, glucose 6-phosphate-dehydrogenase activity of non-viable eggs deposited by flies given a diet containing either 0.08% or 0.25% of apholate. The cuvette contents were: 4μ moles of glucose 6-phosphate (no substrate in endogenous), 0.6μ mole of NADP^+ , 0.2 ml. of egg homogenate and 420μ moles of tris-HCl buffer, pH 7.7; the final volume was 3.0 ml. Glucose 6-phosphate-dehydrogenase activity was determined by measuring the extinction at $340\text{ m}\mu$.

was greatly dependent on larval growth and development within the eggs.

Ionizing radiation, Mitomycin C and nitrogen mustard inhibit the synthesis of DNA in many biological systems. It is now apparent from the present study that the chemosterilant apholate, when given to adult houseflies, also results in the

inhibition of DNA synthesis in eggs deposited by treated insects. It is not clear, however, whether this chemical has a direct or indirect effect on DNA synthesis in the developing eggs. A direct effect could occur by transfer of the chemical from the ovaries to the eggs. On the other hand, ovarian damage, hormonal imbalance, or any one of a number of metabolic abnormalities caused by the chemical, could have an indirect effect on the synthesis of DNA in the developing eggs. It is clear, however, that the increased amount of acid-soluble deoxyribosidic compounds in eggs of apholate-treated insects was not high enough to account for the quantity of DNA that would have been incorporated into the acid-insoluble fractions under normal conditions.

The absence of DNA synthesis in the developing eggs caused by apholate was also accompanied by the loss of the ability of the eggs to form lactate dehydrogenase. However, the activity of glucose 6-phosphate dehydrogenase was unaffected by the chemical, as the amount of enzyme present initially in both types of eggs remained constant during the incubation period. This again suggests that the latter enzyme could have been incorporated into the eggs by the ovaries and was not synthesized to any appreciable extent during the incubation period. It also suggests that the effects on DNA and lactate-dehydrogenase synthesis may not be significant unless the eggs deposited by flies given apholate have other more general metabolic activity.

SUMMARY

1. After an initial lag period, the DNA content in normal housefly eggs increases rapidly during incubation at 37°, whereas the RNA content remains almost constant.

2. The synthesis of lactate dehydrogenase in normal eggs parallels the synthesis of DNA during incubation. However, the activity of glucose 6-phosphate dehydrogenase in the eggs remains unchanged during the same period.

3. Non-viable eggs deposited by insects given a diet treated with the chemosterilant apholate do not synthesize any significant quantities of DNA during incubation.

4. The absence of DNA synthesis in the non-viable eggs deposited by flies given apholate is accompanied by the loss of the ability of the eggs to form lactate dehydrogenase. On the other hand, the enzyme glucose 6-phosphate dehydrogenase is unaffected.

This investigation was supported in part by Research Grant no. CA-06457 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service. We wish to express our appreciation to the Olin Matheson Chemical Corporation for supplying the chemosterilant apholate and to Mrs Mirdza Garskis for technical assistance.

REFERENCES

- Burton, K. (1956). *Biochem. J.* **62**, 315.
 Elson, D., Gustafson, T. & Chargaff, E. (1954). *J. biol. Chem.* **209**, 285.
 Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
 Hoff-Jørgensen, E. (1954). *Colston Pap.* **7**, 79.
 Kilgore, W. W. & Painter, R. R. (1962). *J. econ. Ent.* **55**, 710.
 LaBrecque, G. C. (1961). *J. econ. Ent.* **54**, 684.
 LaBrecque, G. C., Adcock, P. H. & Smith, C. N. (1961). *J. econ. Ent.* **53**, 802.
 Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
 Painter, R. R. & Kilgore, W. W. (1964). *J. econ. Ent.* **57**, 154.
 Plapp, F. W., jun., Bigley, W. S., Chapman, G. A. & Eddy, G. W. (1962). *J. econ. Ent.* **55**, 607.
 West, L. S. (1951). *The Housefly*, p. 193. Ithaca, N.Y.: Comstock Publishing Co.

Biochem. J. (1964), **92**, 357

The Partial Purification and some Properties of Two Sucrases of *Phaseolus vulgaris*

By R. A. COOPER* AND R. N. GREENSHIELDS†
Department of Biochemistry, University of Birmingham

(Received 4 November 1963)

Some members of the raffinose family of oligo-saccharides have been known for many years

* Present address: Department of Biochemistry, University of Leicester.

† Present address: Laboratory, Mitchells and Butlers Ltd., Cape Hill Brewery, Birmingham.

(Johnson, 1843; Planta & Schultze, 1890; Bourquelot & Bridel, 1910), but, since early workers in the field found the isolation and characterization of these sugars difficult, they were thought of as rarities. Recent surveys with chromatographic techniques have shown that they occur more