Protein and Nucleic Acid Changes During Growth and Aging in the Mosquito

By C. A. LANG, H. Y. LAU AND D. J. JEFFERSON

Department of Biochemistry, University of Louisville School of Medicine, Louisville, Ky., U.S.A.

(Received 2 September 1964)

1. Mosquito samples from the entire life span including senescence were analysed for weight, protein, RNA and DNA. 2. The results for each component indicate that maximal values are attained during larval development. No changes were observed during metamorphosis or the adult period. 3. The peak concentration of RNA and DNA was reached on the third day larval age. Protein content and weight were highest on the sixth day. 4. Except for the first 2 days of larval life the DNA/protein ratio was constant. Hence protein content is an index of cell number for this organism. 5. The distribution of the different biochemical components was determined in different body regions and in subcellular fractions. A high concentration of DNA was found in the soluble fraction of larval but not adult homogenates.

The quantitative measurement of growth is based usually on the weight or length of an organism or on estimates of cell number such as the concentration of protein or DNA. The latter biochemical parameters are metabolically stable tissue components and common expressions of reference for enzyme activity. In addition, RNA concentration is an index of protein synthesis and growth in bacteria and mammalian tissue (Davidson, 1960). The initial experiments in our biochemical studies of growth and aging in the mosquito were to determine protein, DNA and RNA concentrations throughout its life span to define the developmental stages in biochemical terms. Further, this information was important as a reference basis for expressing results in our subsequent experiments.

The mosquito is an excellent experimental organism for studies of growth and aging for several reasons. First, the growth and aging periods are discrete and easily discernible. Growth occurs only in the larval stages, and metamorphosis instead of net growth occurs in the pupa. Only ovarian development takes place in the adult, and this requires a blood meal. Since the adult can be maintained adequately in the laboratory on a diet of sucrose and water, thereby preventing ovarian development, the adult stage can be one of aging alone. Secondly, the growth and aging times are relatively short, requiring 7 and 21 days respectively. Thirdly, the nutritional requirements have been determined in detail (Akov, 1962). This information suggests that the intermediary metabolism of the mosquito may be similar to that of

laboratory organisms that have been studied more extensively. Hence results obtained with the mosquito may be also of general and fundamental biochemical importance.

Changes in weight and length, as well as fat, glycogen, nitrogen and protein content, have been investigated in the mosquito (Christophers, 1960; Twohy & Rozeboom, 1957; Rozeboom & Twohy, 1958; Chen, 1958; Lang, 1963). To our knowledge the nucleic acid content of the mosquito has not been determined, although studies in other insects have been reported (Agrell, 1952; Levenbook, 1953; Chino, 1956; Niemierko, Wlodawer & Wojtezak, 1956; Nigon & Daillie, 1958; Wyatt, 1959; Krishnakumaran, 1961; Linzen & Wyatt, 1964). In each case, however, the investigation was restricted to certain age groups. Devi, Lemonde, Srivastava & Sarkar (1963) have determined nucleic acid concentrations in *Tribolium confusum* during a longer age span. In the present experiments protein, RNA and DNA concentrations were determined in the mosquito at specific intervals during the entire life span.

MATERIALS AND METHODS

The mosquitoes were obtained from our own colony of *Aedes aegypti* L., and the larvae were reared according to a standardized procedure (Lang, 1963). The only modification was a different culture medium of 10% (w/v) desiccated hog liver (Wilson and Co. Inc., Chicago, Ill., U.S.A.) suspended in water, which is used currently for our routine cultures. The eggs were hatched under vacuum, and all first-instar larvae were within 15 min. of the same age

With the above rearing technique and at a temperature of 27–28° pupation began 6 days after hatching. The pupae were washed in distilled water and placed into containers of distilled water for adult emergence. Fifty newly emerged male and an equal number of female adults were transferred to lantern chimney cages, which consisted of a water-saturated cotton pad on the bottom for humidification, a raised screen floor above the pad, and a cotton pad saturated with 5% sucrose solution at the top of the cage for food. Samples of mosquitoes of different stages were obtained at intervals, and the age was determined to the nearest 12 hr. interval for larvae and adults and to the nearest 30 min. for pupae. The aged mosquitoes used in these experiments were 28–35 days old and represented the survivors after 50–75% of the original cage population died. The aging temperature was 27–28°.

Samples of larvae and pupae were washed in distilled water for 1–2 hr., cold-inactivated at 0–4° for 3–5 min. and then homogenized in 0.25 M-sucrose solution in an all-glass TenBroeck homogenizer. The homogenate concentration was 15–20% (w/v). Adults were homogenized without washing but in a similar manner. Cellular fractions were obtained by the differential-centrifugation technique of Schneider & Hogeboom (1950). The homogenate was centrifuged successively for 10 min. periods at 600g and 10000g, and the respective sediments were resuspended in sucrose and recentrifuged once at each speed. The washings were pooled with the last supernatant and centrifuged at 105000g for 30 min. to obtain the microsomal and soluble fractions. Centrifugation was carried out at 0–4° in a Lourdes LRA centrifuge and 9RA rotor and a Spinco model L ultracentrifuge and SW 39L swinging bucket rotor.

Fresh-weight values were determined of whole specimens, which were then immediately dried to constant weight at 100°. The difference in the two weights was regarded as the water content. Next these samples were homogenized, and portions taken for protein analysis by the method of Lowry, Rosebrough, Farr & Randall (1951).

For nucleic acid determinations freshly prepared homogenates were fractionated as quickly as possible and kept at 0° to minimize any nuclease effect. The method of Schneider, Hogeboom & Ross (1950) was used with the following modifications. Dilute perchloric acid was added to the homogenate to a final acid concentration of 0.25 N, and the mixture stirred intermittently for 5 min. and centrifuged. The acid-insoluble fraction was washed once with dilute HClO₄, extracted twice with 95% (v/v) ethanol and then extracted twice at 80° for 30 min. with 1.0 N-HClO₄. The 1.0 N-HClO₄ fractions were pooled and analysed spectrophotometrically at 260 m μ and, in addition, by the orcinol method (Schneider, 1957) and the diphenylamine method (Burton, 1956). Standard solutions of RNA and DNA were heated in 1.0 N-HClO₄ before use. Preliminary experiments to validate these procedures indicated that the same values for RNA content were obtained either by orcinol determination or by the difference between the 260 m μ and diphenylamine values. Hence RNA was determined in subsequent experiments by the difference method alone. Spectrophotometric analyses were carried out in a Zeiss PMQ II spectrophotometer.

Chemicals. A standard solution of Cohn fraction V was used for protein analysis. Purified yeast RNA and highly polymerized DNA preparations for standards were obtained from the Worthington Biochemical Corp., Freehold, N.J.,

U.S.A., and standardized on the basis of *E(P)* according to the procedure of Ogur & Rosen (1950). All other chemicals were reagent grade. Statistical analyses were carried out according to the methods given by Snedecor (1946).

RESULTS

The body weights/mosquito during its life span are presented in Fig. 1. The greatest increase occurred during the latter half of larval development from the third to the sixth day. Body weight

Fig. 1. Body weight of the mosquito during its life span: ■, both sexes; ○, female; ●, male. Each point and bar on the graph represents the mean $\pm$ s.e.m. of 11–199 samples. Bars are omitted when the s.e.m. is less than the size of the point. The vertical broken lines delineate the pupal period.

Fig. 2. Water (a) and protein (b) contents of the mosquito during its life span: ■, both sexes; ○, female; ●, male. Each point represents the mean of 5–34 samples. Experimental details are given in the text.

decreased during the pupation and expupation moults but did not change during adult life (including senescence). The greater weight of the female mosquito was apparent in the pupal and adult periods, the only times in which the organisms were separated and analysed according to sex. The decreases in fresh weight during the pupal moults were due to loss of water, as shown in Fig. 2. In Fig. 2 are also presented the protein contents during the life span. This value increased slightly during the pupal period, but the increase is explained by corresponding changes in water content (Fig. 2). Except for the changes during the pupal moults the protein content remained essentially constant. The fresh-weight curve in Fig. 1 therefore also describes a curve showing the amount of protein/organism during the life span.

An important aspect of this investigation was to develop a suitable RNA and DNA determination for mosquito samples. Our preliminary experiments indicated that the method of Ogur & Rosen (1950) was invalid. Further, the orcinol method for the determination of RNA by the Schneider (1957) procedure was not specific for RNA, as DNA interfered. For these reasons the method of Schneider *et al.* (1950) was adopted and evaluated. A typical example of an evaluation experiment is given in Table 1. Each fraction was analysed for extinction at $260m\mu$ and by the diphenylamine reaction of Burton (1956). The difference between these two values was regarded as a measure of RNA. Another portion of each fraction was analysed by the orcinol method (Schneider, 1957). All results were expressed as $\mu\text{g.}$ of nucleic acid equiv./fraction for comparative purposes. As was expected, high extinction values at $260m\mu$ were found in the cold perchloric acid and the 95% ethanol extracts (fractions I-V). In addition, some diphenylamine-positive material was present in these fractions. RNA-like compounds deter-

mined either by the difference or by the orcinol method were present also. Fractions VI-VIII represent cold perchloric acid-insoluble, ethanol-insoluble compounds that are extracted by hot 1.0N-perchloric acid: this was defined as RNA and DNA. Three extractions with hot perchloric acid were sufficient to remove most of the nucleic acid. The RNA values determined by either the difference method or orcinol procedure were in close agreement. This experiment was repeated on mosquito pupae and adults with similar results. The recovery of known amounts of RNA or DNA added to mosquito samples was quantitative. The procedure for the determination of RNA and DNA described in the Materials and Methods section was a result of these preliminary experiments.

The RNA and DNA contents/mosquito throughout its life-span are presented in Fig. 3. These

Fig. 3. RNA and DNA contents/mosquito during its life span: (a) RNA; (b) DNA. Each point represents the mean of 5-9 samples. Experimental details are given in the text.

Table 1. *Example of the determination of RNA and DNA in a mosquito-larval sample*

A 300 mg. sample of fourth-instar mosquito larvae was homogenized in 2.0 ml. of cold 0.25N-HClO₄ and analysed according to the details given in the text. Each fraction was collected and analysed, and the results were expressed as $\mu\text{g.}$ of nucleic acid equiv./fraction.

Extraction medium	Fraction no.	Nucleic acid content ($\mu\text{g. equiv./fraction}$)			
		$E_{260m\mu}$	Diphenylamine	$E_{260m\mu}$ -diphenylamine	Orcinol
Cold 0.25N-HClO ₄	I	400	14	340	565
	II	55	13	40	45
	III	20	12	10	30
95% Ethanol	IV	350	90	212	50
	V	195	98	161	45
Hot 1.0N-HClO ₄	VI	670	162	488	505
	VII	80	28	54	65
	VIII	20	18	7	10

Fig. 4. RNA and DNA contents/mg. of protein during the life span of the mosquito: ●, RNA; ○, DNA. Each point and bar represents the mean±S.E.M. of 5-9 samples. Experimental details are given in the text.

Fig. 5. RNA/DNA ratios during the life span of the mosquito. Each point and bar represents the mean±S.E.M. of 6-10 samples. Experimental details are given in the text.

curves are similar to the weight curve shown in Fig. 1 and demonstrate that the increase in nucleic acid occurs during the larval stages. The decrease in nucleic acid concentration of approx. 50% from the late larva to the adult is correlated with the decrease in weight that occurs at this time (Fig. 1). The concentrations of both RNA and DNA were constant during metamorphosis and in the adult stage including senescence.

In Fig. 4 are presented the RNA and DNA contents/mg. of protein of the mosquito. Decreases in both nucleic acids were observed during larval development. The decrease in RNA occurred during the second to the fifth day of larval development, whereas the decrease in DNA occurred earlier, during the first 2 days of larval development. The RNA content decreased from 167 to 77 µg./mg. of protein, and the DNA content from 53 to 18 µg./mg. of protein. A small decrease in RNA but not DNA content was also observed during the first 6 days of adult life. No sex difference in either RNA or DNA content was found.

The RNA/DNA ratios during the life span are shown in Fig. 5. A maximum value of 8 was reached on the third day of larval development and this decreased to about 4 in the late larvae. As in the other graphs no changes were observed during metamorphosis. The RNA/DNA ratio showed a slight increase in the newly emerged adult, but dropped to a constant value by the fifth day of adult life.

The distribution of nucleic acid in different regions of the larva and the adult was determined (Table 2). Individual fourth-instar larvae and adults were trisected, and the different body regions were pooled to obtain sufficient amounts of sample for analysis. The various parameters were expressed as percentages of whole organism found in the head, thorax or abdomen. In the larva the percentages of weight, protein and RNA in the different regions were approximately the same. However, a relatively high concentration of DNA was found in the head, and a lower concentration in the abdomen. The distribution pattern in all regions was different in the adult. Although

Table 2. *Distribution of nucleic acids in different regions of the mosquito*

Experimental details are given in the text.

Stage	Region	Distribution (% of that in whole organism)			
		Weight	Protein	RNA	DNA
Larval	Head	14	11	11	21
	Thorax	21	26	30	33
	Abdomen	65	63	59	46
Adult	Head	7	6.5	20	21
	Thorax	57	66	42	39
	Abdomen	36	28	38	39

Table 3. *Intracellular distribution of nucleic acids in the mosquito*

Experimental details are given in the text. Each value is the average of three or four experiments. The range of recovery was 86–107%.

Developmental stage	Fraction	Distribution (% of that in homogenate)		
		Protein	DNA	RNA
Larval	Homogenate	100	100	100
	600g × 10 min. Residue	24	39	25
	10000g × 10 min. Residue	15	13	16
	105000g × 30 min. Residue	12	3.1	23
	105000g × 30 min. Supernatant	56	31	41
Adult	Homogenate	100	100	100
	600g × 10 min. Residue	46	74	44
	10000g × 10 min. Residue	9.2	7.6	11
	105000g × 30 min. Residue	11	2.2	13
	105000g × 30 min. Supernatant	29	2.5	19

the percentages of weight and protein were similar, the head contained 20% of both the RNA and the DNA content but only 7% of the weight and protein. In the thorax, however, about 40% of both nucleic acids was observed compared with 57% of the weight and 66% of the total protein. In the abdomen, about 38% of the weight, RNA and DNA was found compared with 28% of the protein.

The intracellular distribution of RNA, DNA and protein was determined in samples of larvae and adults (Table 3). In the larva 39% of the homogenate DNA was present in the 600g × 10 min. residue, 13% was found in the 10000g × 10 min. residue, and 31% in the 105000g × 30 min. supernatant. The high concentration of DNA in the last-named fraction has been investigated further by Meins & Lang (1963, and unpublished work). RNA was distributed similarly, but with a higher concentration in the 105000g × 30 min. residue, due presumably to ribosomal RNA. In the adult, however, 74% of the total homogenate DNA was found in the 600g × 10 min. residue, and only 7.6 and 2.5% were found in the 10000g × 10 min. residue and 105000g × 30 min. supernatant respectively. On the other hand, 44% of the total RNA was concentrated in the 600g × 10 min. residue, and the remainder was distributed in the other fractions. This distribution was similar to that of protein.

DISCUSSION

The various methods for the determination of RNA and DNA and their pitfalls have been reviewed by Hutchison & Munro (1961). It is essential that the particular method adopted is evaluated for the tissue under study, and a discussion of these points and a procedure for silkworm tissues has been presented by Linzen & Wyatt (1964). The procedures we used are simpler than

their method, since the mosquito has only a small fat body and low amounts of uric acid, which interfered with their determinations. Evidence for the validity of our method was that the ultra-violet-absorption spectra of the acid extracts were identical with those of calf-thymus DNA and yeast RNA standards. Further, consistent results were obtained on replication. Although low DNA values when perchloric acid is used have been reported by Løvtrup & Roos (1961) with dried liver powder, our experiments indicate that over 95% of the RNA or DNA added to mosquito samples could be recovered under our conditions. Our results also demonstrate that the fractionation step involving ethanol or a similar organic solvent is necessary to remove a considerable amount of diphenylamine-, orcinol- and $E_{260m\mu}$ -positive compounds that are not RNA or DNA. This step has been omitted in the work of Devi *et al.* (1963).

The samples used in the present investigation included homogenates of whole organisms as well as of body regions. In such preparations only changes of large magnitude are observed, involving a large proportion of the total cell types in the organism. An advantage is that the changes represent a general phenomenon of the organism rather than of an isolated and perhaps atypical cell type.

The patterns of several biochemical parameters were determined in the mosquito throughout its life span. In the larval period occurred the greatest changes in concentration of fresh weight, dry weight, protein, RNA and DNA/organism. During the moults that define the pupal period decreases in water content as well as dry weight/organism were observed. These changes are attributed to the loss of moulting fluid, larval skins and pupal casts.

The contents of all components investigated throughout the life span on a per organism basis

appear qualitatively the same during larval development, and the protein and the weight curves are identical, as evidenced by the constant values for protein content. However, the times of attaining maximal concentration are different: RNA and DNA on the third day, and protein and weight on the sixth day.

The DNA/protein ratio is highest in the newly hatched larva and falls to 20% of that value by the second day, where it remains up to and during senescence. In contrast, the RNA/protein ratio is highest in the early larva but does not reach a plateau until late larval development. These decreasing curves are due to the fact that protein is increasing more rapidly than the nucleic acids.

RNA content can be considered an index of the capacity for protein synthesis, and DNA content an estimate of cell number. The RNA/DNA ratio is therefore an index of the protein-synthesis capacity/cell. The highest ratio was found on the third day of larval development, and, as expected, preceded the peak of protein concentration. The slight rise in the RNA/DNA ratio during the first days of adult life is correlated with the increase in protein content and in the activity of electron-transport enzymes that occur at that time (Lang, 1959).

No marked changes in concentration of any of the components were observed during the pupal period or in the adult through senescence. These results suggest that neither metamorphosis nor physiological aging is accompanied by significant decreases in cell number or protein-synthesis potential.

The distribution of DNA in the different regions of the fourth-instar larva was similar to that of the adult, unlike weight, protein and RNA. The DNA pattern indicates that the cell number is the same in these two stages. Further, the changes in the other components may reflect differential increases in cell size taking place in the various regions. However, in the whole organism the DNA/protein ratio is constant except for the first 2 days of larval life. Hence the protein content, which is easier to determine, is also a useful measurement of cell number in this organism. The relatively high DNA content in the head region is explained by the large number of cells in the eyes, assuming that the DNA/cell is constant.

The most striking feature of the distribution of nucleic acid in the cell fractions was the high DNA content found in the soluble fraction of the larva, which fell to less than 10% of that value in the adult. Similarly, the soluble protein, microsomal RNA and soluble RNA contents were about 50%

lower in the adult than in the larva. These changes may be causally related to growth. However, the decrease in both microsomal and soluble RNA are not in accord with the findings of Kjeldgaard & Kurland (1963), who found in bacterial cells that growth rate was correlated with ribosomal and not soluble RNA.

The authors are indebted to Dr Claire Graham of the Wilson Co. for a gift of desiccated hog liver. This investigation was supported by a research grant from the National Institutes of Health, U.S. Public Health Service (RG-6748).

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