Nicotinamide-Adenine Dinucleotide Phosphate Enzymes in the Mosquito during Growth and Aging

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1. Optimum conditions were established for determining the activities of the NADP⁺-linked enzymes, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase, in mosquito tissues. 2. The activity of each dehydrogenase was determined in samples of mosquitoes of different ages throughout the life-span. The specific-activity curves attained maximal values in the pupal or early adult period. From these maxima an 81% decrease in glucose 6-phosphate-dehydrogenase and 67% decrease in 6-phosphogluconate-dehydrogenase activities occurred after the tenth day of adult life; a 77% decrease in isocitrate-dehydrogenase activity occurred before the fifth day. 3. The activity differences were found in different body regions as well as in whole organisms. 4. Starvation of the larva or adult did not result in decreases in enzyme activity. 5. These findings support the hypothesis that the activities of enzymes that form NADPH are related to the biosynthetic activity, for the enzyme activities increased during the period of cellular growth and decreased during the aging period.

NADPH is the coenzyme required for many biosynthetic reactions, such as fatty acid synthesis and steroid hydroxylation (Langdon, 1955; Engel & Langer, 1961). Because of this relationship it has been postulated that the concentration of NADPH can act as a regulatory mechanism by controlling the rate of these biosynthetic reactions (Horecker & Hiatt, 1958*a,b*; Scott, 1958; Potter & Niemeyer, 1959; Lowenstein, 1961).

Our hypothesis is that the activities of NADP⁺-linked enzymes, which generate NADPH, play a role in metabolic regulation. To test the hypothesis in the present investigation the activities of several NADP⁺-linked enzymes were determined in tissue samples of differing biosynthetic activity, such as found in the different developmental stages occurring during the life-span of the mosquito. Particular attention was focused on the period of growth, when biosynthesis is at a maximum, and on the period of aging, when net biosynthesis is minimal or zero. The basis for this view was the relatively high activity of NADPH-cytochrome *c* reductase found in mosquito larvae and not in pupae or adults (Lang, 1959).

The enzymes investigated were glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate-NADP⁺ oxidoreductase, EC 1.1.1.43) and isocitrate dehydrogenase (L-isocitrate-NADP⁺ oxidoreduct-

ase, EC 1.1.1.42). The presence of all three enzymes was reported in the larval and adult blowfly (McGinnis, Cheldelin & Newburgh, 1956), and glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were studied in detail in the adult housefly (Chefurka, 1957).

The choice of the mosquito as the experimental organism was based on its short life-span, which permitted the study of an entire life-cycle in about 6 weeks. Further, our previous studies demonstrated that some enzymes which control NADPH concentration in other organisms are absent in the mosquito, such as NAD⁺-NADP⁺ transhydrogenase (Lang, 1961), or present at low activities, such as NAD⁺ pyrophosphatase (Anderson & Lang, 1966).

MATERIALS AND METHODS

The mosquitoes were obtained from our colony of *Aedes aegypti* L. reared according to a standardized procedure (Lang, Lau & Jefferson, 1965). Samples of mosquitoes of different stages were obtained at intervals, and the ages were 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 had died. The aging temperature was 27–28°. To obtain samples of head, thorax and abdomen, whole mosquitoes were cold-inactivated in a Petri dish and then trisected at the head-thorax and thoraco-abdominal junctions with stainless-steel needles. Pooled samples of each body region were homogenized.

Homogenization. Samples of larvae and pupae were suspended in several changes of distilled water for 1–3 hr., counted into an all-glass Ten-Broeck homogenizer, and homogenized in 0.25 M-sucrose at 0°. Unwashed adults were cold-inactivated at 0–4° for 3–5 min. and then homogenized in a similar manner. Samples from different age groups were included in each daily experiment.

Centrifugation. For routine procedures, homogenates were centrifuged at 19000g for 10 min. at 0–4°, and the supernatant fractions were analysed.

Subcellular 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 0.25 M-sucrose and centrifuged again once at each speed. The washings were combined with the last supernatant and centrifuged at 124000g for 30 min. to obtain the microsomal and soluble fractions. All centrifugation procedures were 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. The rotors were equipped with micro-adapters to enable the centrifugation of samples of 1 ml. volume.

Fresh solutions of NADP⁺ were prepared daily and standardized by measuring the extinction at 259 m μ and determining the concentration with the extinction coefficient 18×10^6 cm.²/mole (Siegel, Montgomery & Bock, 1959). In the same way, the concentration of NADPH solutions was determined with the extinction coefficient 6.22×10^6 cm.²/mole (Horecker & Kornberg, 1948).

The procedures for the determination of each enzyme activity were established initially with purified enzyme preparations obtained commercially, before applying the methods to mosquito samples.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase activities were determined by the method of Glock & McLean (1953) modified for mosquito tissues. The reaction mixture of 1.00 ml. final volume contained 35–40 μ moles of tris buffer, pH 7.4, 4.0 μ moles of glucose 6-phosphate or 0.34 μ mole of 6-phosphogluconate, 15 μ moles of MgCl₂, 0.84 μ mole of NADP⁺ and 0.02–0.10 ml. of the 19000g supernatant from a 20% (w/v) homogenate. The change in extinction when substrate was omitted was less than 20% of the total rate in all cases. The spectrophotometric measurements were carried out in a Zeiss PMQII spectrophotometer at 23–25° with 1 ml. quartz cuvettes with a light-path of 1.0 cm.

Isocitrate dehydrogenase. Isocitrate-dehydrogenase activity was determined by the method of Plaut & Sung (1954). The reaction medium of 1.00 ml. final volume contained 37.5 μ moles of tris buffer, pH 7.4, 5.0 μ moles of DL-isocitrate, pH 7.4, 15 μ moles of MnCl₂, 0.84 μ mole of NADP⁺ and 0.02–0.05 ml. of the 19000g supernatant from a 4% (w/v) homogenate. To determine NAD⁺-specific isocitrate dehydrogenase, NAD⁺ was substituted for NADP⁺ in the reaction mixture. The change in extinction when substrate was omitted was less than 5% of the total rate in all cases.

For the determination of enzyme activity supernatants were assayed within 15 min. after they were obtained. Excess of substrate and coenzyme concentrations were used in all enzyme determinations, and the results were corrected for the rate without substrate. The initial rate of change in extinction at 340 m μ was proportional to protein con-

centration determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with human serum albumin (Cohn fraction V) as the standard. The specific activity of these dehydrogenases was defined as μ moles of NADPH formed/min./mg. of protein.

The results were analysed statistically by the methods given in Snedecor (1946).

Chemicals. NAD⁺ and NADP⁺ were of greater than 90% purity (Sigma Chemical Co.). D-Glucose 6-phosphate (disodium salt, 98–100% purity), 6-phosphogluconic acid (sodium salt, Sigma grade) and DL-isocitric acid (trisodium salt, 93–98% purity) were obtained from the Sigma Chemical Co. Other chemicals were of reagent grade, and double-distilled water was used.

RESULTS

Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase have not been investigated previously in the mosquito. For this reason the optimum conditions for the quantitative determination of maximal enzymatic activity in the mosquito were established.

The determination of these dehydrogenase activities was based on the rate of NADPH formation measured by the increase in extinction at 340 m μ . In the larva and pupa, but not the adult, extinction changes in the absence of substrate were observed. To determine whether these changes were specific for NADP⁺ reduction, samples of mosquito homogenate and tris buffer only were analysed at two wavelengths. The increase in extinction at 340 m μ was identical with that obtained at 400 m μ where NADPH does not absorb, and this result indicated that the change without substrate was non-specific and may reflect the blackening due to polyphenol-oxidase activity.

The pH curves for the larval dehydrogenase activities with tris and glycylglycine buffers were determined. Glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase activities were similar for both buffers, but isocitrate-dehydrogenase activity was different. A pH optimum 6.5–7.5 was observed for both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. At pH 7.4 isocitrate-dehydrogenase activity was about 50% of maximum. Similar results were obtained for all enzymes with adult homogenates. For convenience a tris buffer, pH 7.4, was always used for all enzyme determinations.

The metal and coenzyme requirements for the pentose phosphate-cycle enzymes were investigated. Maximal activity was observed with Mg²⁺ and NADP⁺, and no activity could be detected with either Mn²⁺ or NAD⁺. Maximal activity of isocitrate dehydrogenase required Mn²⁺. No activity was observed in the absence of Mn²⁺, and only minimal activity was observed with the substitution of Mg²⁺.

Table 1. *Subcellular distribution of dehydrogenase activities in the mosquito larva*

A 20% (w/v) homogenate of 5-day-old mosquito larvae was used for the glucose 6-phosphate-dehydrogenase and the 6-phosphogluconate-dehydrogenase determinations, and a 5% (w/v) homogenate was used for the isocitrate-dehydrogenase determination. One unit of enzyme activity equals $10^3 \Delta E_{340 \text{ m}\mu} / \text{min}$. Methods for the preparation of fractions and their analyses are described in the Materials and Methods section.

Fraction	Glucose 6-phosphate dehydrogenase		6-Phosphogluconate dehydrogenase		Isocitrate dehydrogenase	
	Total units	Units/mg. of protein	Total units	Units/mg. of protein	Total units	Units/mg. of protein
Whole homogenate	4600	52.3	4400	55.0	14600	166
Residue (600g, 10 min.)	< 25	< 5	< 25	< 5	< 50	< 10
Residue (10000g, 15 min.)	< 50	< 10	< 50	< 10	3200	640
Residue (124000g, 30 min.)	< 20	< 1	< 20	< 1	< 20	< 1
Soluble (124000g, 30 min.)	18816	267	2244	31.8	33369	473

The distribution of all three dehydrogenase activities was studied in the subcellular fractions of the mosquito (Table 1). A decrease in extinction with time was observed consistently in control cuvettes containing whole homogenates of larval samples and glucose 6-phosphate, 6-phosphogluconate or isocitric acid but lacking NADP. This negative endogenous rate was absent in the soluble fraction. The cause of this is unknown, and it could not be prevented by the addition of cyanide.

The homogenate activity for glucose 6-phosphate dehydrogenase and isocitrate dehydrogenase was less than the sum of the activities in the fractions, and thus the percentage recovery could not be based on the homogenate activity. All of the glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase and 89% of the sum of the isocitrate-dehydrogenase activities were localized in the 124000g supernatant fraction. The remainder of the isocitrate-dehydrogenase activity was in the 10000g residue, and upon freeze-thaw treatment of this residue 91% of the activity was solubilized. The 19000g supernatant fraction, which had essentially the same specific activities for all enzymes as the 124000g supernatant and accounted for 90–100% of the total activity, was always used.

In other organisms, isocitrate-dehydrogenase specificity for two coenzymes, NAD⁺ and NADP⁺, has been reported (Kornberg & Pricer, 1951; Plaut & Sung, 1954). In addition, isocitrate-dehydrogenase activity was enhanced by the addition of adenosine phosphate compounds (Kornberg & Pricer, 1951; Chen & Plaut, 1962). Mosquito isocitrate dehydrogenase was specific for NADP⁺, and the activity was not increased upon addition of 0.3–15mM-AMP, -ADP or -ATP. Moreover, NADP⁺-specific isocitrate-dehydrogenase activity could not be demonstrated in any subcellular fraction.

When the 10000g residue fraction was treated with digitonin, deoxycholate or Triton X-100, or subjected to repeated freeze-thaw treatment, and then centrifuged, an increased protein content was found in the supernatant. Yet NAD⁺-specific isocitrate-dehydrogenase activity could not be demonstrated in the residue or supernatant fractions of the solubilized mitochondria even upon addition of AMP, ADP or ATP.

The stability of the dehydrogenase activities in the larva at 0° was investigated. From the time of tissue homogenization to removal of the supernatant fraction after centrifugation of the samples 15 to 20 min. elapsed. During the 30 min. thereafter glucose 6-phosphate-dehydrogenase activity decreased 33%, 6-phosphogluconate-dehydrogenase activity decreased 10% and isocitrate-dehydrogenase activity remained constant. The pentose phosphate-cycle enzymes showed no further decrease up to 2 hr., whereas isocitrate-dehydrogenase activity decreased 16% in the 30–60 min. period and remained at this value up to 2 hr. In pupal and adult samples the activities changed less than 2%.

The sequential reactions catalysed by glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are separated by the reaction catalysed by lactonase. The 6-phosphogluconate formed by glucose 6-phosphate dehydrogenase and lactonase could react further with 6-phosphogluconate dehydrogenase. This would contribute to the formation of NADPH in the glucose 6-phosphate-dehydrogenase determination and result in an erroneous and increased glucose 6-phosphate-dehydrogenase activity value, since the measurement of both activities was based on the rate of NADPH formation. To examine this possibility the concentrations of glucose 6-phosphate and 6-phosphogluconate required to give one-half maximal activity of their respective enzymes were determined, and these amounts were added to a

single reaction mixture. Presence of lactonase would result in an observed activity greater than the sum of the individual activities. However, the resultant enzymic activity was approx. 70% of this sum, indicating that the determined glucose 6-phosphate-dehydrogenase activity did not include 6-phosphogluconate-dehydrogenase activity under our conditions. Further, the results suggested that the glucose 6-phosphate-dehydrogenase activity was inhibited by 6-phosphogluconate. This inhibition was not a significant factor in our routine determinations since excess amounts of the individual substrates were used and initial rates of reaction were measured, which minimized the possibility of product inhibition.

The various enzyme-activity patterns during the life-span of the mosquito were similar, showing an increase to a maximal activity in the pupal or early adult stages and a 67–81% decrease from the maxima during the adult period (Figs. 1, 2 and 3). The main differences in the patterns were in the time of occurrence and the magnitude of the maximal activities. In all stages isocitrate-dehydrogenase activity was highest, followed by glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase activities.

To localize these dehydrogenase-activity changes in the mosquito, different body regions were analysed for enzyme and protein content (Table 2). In the larval (but not the adult) head the percentage total activities of all dehydrogenases were much greater than the proportion of protein

present. The distribution in the other regions of both larva and adult varied with the enzyme, but in general more than 50% of the total activity was in the abdomen. In the adult the specific activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in all regions were 2 to 14 times greater than the values in the larva, and the specific activities of isocitrate dehydrogenase were the same.

Fig. 2. 6-Phosphogluconate-dehydrogenase activity during the life-span of the mosquito. Dehydrogenase activities were determined as described in the Materials and Methods section. Specific activity was described as μmoles of NADPH formed/min./mg. of protein. The vertical broken lines delineate the pupal period. Each point and bar on the graph represents the mean $\pm$ S.E.M. of four to nine samples. Except for the 40-day point, the points without bars represent the mean of two to four samples.

Fig. 1. Glucose 6-phosphate-dehydrogenase activity during the life-span of the mosquito. Dehydrogenase activities were determined as described in the Materials and Methods section. Specific activity was defined as μmoles of NADPH formed/min./mg. of protein. The vertical broken lines delineate the pupal period. Each point and bar on the graph represents the mean $\pm$ S.E.M. of four to nine samples. Except for the 40-day point, the points without bars represent the mean of two to four samples.

Fig. 3. Isocitrate-dehydrogenase activity during the life-span of the mosquito. Dehydrogenase activities were determined as described in the Materials and Methods section. Specific activity was defined as μmoles of NADPH formed/min./mg. of protein. The vertical broken lines delineate the pupal period. Each point and bar on the graph represents the mean $\pm$ S.E.M. of 5–12 samples.

Table 2. *Distribution of NADP⁺-linked dehydrogenase activities in different body regions of the mosquito*

Larval samples were composed of 4- and 5-day-old mosquitoes and the adult samples of ages from 5 to 10 days. Each body region represented a pooled sample of 200 mosquitoes for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and 100 mosquitoes for isocitrate dehydrogenase. Methods of dissection and determination of activities are described in the Materials and Methods section.

Stage	Body region	Pentose phosphate-cycle enzymes						
		Protein (mg./ region)	Glucose 6-phosphate dehydrogenase		6-Phospho- gluconate dehydrogenase		Isocitrate dehydrogenase	
			% Total activity	% Specific activity	% Total activity	% Specific activity	Protein (mg./ region)	% Total activity
Larva	Head	1.6	34	88	17	19	1.1	1800
	Thorax	2.5	18	31	28	20	4.0	970
	Abdomen	5.4	48	37	55	19	5.9	1300
Adult	Head	0.68	3.8	150	2.0	34	0.68	4.7
	Thorax	5.6	22	210	45	190	5.6	35
	Abdomen	3.8	75	530	53	170	3.8	60

To eliminate starvation as a causative factor the enzyme activities were determined in larvae which had been starved and re-fed. Three-day larvae were removed from the culture medium, washed and placed in distilled water for 2 days. The organisms were then resuspended in culture medium. At daily intervals mosquito samples were removed and processed, and the soluble protein concentration and enzyme activities were determined (Fig. 4). In the starved organisms the enzyme activities remained at the pre-starvation value even though the protein content decreased 50%. Upon re-feeding, the glucose 6-phosphate-dehydrogenase and isocitrate-dehydrogenase activities and the protein concentration increased almost to the control values whereas the 6-phosphogluconate-dehydrogenase activity decreased slightly. Analogous starvation experiments were performed with adult organisms, but no changes in enzyme activity or protein concentration were observed either upon starvation or re-feeding.

DISCUSSION

The protein, RNA and DNA content per organism are biochemical parameters of cell mass or number. These parameters increased to maximal values in the larval stages of the mosquito and remained essentially constant from that time through the aged adult stage (Lang *et al.* 1965). Such results indicated that net growth occurs only in the larva and not in the adult. The adult stage is a period of aging as defined by Comfort (1964) as one of increased rate of mortality.

Although the increasing activities of pentose phosphate-cycle enzymes in the growing mosquito larva are in contrast with the constant activities found in tissues of newborn and adult rats, these results may not be strictly comparable because of the limited age span studied in the rat (Glock & McLean, 1954; Weber & Cantero, 1957; Burch *et al.* 1963). To our knowledge no information is available on isocitrate-dehydrogenase patterns during growth or on all three enzymes during aging.

The enzyme patterns in the pupal period were different from the U-shaped curves of the cytochrome *c* reductases observed in the mosquito (Lang, 1959) and may reflect a difference between mitochondrial and soluble enzymes.

During the early adult period the pentose phosphate-cycle enzyme activities increased to maxima at about 10 days and were similar to the increases in dry weight and cytochrome *c* content of flight muscles in other dipterans which occur at this time (Levenbook & Williams, 1956). However, the development of flight muscles does not explain the decrease in isocitrate-dehydrogenase activity that occurred during this period in the mosquito.

The marked decreases in NADP⁺-linked-dehydrogenase activities during aging can be interpreted in several ways. With glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase the fall in activity may indicate a decreased ability of the aged cell to synthesize ribose from glucose and result in an impaired synthesis of nucleic acid. Further, the lower isocitrate-dehydrogenase activity may indicate a decreased metabolism of the Krebs cycle in the aging organism.

Fig. 4. Effect of starvation on protein content and dehydrogenase activities in the larva. On the abscissa are plotted the days of larval life after their removal from the rearing medium. The last points for each curve represent larvae within 1 day of pupation. O, Control organisms; ●, starved organisms. The arrow after the second day indicates the point at which the starved organisms were re-fed. (a) Soluble protein/larva; (b) specific activity of glucose 6-phosphate dehydrogenase; (c) specific activity of 6-phosphogluconate dehydrogenase; (d) specific activity of isocitrate dehydrogenase.

The values reported were obtained with samples of whole organisms. This procedure is advantageous in growth and aging investigations because the results reflect a general phenomenon of all cells rather than of an isolated and perhaps atypical cell type. This view was supported by the evidence that changes in enzyme activity of different body regions also occurred with age.

The enzyme activities were determined under optimum assay conditions to ensure that the amount of enzyme present would be the limiting factor. Further, the assay of combined samples of high and low activities gave additive results indicating the absence of activators and inhibitors. It is pertinent that the percentage protein content was essentially constant in these samples (Lang *et al.* 1965), and the specific enzyme activity was expressed per mg. of protein. For these reasons it

was concluded that the changes in enzyme activity represent changes in apoenzyme concentration.

Investigation of assay conditions also revealed differences between the enzyme systems of mosquitoes and of mammals or bacteria. These differences include a lower pH optimum for 6-phosphogluconate dehydrogenase, the localization of NADP⁺-specific isocitrate-dehydrogenase activity in the soluble fraction, and the absence of NAD⁺-linked isocitrate dehydrogenase from the mosquito.

Starvation can alter some enzyme activities, and a decrease in glucose 6-phosphate-dehydrogenase activity in rat liver with starvation and a 100-fold increase upon re-feeding have been reported (Potter & Ono, 1961; Tepperman & Tepperman, 1963). Such changes were not found in the mosquito, indicating that the changes in specific activity with age were not manifestations of starvation conditions.

The results of these experiments indicated that increasing activities of NADP⁺-linked enzymes coincided with the period of cellular growth, thereby supporting the hypothesis that NADPH concentration is high during periods of greatest biosynthetic activity. In addition, the decreases in NADP⁺-linked dehydrogenases during the aging period indicated that the capacity for biosynthesis in aged organisms is decreased.

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