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*RESPIRATORY ENZYME CHANGES DURING FROG EMBRYOGENESIS**

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Marked differential changes in several respiratory enzyme activities have been demonstrated in the mosquito during its post-embryonic life span.[1] Of particular interest was the finding that the ratio of DPNH-cytochrome *c* reductase to TPNH-cytochrome *c* reductase was about 2 during the growing stages and increased to over 20 in the adult stage. This latter ratio was the result of both an increase in the DPNH-linked enzyme and a decrease in the TPNH-reductase activities. We were curious as to whether similar changes in these enzyme patterns occur during frog embryogenesis and are related to known physiological and morphological events.

Many studies have been reported on respiration rates of whole amphibian embryos and brei preparations as measured by oxygen uptake and individual enzymes. To our knowledge, however, the activities of several respiratory enzymes during embryogenesis have not been measured in the same sample. To this end the activities of DPNH-, TPNH-, and succino-cytochrome *c* reductases and cytochrome oxidase were determined in frog embryos of different developmental stages.

Materials and Methods.—Eggs were obtained from *Rana pipiens* females by the usual method of pituitary injection.[2] Fertilized eggs, in groups of approximately 100, were placed in finger bowls containing charcoal-treated tap water and allowed to develop at 17–19°C. At various time intervals, samples were taken, and the jelly was removed. Embryos of stages 1–15 (Shumway)[3] were used with the vitelline membrane intact, but this membrane and perivitelline fluid were removed from embryos of stages 16–25. The above procedure was replicated using several batches of eggs obtained from eight different frogs. In each experiment samples from early, middle, and late stages of development were analyzed.

Ten embryos were homogenized in 1.0 ml of 0.25 *M* sucrose using a loose-fitting Ten Broeck homogenizer, and the homogenate was centrifuged at 600 × *g* for 10 min. The resultant residue was washed twice with sucrose, and the washings together with the original supernatant fraction were saved for analysis. The above sample preparation was carried out at 0–4°C and kept at this temperature until assayed. Microscopic examination indicated that a few homogenates (usually of stages 20–25) contained a small number of whole cells, and all preparations were completely free of yolk granules.

Each sample was assayed for the different enzymatic activities and protein content. The cytochrome *c* reductases were determined spectrophotometrically at 23–25°C using the method of Lehman and Nason,[4] and the reduction of cytochrome

c measured at 550 mμ in the presence of DPNH, TPNH, or sodium succinate was used as a measure of the different reductases. Cytochrome oxidase was assayed by the method of Cooperstein and Lazarow;[5] cyanide controls were run for each sample. A unit of both reductase and oxidase activity was defined as a change of 0.001 optical density units at 550 mμ per min, and the initial rate of change was determined and was proportional to protein concentration determined by the method of Lowry *et al.*[6] Conditions of substrate saturation were used in the enzyme assays, and all results were corrected for the endogenous rate which was generally less than 20% of the total activity. Cytochrome c and the reduced pyridine nucleotides were of greater than 90% purity and obtained from the Sigma Chemical Company.

Results.—Since the enzymatic activities are expressed in terms of milligram protein, it was necessary to determine the protein content of the samples during development. The data in Figure 1 indicate that the protein content, 410 ± 15

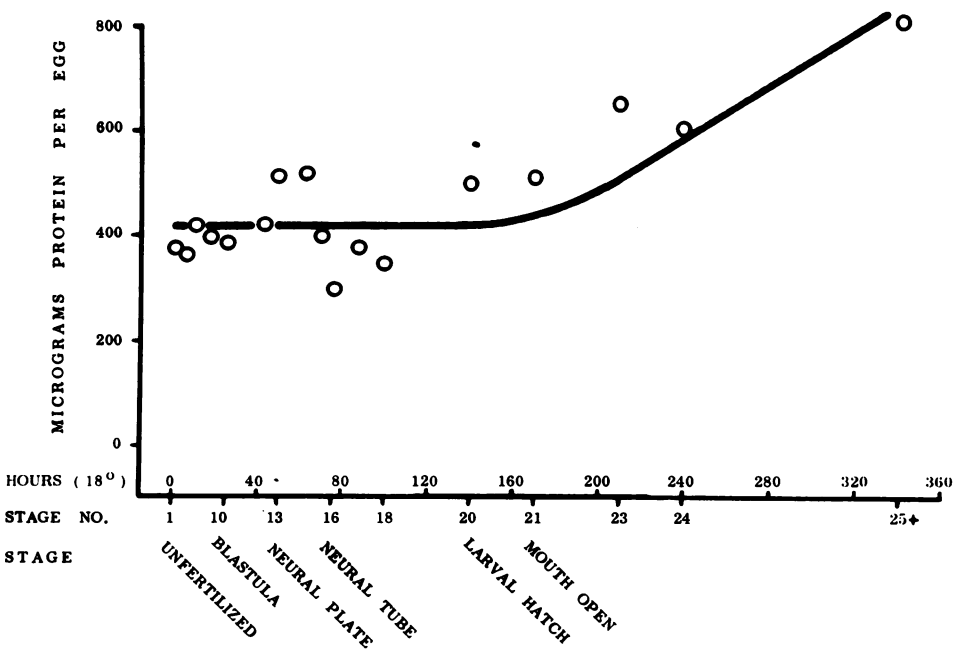

FIG. 1.—Protein concentration during frog embryogenesis. Method of analysis is given in the text. Each point represents the average value of 3–6 samples with the exception of stages 24 and 25 in which only one sample each was analysed.

mcg per embryo, is constant until hatching. From that time through stage 25 the content increases. Consequently differences in specific activity during embryogenesis are due to changes in enzymatic activity rather than protein concentration. Since the protein level is constant, the index, units per embryo, can also be used as an expression of specific activity.

The reductase activities are presented in Figure 2. DPNH-cytochrome c reductase was high in the unfertilized egg and in the early embryonic stages, declining slightly from stages 1 to 14. From stages 15 through 18, however, the activity decreased 75 per cent and continued at this low level until hatching, stage 21.

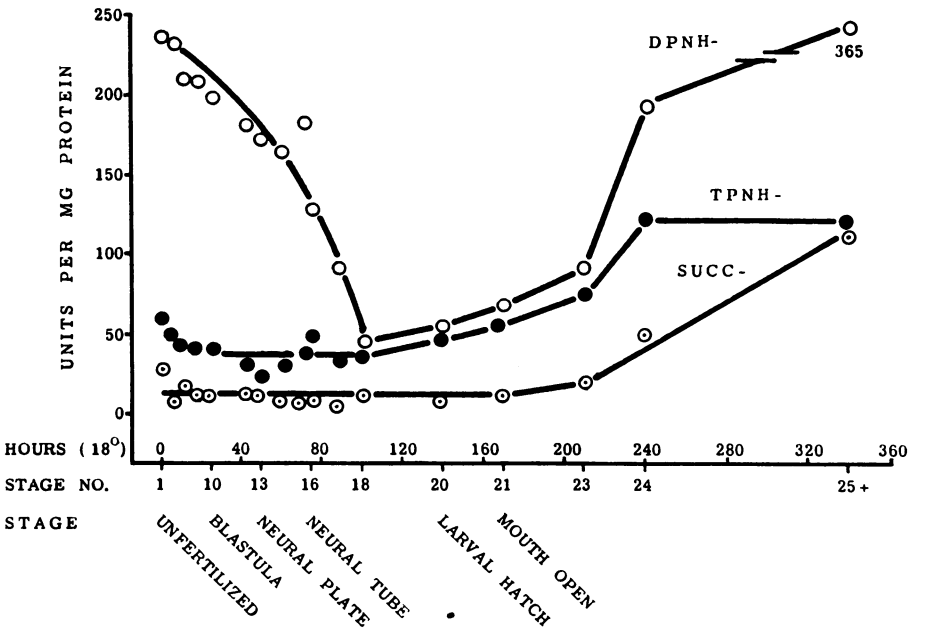

FIG. 2.—DPNH-, TPNH-, and succino-cytochrome *c* reductase activities during frog embryogenesis. Methods of analysis are given in the text. Each point represents the average value of 3–6 samples with the exception of stages 24 and 25 in which only one sample each was analysed.

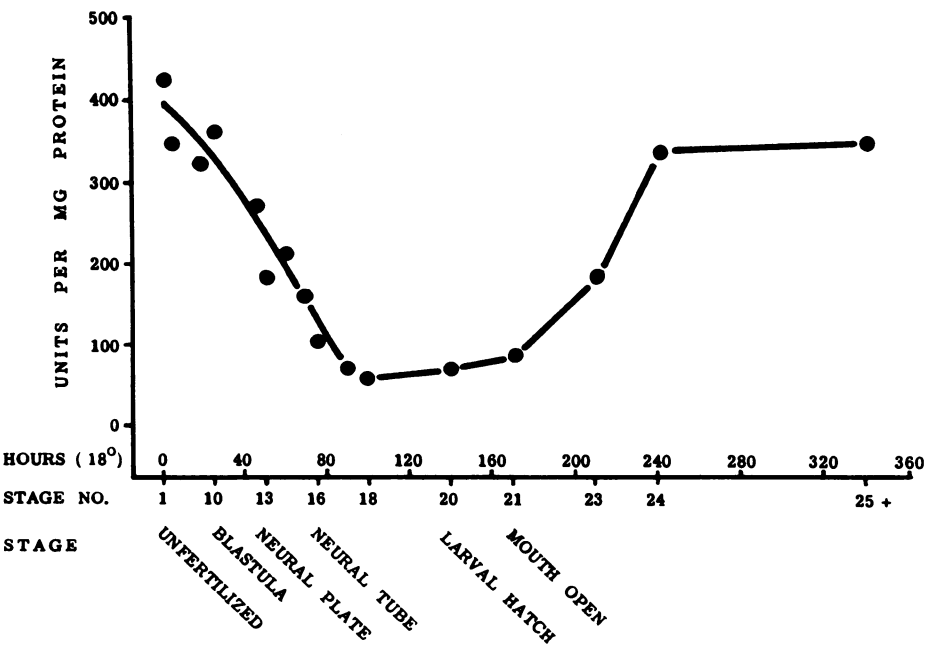

FIG. 3.—Cytochrome oxidase activity during frog embryogenesis. Methods of analysis are given in the text. Each point represents the average value of 2–6 samples with the exception of stages 24 and 25, in which only one sample each was analysed.

Henceforth the activity increased and at stage 25 was higher than the initial value. A similar pattern was observed for cytochrome oxidase as shown in Figure 3, except that the increase in post-embryonic stages was less. The marked decreases in activity of these two enzymes during neurulation (stages 15–18) were observed consistently in separate batches of eggs from five different females.

The TPNH-cytochrome *c* reductase level was lower than that of the DPNH-enzyme and remained constant until the time of hatching, when it increased almost threefold at stages 24 and 25. Succino-cytochrome *c* reductase activity was barely detectable during embryogenesis but likewise increased after hatching.

In view of the variation observed between batches of eggs, the validity of the slight decrease in DPNH-cytochrome *c* reductase and cytochrome oxidase during early embryogenesis was questioned. The data for these two enzymes were grouped as shown in Table 1 and analyzed by standard statistical procedures. It was found

TABLE 1

CYTOCHROME REDUCTASE AND OXIDASE DURING FROG EMBRYOGENESIS

Stage	Stage (no.)	Age (hr)	No. of samples	DPNH-cyt. *c* reductase (Units/mg. protein)	Cytochrome oxidase (Units/mg. protein)
Unfertilized: mid-gastrula	1–11	0–35	23	221 ± 13.0*	366 ± 15.2
Late gastrula: rotation	12–15	37–75	16	172 ± 10.0	208 ± 15.2
Neural tube and tail bud	16–17	72–94	9	117 ± 24.4	94.4 ± 13.3
Muscular response: hatching	18–21	93–173	12	54.8 ± 4.69	70.9 ± 8.25
Larval	22–23	192–216	5	87.0 ± 13.4	179 ± 50.3
	24	240	1	190	334
	25	334	1	365	344

* Mean, ± standard error of the mean except stages 24 and 25.

that the initial DPNH-cytochrome *c* reductase level up through gastrulation (stages 1–11) is different from that in stage 12–15 ($P < 0.01$) and in stages 16–17 or 18–21 ($P < 0.001$). Similarly the cytochrome oxidase concentration in the same initial stages is significantly decreased in the subsequent stages (all having a $P < 0.001$). These data demonstrate, therefore, that these two enzymatic activities decrease slightly during stages 1–15 and markedly during stages 15–18.

The relative activities of DPNH-cytochrome *c* reductase and cytochrome oxidase in reference to TPNH-cytochrome *c* reductase are shown in Table 2. During early development until neural tube closure (stages 1–15) the DPNH-reductase activity is approximately 5 to 6 times greater than the TPNH-reductase activity. During neural tube closure and tail bud formation (stages 16 and 17), the DPNH-reductase

TABLE 2

RELATIVE CYTOCHROME REDUCTASE AND OXIDASE ACTIVITIES DURING FROG EMBRYOGENESIS

Stage	Stage no.	Age (hr)	Relative Activity*: DPNH-cyt. *c* reductase	Relative Activity*: TPNH-cyt. *c* reductase	Relative Activity*: Cytochrome oxidase
Unfertilized: mid-gastrula	1–11	0–35	5.01	1.00	9.25
Late gastrula: rotation	12–15	37–75	6.30	1.00	8.22
Neural tube and tail bud	16–17	72–94	2.68	1.00	2.17
Muscular response: hatching	18–21	93–173	1.18	1.00	1.53
Larval	22–24	192–240	1.25	1.00	2.55
	25	334	3.04	1.00	2.86

* Data represent averages of 6–23 samples per group except for stage 25 which is a single sample.

was only 2 to 3-fold greater, and from tail bud until post hatching (stages 18–24), the two reductase activities were approximately the same. Although an increase in DPNH-reductase activity is shown at stage 25, its significance is questionable since only one determination was made. The relative activity of cytochrome oxidase changed in a similar manner. It is of interest that succino-cytochrome *c* reductase was less than 10–30 per cent of the TPNH-reductase activity throughout development.

Factors affecting enzymatic activities: The observed changes in enzymatic activity may be attributable to various factors. In addition to obvious changes in apoenzyme content, differences in the intracellular distribution and structural organization may alter the measurable activity. It is also possible that activators and inhibitors may be present and will affect activity in a manner similar to that due to changes in amount of enzyme. A series of experiments was undertaken in an attempt to resolve some of the more obvious factors which may be responsible for the observed activity changes. In these experiments samples of high activity (stages 12 and 13) were compared with those of low activity (stages 17 and 18).

The reductase activities were unaffected by homogenization in water, 0.25 *M* sucrose, or 0.50 *M* sucrose; or by the addition of the surfactant, Triton X-100. However, freeze-thaw treatment of homogenate and subcellular fractions resulted in a lowering of reductase activities, and storage of the homogenate at 0°C for 30 hr or 23°C for 4 hr produced a 60 per cent reduction of DPNH- and TPNH-cytochrome *c* reductase activities.

To determine whether activators or inhibitors were present, homogenates prepared from stages of high and of low activity were combined and compared with their individual activities. The results revealed that the mixture expresses a summation of the individual activities. Since treatment of these samples by techniques designed to demonstrate the influence of structural organization, latency, and activators or inhibitors were ineffectual, the data suggest that changes in apoenzyme content are responsible for the activity changes.

Distribution of activity in the embryo: One objective of the localization experiments was to determine a possible relationship between the marked decrease in enzymatic activity and the morphological change occurring during neurulation. In addition, information on the distribution of activity in the embryo would be obtained. Samples of embryos of stages 13, 16, and 18, which include the period of neurulation, were dissected as follows: The dorsal region was separated from the ventral, yolk-filled area, and both sections of the same eggs were compared to whole eggs. The dorsal region of stage 13 consisted primarily of the neural plate whereas the dorsal section of stages 16 and 18 included head, neural tube, and somites. Each sample was analyzed for DPNH- and TPNH-reductase activities. The results are shown in Table 3.

The specific activities (units per milligram protein) of both enzymes were higher in the dorsal regions of all stages than in either the ventral section or the whole embryo. This greater concentration of activity is consistent with the fact that at this developmental stage the most active portion of the embryo from a morphogenetic viewpoint is the dorsal region which contains the neural tissue. However, the enzymatic activities found in each region (units per embryo) are approximately equal and indicate that the low specific activity found in the ventral areas is due

TABLE 3

DISTRIBUTION OF CYTOCHROME *c* REDUCTASES IN THE FROG EMBRYO

Stage no.	Sample	Cytochrome *c* reductases			
		DPNH- (Units/mg. protein)	TPNH- (Units/mg. protein)	DPNH- (Units/embryo)	TPNH- (Units/embryo)
13	Whole	67	14	56	12
	Dorsal region* (neural plate)	137	.91	19	11
	Ventral region*	103	30	24	6
16	Whole	28	20	22	16
	Dorsal region (neural tube and brain)	98	60	12	7
	Ventral region	32	25	11	9
18	Whole	14	7	12	4
	Dorsal region (neural tube, head, and somites)	96	36	11	4
	Ventral region	36	0	7	0

* Vitelline membranes and perivitelline fluids were removed.

to a higher protein content rather than lower activity. Since the ventral region has a greater concentration of yolk, the high protein content in this region may be due to soluble yolk protein which would be included in our sample preparations. It is possible, therefore, that the concentration of enzymatic activity per milligram protein of completely yolk-free embryos may be actually the same.

Intracellular localization: The intracellular distribution of the reductases was determined in stages 12, 13, and 18. Differential centrifugation procedures using 0.25 *M* sucrose were employed to obtain nuclear, mitochondrial, microsomal, and supernatant fractions. These data, in general, reflect a distribution common to that reported for mammalian tissues; namely, DPNH- and succino-cytochrome *c* reductase were concentrated in the mitochondrial fraction, and TPNH-cytochrome *c* reductase, in the microsomal fraction. The data are insufficient to evaluate any changes in distribution during development.

Discussion.—Embryonic development cannot be defined easily by usual biochemical parameters. Deoxyribonucleic acid content and cell number, for example, are described by an increasing curve of exponential nature.[7] On the other hand the total nitrogen[8] and protein concentration remains constant during frog embryogenesis. Our data on DPNH-cytochrome *c* reductase and cytochrome oxidase activities in terms of units per milligram protein present still another pattern. This activity curve decreases during the early developmental stages, falls markedly during neurulation, and remains at a low level until larval emergence when the activity increases and attains a magnitude at least as high as the initial values. This decrease in two enzymes of metabolic importance is particularly unusual, for the general pattern of enzyme development during embryogenesis is an increasing curve. This emphasizes the possible importance of losses as well as gains in enzymatic activity as a phenomenon of embryonic differentiation.

Previous studies of respiratory enzymes in the amphibian embryo have been concerned primarily with cytochrome oxidase and succinoxidase. Since succinoxidase is an over-all measurement of an electron transport pathway in contrast to succino-cytochrome *c* reductase as determined in this study, comparison is difficult. Spiegelman and Steinbach[9] found that cytochrome oxidase was constant throughout development of *R. pipiens*, whereas Boell and Weber[10] observed an increasing activity in *Xenopus laevis*. This discrepancy may be due to differences in experimental procedure or biological species. Although both groups used manometric

methods of assay with *p*-phenylenediamine and cytochrome *c* as substrates, the former prepared their samples at temperatures "below 25°C" and assayed at pH 6.5. No data were presented to indicate the validity of these conditions.

Our cytochrome oxidase data which describe a different pattern from either of the forementioned, may perhaps also be explained by differences in assay method. The microspectrophotometric method employed by us is sensitive, utilizes a non-autoxidizable and naturally occurring substrate, has a small endogenous rate correction, and requires less than two minutes' exposure of the samples to room temperature. This procedure enabled the rapid determination of initial oxidation rates and avoided the possible inactivating effects of incubation at 25° or higher, an intrinsic characteristic of the manometric methods used above. Our own data on other tissues, as well as that of others,[11] indicate that cytochrome oxidase activity is rapidly destroyed in less than one hour at 25°, but is stable for at least six hours at 0°.

In order to measure total activity of the various enzymes, which are localized in different intracellular fractions, homogenates instead of mitochondrial preparations were studied. However, these homogenates were cleared of cell wall debris, nuclei, and yolk granules to remove as much metabolically inactive material as possible. Preliminary experiments comparing cleared homogenates with dissected components of the egg, such as the jelly coat and perivitelline membranes and fluid, and with whole egg homogenates indicated little if any difference in activity.

One objective of this investigation was to examine the relative contribution of several individual respiratory enzymes to electron transport. According to the results, the main pathway appears to be via DPNH-cytochrome *c* reductase and cytochrome oxidase, for the activities of TPNH- and succino-reductase are very low throughout embryogenesis. The rate-limiting step under our assay conditions of substrate saturation is probably between DPNH and cytochrome *c* except during stages 16 and 17 (see Table 1) when the DPNH-reductase and cytochrome oxidase activities are approximately equal. We were unable to demonstrate pyridine nucleotide transhydrogenase activity using depleted mitochondria or DPN-analogues.

The U-shaped enzyme curves described by DPNH-cytochrome *c* reductase and cytochrome oxidase in Figures 2 and 3 are analogous to those found in our earlier experiments on the mosquito pupa. This metamorphic insect stage is a period of active differentiation and has some other similarities to the frog embryo. Although a correlation between the ratio of DPNH- to TPNH-reductase and growth stages was observed in the mosquito, the significance of the ratio changes in regard to the various differentiation processes of frog embryogenesis is difficult to interpret.

The significant and considerable decrease in DPNH-cytochrome *c* reductase and cytochrome oxidase activities during neurulation is of particular interest. It could be assumed on the basis of the failure to demonstrate latency or inhibitors and activators and the presence of excess amounts of substrates and cofactors in the assay medium that the observed activities are due to changes in apoenzyme concentration. Although such a conclusion requires some temerity and may be proved to be fallacious upon further investigation, it seems reasonable at the present time.

The decreases correlate very well with the fall in oxygen consumption of *R. pipiens* breis during the same developmental period as described by Spiegelman

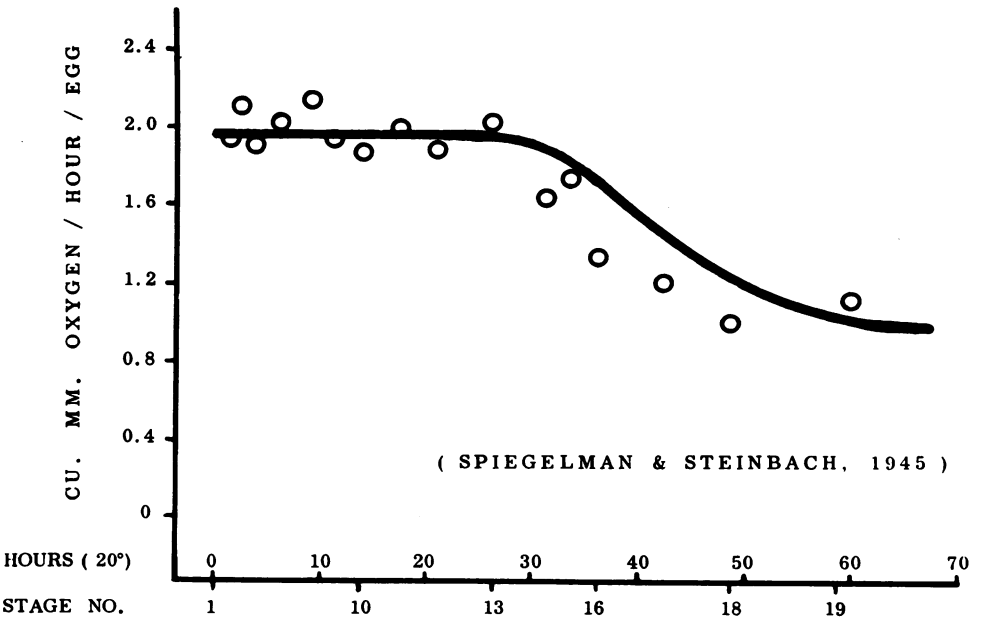

FIG. 4.—Respiration rates during frog embryogenesis. The data of Spiegelman and Steinbach[9] have been replotted but are otherwise unaltered.

and Steinbach[9] and shown in Figure 4. In their experiments the frog embryos were incubated at a temperature the same as ours. Our findings may provide a partial explanation for the diminished oxygen uptake. A different oxygen consumption curve was observed by Gregg and Ray[12] who used embryos incubated at temperatures ranging from 8–25°C. Their curve indicated a low oxygen-consumption rate during early embryogenesis and might be explained by the data of Hopkins and Handford,[13] which demonstrated that oxygen uptake is reduced markedly in cold-reared early embryos.

According to the present results, the enzymatic pathways for producing energy by means of electron transport and oxidative phosphorylation are at a low level during later embryogenesis. It is interesting to note, however, that this low activity appears to be concentrated in the dorsal region, which is a morphogenetically active center during this period of neural tube closure and initiation of muscular activity. Whether or not the decrease in energy-producing enzymes can be interpreted as a diminished requirement for energy during this embryonic period is an open question.

The results presented are of a preliminary nature. Elucidation of the biochemical mechanisms involved must await further experimental inquiry.

Summary.—The enzymatic activities of DPNH-, TPNH-, and succino-cytochrome *c* reductases and cytochrome oxidase were determined in homogenates of *Rana pipiens* of different stages of embryonic and early larval development. Similar U-shaped activity curves were observed for DPNH-cytochrome *c* reductase and cytochrome oxidase, and the lowest activity levels occurred during the period from neurulation to larval hatching. These changes coincide with oxygen consumption data obtained by others. TPNH- and succino-cytochrome *c* reductase

activities were low throughout embryogenesis and increased after larval hatching. Other results describe the localization of the different activities and provide presumptive evidence that the activity differences are due to changes in apoenzyme concentration. The possible significance of these findings is discussed.

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The following abbreviations are used: DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

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