Respiratory Enzymes in the Heart and Liver of the Prenatal and Postnatal Rat

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

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1. Heart and liver tissue samples were obtained from rats in various developmental stages from the 12-day-old embryo to the 120-day-old postnatal animal. 2. The body, heart and liver weights and percentage protein in the liver and heart of the prenatal and postnatal rat were determined. 3. The activities of NADH–, NADPH– and succinate–cytochrome *c* reductases and cytochrome oxidase were determined also. 4. The specific activities of all the enzymes increased in both heart and liver during late foetal development (16 days to term). The NADH– and succinate–cytochrome *c*-reductase activities in the heart increased threefold during the neonatal period (0 to 25 days *post partum*) and then remained constant to 120 days. All reductase activities increased in the liver three- to six-fold during the neonatal period. Cytochrome-oxidase activity in both tissues increased sixfold during this time but plateaued in the liver at 12 days rather than 25 days. 5. A sex difference was observed in NADH–cytochrome *c*-reductase activity in the liver. Up to 25 days *post partum* the activity was the same in both sexes, but from that time on the activity continued to increase in the female but remained unchanged in the male. 6. NADPH–cytochrome *c*-reductase activity increased only in the liver. 7. These results indicate that different electron-transport pathways predominate according to the tissue, developmental stage and sex of the animal.

The morphological and physiological changes that occur during mammalian development are better known than the biochemical and molecular events that effect these changes. Some of the biochemical changes and correlations between enzymic activity and developmental status have been reviewed by Moog (1952), Knox, Auerbach, & Lin (1956), Flexner (1955) and Herrmann & Tootle (1964).

In the present study, attention has been centred on the electron-transport system that plays a fundamental metabolic role in producing ATP required by many enzymic processes. Different pathways of electron transport have been described and have different capabilities in the production of ATP. The P/O ratios are: for the NADH pathway, 3:1; for the succinate pathway, 2:1; for the NADPH pathway, 0–1:1 (Kaplan, Swartz, Frech & Ciotti, 1956; Potter, 1958; Vignais & Vignais, 1957). Since oxidative phosphorylation occurs at a relatively low level if at all in the NADPH pathway, the primary role of the NADPH electron-transport scheme may be as a $NADP^+$-generating system or recycling step to provide $NADP^+$ for other enzyme systems. NADPH is required for reductive steps in a number of biosynthetic processes. For this reason high concentrations of these enzymes may be present during development (Horecker & Hiatt, 1958*a*, *b*). On the basis of the foregoing data it was postulated that the pattern of respiratory enzymes representative of these three pathways and hence the energy-producing capacity would vary with the developmental stage of the organism.

Studies of the activities of electron-transport enzymes during embryonic and postnatal development have been restricted mainly to succinoxidase and cytochrome oxidase in the chick and amphibian embryo (Sippel, 1954; Boell, 1955; Davidson, 1957; Herrmann & Tootle, 1964) and in the mammal (Potter, Schneider & Liebl, 1945; Flexner, 1955; Shen, 1955; Dawkins, 1959; Richter, 1961). In none of these investigations were the experiments designed to study enzymes representing several pathways of electron transport, and usually the age span of these studies was limited. Previous investigations on the frog embryo and the postembryonic mosquito described different profiles for several electron-transport enzymes during embryogenesis and over the entire life span (Lang & Grant, 1961; Lang, 1959).

In the present study the activities of NADH–, NADPH– and succinate–cytochrome *c*-reductases and of cytochrome oxidase were determined in

heart and liver tissues obtained from rats ranging in age from the 12-day-old embryo to the 120-day-old postnatal animal. The purpose was to determine the enzyme patterns during embryogenesis and postnatal development and to compare the alternative pathways of electron transport represented by these enzymes. Two functionally different types of tissue, heart and liver, which differ in their biosynthetic capacity and contractile activity, were also studied.

MATERIALS AND METHODS

Animals. Sprague–Dawley rats from the colony at this Department were used in all experiments. The foetal age was estimated from the time of conception and was accurate to within 12hr. The design of daily experiments included animals from two or more age groups. Each animal was killed by cervical dislocation, the head removed and the animal exsanguinated. The liver and heart were excised rapidly and immediately rinsed in ice-cold 0·25M-sucrose. Heart tissue was dissected free of the atria, valves and connective tissue. The fresh weight of blotted tissue was determined. All homogenates were prepared from single organs, except for the samples obtained from 12-day-old and 15-day-old rat foetuses in which 12–15 organs were pooled to obtain sufficient amounts for analysis.

Homogenization procedure. The tissue homogenates (10%, w/v) were prepared in 0·25M-sucrose in an all-glass Ten-Broeck homogenizer. Samples were kept at 0° throughout sample preparation.

Enzyme assays. Each sample was assayed for the different enzymic activities and for protein content. The cytochrome *c* reductases were determined spectrophotometrically at 23–25° by the method of Lehman & Nason (1956); the rate of cytochrome *c* reduction measured at 550mμ in the presence of NADH, NADPH or sodium succinate was used as a measure of the different reductases. Cytochrome oxidase was assayed by the method of Cooperstein & Lazarow (1951); cyanide controls were run for each sample. Enzymic activity was expressed as the mμmoles of cytochrome *c* reduced or oxidized at 550mμ/min. based on the millimolar extinction coefficient of 27·7 (Margoliash, 1954). The initial rate of change was determined and was proportional to protein concentration determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Specific activity was expressed as enzymic activity/mg. of protein. Conditions of substrate saturation were used in the reductase assays, and all results were corrected for the endogenous rate, which was less than 20% of the total rate. The spectrophotometric measurements were carried out in a Zeiss PMQII spectrophotometer.

Chemicals. Cytochrome *c*, NADH and NADPH were of greater than 90% purity (Sigma Chemical Co., St Louis, Mo., U.S.A.). Solutions of NADH and NADPH were prepared fresh before use (Lehman & Nason, 1956). Other chemicals were of reagent grade.

The results were analysed by standard statistical procedures (Snedecor, 1946).

RESULTS

Weight changes. The body, liver and heart weights of the prenatal rat are presented in Figs. 1 and 2. An appreciable increase in body weight did not occur until after the fifteenth day of gestation. From that time to birth the body weight increased over tenfold. A similar pattern of increase of the same magnitude was observed in the heart and liver.

In the postnatal rat similar increases were observed in body, heart and liver weights, and maximum values were attained about 75 days *post partum* (Figs. 3, 4 and 5). The increase during this period was greater than 20-fold in all cases. A sex difference was observed, for the body weights and tissue weights of the male were significantly

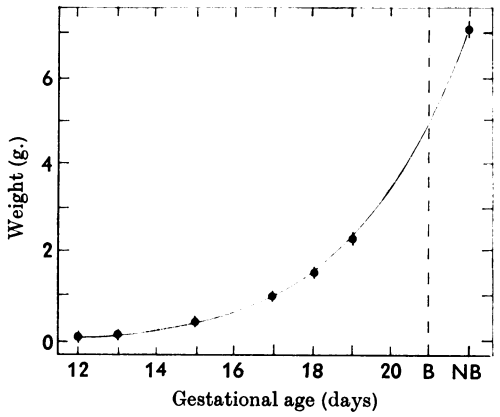

Fig. 1. Body weight of the prenatal rat. Each point and bar on the graph represents the mean ± S.E.M. of 3–5 samples. The vertical broken line at B indicates the time of birth; NB stands for newborn animals less than 24hr. old.

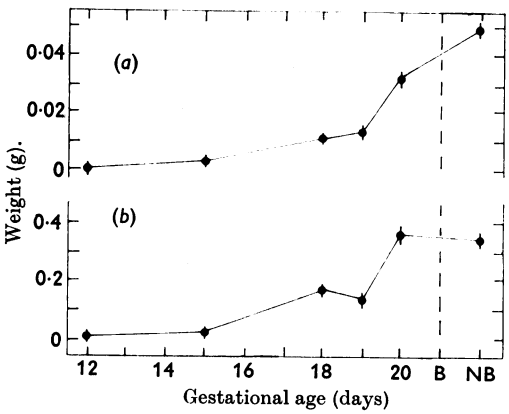

Fig. 2. Heart and liver weights of the prenatal rat: (*a*) heart; (*b*) liver. Each point with bar represents the mean ± S.E.M. of 3–8 samples. The vertical broken line at B indicates the time of birth; NB stands for newborn animals less than 24hr. old. Experimental details are given in the text.

greater than the female. This difference became apparent at about 50 days of age.

Protein concentration. In the prenatal rat the protein content based on wet weight in the liver and in the heart was constant. The mean values ± S.E.M. were: for the liver, 7·14 ± 0·61%; for the heart, 4·63 ± 0·43%. In the postnatal rat higher values for both tissues were found, and the values were constant throughout the postnatal period. The protein content in the liver was 16·9 ± 1·2% and in the heart was 11·1 ± 0·59%.

Enzyme patterns in the prenatal rat. The patterns of cytochrome *c*-reductase activities in the heart are presented in Fig. 6. Although there appeared to be a slight increase from the twelfth day to birth, the increase was less than threefold and was not significant statistically. The activity of NADH–cytochrome *c* reductase was sixfold greater than that of both the succinate and NADPH enzymes, which were the same.

A different pattern was observed in the liver during the same period (Fig. 7). A significant threefold

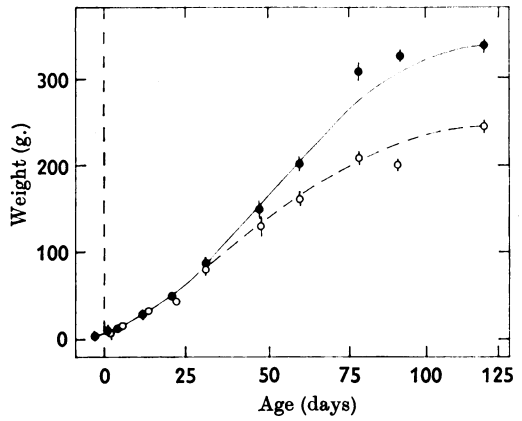

Fig. 3. Body weight of the postnatal rat: ●, male; ○, female. Each point with bar represents the mean ± S.E.M. of 3–12 samples; points without bars represent averages of two samples. Experimental details are given in the text.

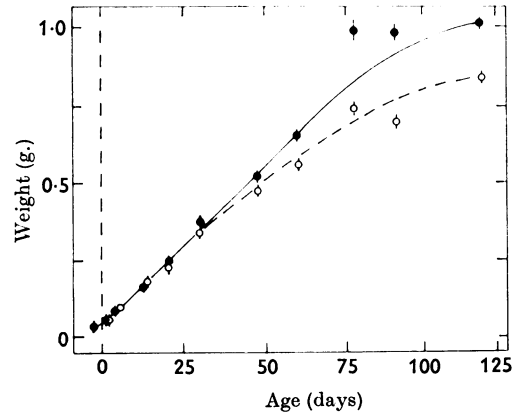

Fig. 4. Heart weight of the postnatal rat: ●, male; ○, female. Each point with bar represents the mean ± S.E.M. of 3–14 samples; points without bars represent averages of two samples. Experimental details are given in the text.

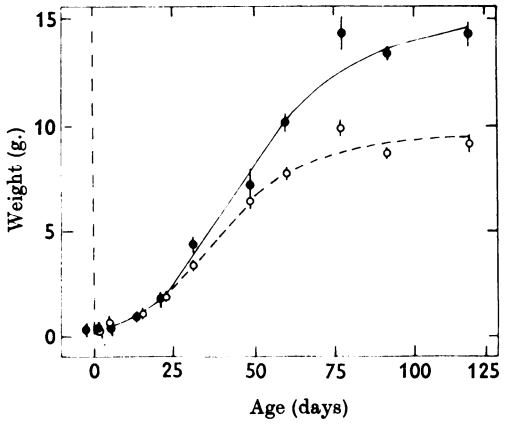

Fig. 5. Liver weight of the postnatal rat: ●, male; ○, female. Each point with bar represents the mean ± S.E.M. of 3–14 samples. Experimental details are given in the text.

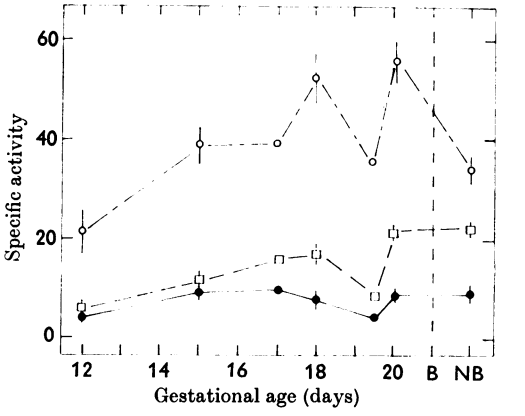

Fig. 6. Cytochrome *c*-reductase activities in the heart of the prenatal rat: ○, NADH–cytochrome *c* reductase; □, succinate–cytochrome *c* reductase; ●, NADPH–cytochrome *c* reductase. Specific activity is given as mμmoles of cytochrome *c* reduced/min./mg. of protein. Each point with bar represents the mean ± S.E.M. of 3–6 samples; points without bars represent averages of two samples. The vertical broken line at B indicates the time of birth; NB stands for newborn animals less than 24 hr. old. Experimental details are given in the text.

increase was observed in NADH–cytochrome *c*-reductase activity between day 18 and day 19. A slight increase was observed in succinate–cytochrome *c*-reductase activity at this time also. Again the specific activity of the NADH enzyme was five- to six-fold higher than that of the other two enzymes.

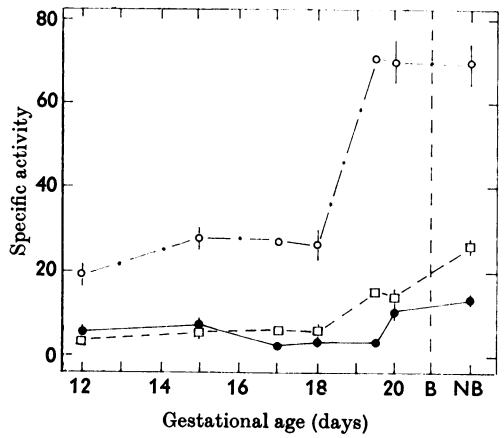

Fig. 7. Cytochrome *c*-reductase activities in the liver of the prenatal rat: ○, NADH–cytochrome *c* reductase; □, succinate–cytochrome *c*-reductase; ●, NADPH–cytochrome *c* reductase. Specific activity is given as mμmoles of cytochrome *c* reduced/min./mg. of protein. Each point with bar represents the mean ± S.E.M. of 3–8 samples; points without bars represent averages of two samples. The vertical broken line at B indicates the time of birth; NB stands for newborn animals less than 24 hr. old. Experimental details are given in the text.

Cytochrome-oxidase activity patterns in both heart and liver tissues are presented in Fig. 8. In the heart a peak of activity was observed on day 18 with a decrease in activity to less than half on day 19. A similar but less prominent peak of activity was observed in the liver at this time. Cytochrome-oxidase activity in the liver increased over twofold during the period between the 20-day foetus and newborn animal.

Enzyme patterns in the postnatal rat. In the heart during the first 25 days of postnatal development the activities of both NADH– and succinate–cytochrome *c*-reductase activities increased three-fold to a maximum. NADPH–cytochrome *c*-reductase activity was very low during the 120 days of this investigation (Fig. 9).

A sex difference was observed in the liver NADH–cytochrome *c*-reductase activity (Fig. 10). In both sexes the activity increased fourfold during the first 25 days. However, from 25 to 120 days only a slight increase in males occurred, whereas a two- to three-fold increase in females was observed.

In Fig. 11 are presented the results for succinate– and NADPH–cytochrome *c*-reductase activities in the liver. A three- to four-fold increase in activity was again observed during the 0–25 day period, and the activity remained at this level through to 120

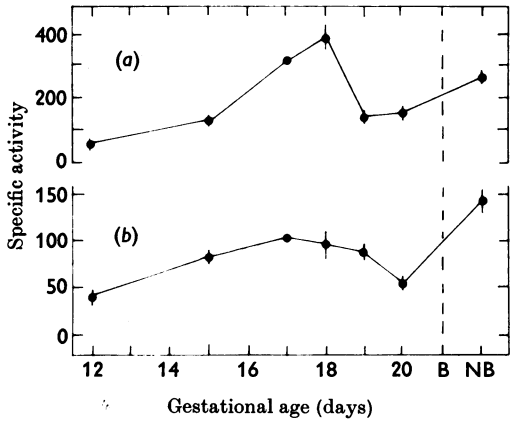

Fig. 8. Cytochrome-oxidase activities in the heart and liver of the prenatal rat: (*a*) heart; (*b*) liver. Specific activity is given as mμmoles of cytochrome *c* oxidized/min./mg. of protein. Each point with bar represents the mean ± S.E.M. of 3–9 samples; points without bars represent averages of two samples. The vertical broken line at B indicates the time of birth; NB stands for newborn animals less than 24 hr. old. Experimental details are given in the text.

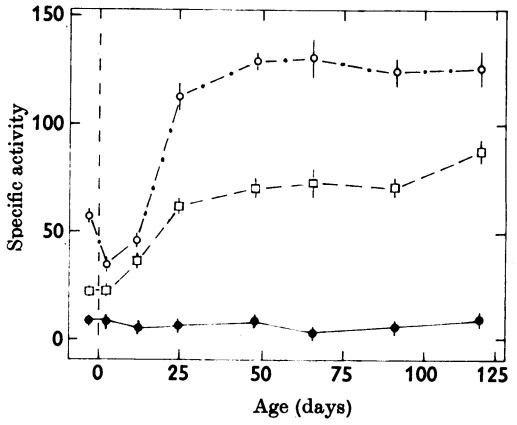

Fig. 9. Cytochrome *c*-reductase activities in the heart of the postnatal rat: ○, NADH–cytochrome *c* reductase; □, succinate–cytochrome *c*-reductase; ●, NADPH–cytochrome *c* reductase. Specific activity is given as mμmoles of cytochrome *c* reduced/min./mg. of protein. Each point with bar represents the mean ± S.E.M. of 6–10 samples. Experimental details are given in the text.

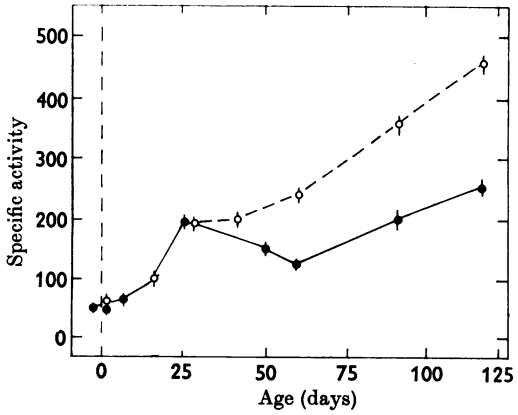

Fig. 10. NADH–cytochrome *c*-reductase activity in the liver of the postnatal rat: ○, female; ●, male. Specific activity is given as mμmoles of cytochrome *c* reduced/min./mg. of protein. Each point represents the mean ± S.E.M. of 3–5 samples. Experimental details are given in the text.

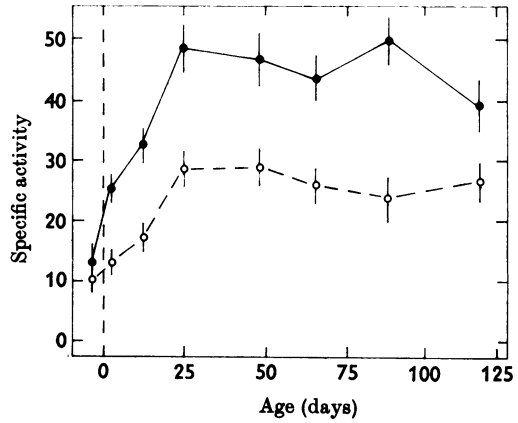

Fig. 11. NADPH– and succinate–cytochrome *c*-reductase activities in the liver of the postnatal rat: ●, succinate–cytochrome *c*-reductase; ○, NADPH–cytochrome *c* reductase. Specific activity is given as mμmoles of cytochrome *c* reduced/min./mg. of protein. Each point with bar represents the mean ± S.E.M. of 3–5 samples. Experimental details are given in the text.

days. The activity of the NADH–cytochrome *c* reductase at 25 days (Fig. 10) was about four times that of the succinate enzyme, which in turn was twice that of the NADPH enzyme. NADPH–cytochrome *c*-reductase activity in the liver was three times that in the heart.

The profiles of cytochrome-oxidase activity in the heart and liver are presented in Fig. 12. A five- to

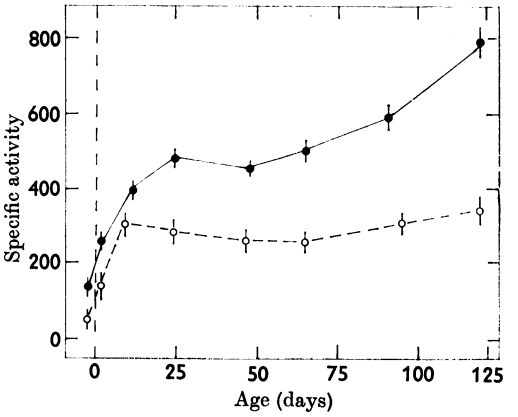

Fig. 12. Cytochrome-oxidase activity in the heart and liver of the postnatal rat: ●, heart; ○, liver. Specific activity is given as mμmoles of cytochrome *c* oxidized/min./mg. of protein. Each point with bar represents the mean ± S.E.M. of 4–13 samples. Experimental details are given in the text.

six-fold increase in activity was observed during the first 25 days of postnatal development. However, maximal cytochrome-oxidase activity in the liver was reached at 12 days, whereas the activity in the heart continued to increase to 120 days.

DISCUSSION

The profiles of several electron-transport enzymes representing various pathways have been determined in the heart and liver during development in the pre- and post-natal rat. The general pattern is an increase in enzymic activity, but the individual enzymes differ in the magnitude of increase and the time that these increases occur.

Specific activities of all enzymes in both heart and liver increased two- to three-fold during late embryogenesis (16 to 21 days after conception). Further increases of three- to six-fold occurred during the postnatal period, with the exception of the activity of NADPH–cytochrome *c* reductase in the heart, which remained at a very low level. These findings are in accordance with those reported by Flexner (1955) in the guinea pig and Potter *et al.* (1945) in the rat, who found threefold increases in liver succinate-dehydrogenase activity shortly after birth. Flexner (1955) has described a critical period of biochemical development during the late foetal stage of the guinea pig. At this time marked increases in nicotinamide–adenine dinucleotidase occur (Nemeth & Dickerman, 1960). Our results indicate that the time of great increase in respiratory-enzyme activities occurs in the 0–25-day period

after birth in the rat. This discrepancy in critical periods may be explained by differences in the enzymes studied or by the fact that the guinea pig is further developed at birth than the rat.

Dawkins (1959) has reported that the mitochondrial nitrogen content of the newborn rat increases rapidly and reaches nearly adult levels by the fifth day. Further, he stated that a qualitative difference was observed in the number of liver mitochondria as seen in the phase-contrast microscope. Our investigation covered a longer period and indicated that the increase in mitochondrial enzymes continues beyond the fifth day to the twelfth or twenty-fifth day depending on the enzyme. The specific activities of all electron-transport enzymes increased during the suckling period of the rat and reached a maximum at the time of weaning. The possible relationship between this enzyme pattern and the change in diet from milk to solids is of interest.

The increases in specific activity were due to increases in enzyme activity rather than to decreases in total protein content, which was constant. This latter fact also precludes the possibility of tissue dehydration as a factor. Further, these results indicate that the rate of increase of these enzyme proteins is higher than that of proteins in general. Also, the times of attaining maximal activity differed. For example, liver cytochrome-oxidase activity required 12 days and the cytochrome reductases 25 days; heart, liver and body weights plateaued at 75 days.

The increase in specific activity during the neonatal period may be due to a change in cell type, but this explanation is inadequate for the liver, where the transition from diploid to tetraploid cells occurs later and after 28 days of age (Alfert & Geschwind, 1958). Another explanation is the possibility that the percentage of parenchymal cells, which may have a high enzyme content, increases during this time and thus could account for the increase in enzymic activity.

The relative activities of these enzymes are of interest. Cytochrome-oxidase activity in general was from one to four times as great as the combined cytochrome *c*-reductase activities. This result may be expected, since this enzyme is a common step in electron transport for several cytochrome reductases. In all tissues the next highest specific activity was that of NADH–cytochrome *c* reductase followed by the succinate enzyme and then the NADPH enzyme.

These findings indicate that the respiratory-enzyme activities and hence capacity for electron transport are low in the prenatal tissues and increase during late foetal development and postnatal growth. This enzymic profile could be interpreted as a reflexion of the capacity for oxidative phosphorylation and ATP production. Teleologically these findings may be associated with an increased energy requirement of postnatal tissues. Although the contribution of each electron-transport pathway to fulfil the energy requirement is unknown, the higher concentration of NADH–cytochrome *c* reductase in both tissues at all ages suggests a predominant role for the NADH pathway. Other factors that can regulate electron transport include the concentration of substrates, coenzymes and other enzymes, e.g. transhydrogenase, that control these concentrations.

The high NADPH–cytochrome *c*-reductase activity in liver is correlated with the greater biosynthetic and regenerative capacities of this organ compared with the heart. This observation is in accord with the interpretation that the role of this enzyme is to regenerate $NADP^+$, which is required by glucose 6-phosphate dehydrogenase and thus could be important for ribose synthesis. On the other hand, this observation is in contrast with the proposal that NADPH and not $NADP^+$ is required by many biosynthetic reactions (Horecker & Hiatt, 1958*a*,*b*). Our present state of knowledge is insufficient to explain these interrelationships.

These two tissues were also compared with respect to their total cytochrome *c*-reductase activity and cytochrome-oxidase activity. In the 50-day-old animal the total cytochrome *c*-reductase activity is 50% greater in the liver than in the heart; the cytochrome-oxidase activity is 50% greater in the heart than in the liver. Since the rate-limiting portion of electron transport, as demonstrated in these experiments, is in the region of the cytochrome *c* reductases, these results indicate that liver tissue may have a greater capacity than heart tissue. Further, these findings may be an indication that the energy requirements for biosynthesis in the liver are greater than for muscle contraction in the heart. It is realized that differences in mitochondrial permeability to nucleotides in the two issues may account for the different enzyme activities.

A sex difference in the specific activity of NADH–cytochrome *c* reductase in the liver was noted. The difference was observed only in this reductase, and suggests a sex-hormone control mechanism associated with this enzyme. Support for this view is the observation that the time of occurrence of the enzyme difference is correlated approximately with the time of gonad maturation.

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