6. Holman, H., Nickel, W., Sleisenger, M., *Am. J. Med.*, 1959, v27, 963.
7. Armstrong, F. B., Margen, S., Tarver, H., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 592.
8. Borgstrom, B., Dahlquist, A., Lundh, G., Sjovall, J., *J. Clin. Invest.*, 1957, v36, 1521.
9. Parkins, R. A., Nimitriadou, R., Booth, F. C., *Clin. Sci.*, 1960, v19, 595.
10. Katz, J., Rosenfeld, S., Sellers, A. L., *Am. J. Physiol.*, 1960, v200, 1301.
11. Beeken, W. L., Imredy, K., *Biochim. Biophys. Acta*, 1962, v62, 579.
12. McFarlane, A. S., *Nature*, 1958, v184, 53.
13. Dixon, W. J., Massey, F. J., *Introduction to Statistical Analysis*, McGraw Hill, N. Y., 1957, 119.
14. Small, M. D., Bezman, A., Longavini, A. E., Fennell, A., Zamcheck, N., PROC. SOC. EXP. BIOL. AND MED., 1960, v106, 450.
15. Pelot, D., Grossman, M. I., *Am. J. Physiol.*, 1962, v202, 285.

Received January 15, 1964. P.S.E.B.M., 1964, v117.

A Histochemical Study of Succinic and Lactic Dehydrogenases in the Regenerating Forelimb of the Adult Newt, *Triturus*.*† (29488)

ELEANOR A. JOHNSON AND MARCUS SINGER (Introduced by H. A. Schneiderman)
Department of Anatomy, School of Medicine, and Developmental Biology Center, Western Reserve University, Cleveland, Ohio

There are few biochemical or histochemical studies on the metabolism of regenerating tissues in amphibians. Okuneff(1) and Vladimirova(2) demonstrated that lactic acid increases in the early regenerate and then declines to normal during differentiation. Associated with the increase in lactic acid is a decrease in pH(3) and respiratory quotient (4).

In the absence of oxygen, pyruvic acid formed during glycolysis is reduced to lactic acid by lactic dehydrogenase instead of being converted to acetyl CoA, one of the first compounds in the Krebs Cycle. The accumulation of lactic acid in the regenerate bud suggests that glycolysis is the principal source of energy and would explain the lowered RQ and pH of these tissues. However, Gezsik and Wolsky(5) found that after an initial decline in the levels of succinic, malic and lactic dehydrogenases in early tail regenerates, the activities rose during "progressive" stages of tail regeneration, particularly for succinic dehydrogenase, indicating that respiratory metabolism via the Krebs cycle might be important. Furthermore, Niwelinski (6) found that the blastemal cells of the regenerating forelimb of the newt reacted intensely when tested histochemically for succinic dehydrogenase and DPNH diaphorase and reached adult levels by the fourth week.

On the other hand, in a more recent study published after the present work was completed, Wolfe and Cohen(7) reported that little or no succinic dehydrogenase and isocitric dehydrogenase were present in the undifferentiated blastema but appeared during later differentiation. However, the blastema reacted strongly for DPNH diaphorase, lactic dehydrogenase and malic dehydrogenase. Furthermore, they reported increased levels of glucose-6-phosphate dehydrogenase and TPNH diaphorase during the undifferentiated blastemal stages.

In the present study several stages during the regeneration of the forelimb were tested histochemically for succinic dehydrogenase (SDH) and lactic dehydrogenase (LDH), the former because of its significance in the respiratory Krebs cycle, and the latter because of its role in glycolysis.

* Supported by grants from Am. Cancer Soc. and Nat. Inst. Health.

† The present work was presented at Am. Soc. of Zool. meetings (Johnson, E. A., Singer, M., *Am. Zool.*, 1963, v3, 511). On reading the abstract of the presentation, Dr. Anthony Schmidt kindly sent us the manuscript of his unpublished paper dealing with histochemical tests for oxidative enzymes in the regenerating limb, among which glucose-6-phosphate dehydrogenase increased and dehydrogenase and aldolase decreased in the blastema.

Materials and methods. Adult Triturus kept at 25°C were anaesthetized in a chloretone solution and their forelimbs amputated just proximal to the elbow. At selected days after amputation and in subsequent selected regenerate stages(8, p. 170), the right stumps with the regenerate were removed and the bone of the stump excised; the tissues were quick frozen, and then sectioned in a cryostat at 10 μ. The method of Nachlas *et al*(9) was used for histochemical demonstration of SDH; and for LDH that of Nachlas, Walker and Seligman(10), and Hess, Scarpelli and Pearse(11; see Pearse, 12). The left stump and regenerate served as a control in which the sections were incubated in the same manner as the experimental but without the substrate. In some cases the stump and regenerate of the same stage as the experimental were taken from another animal to serve as control. At least 4 animals were used to test the activity of each enzyme at a given stage of regeneration. Activity was recorded according to the relative number of cells and to the number and intensity of stained sites in each cell.

Results. By 5 days after amputation, the amputation surface is covered by a layer of epidermis and is underlaid by a fibrocellular scar. The wound epidermis was heavily stained for SDH and was sharply distinguished from the less reactive normal epidermis of the stump. Connective tissue cells of both the stump and the wound site were weakly reactive while the nerve showed little or no reaction. Stump muscle was highly reactive with a pattern of formazan deposits corresponding to the pattern of mitochondrial distribution. The distal stump muscle and both stump and wound epidermis stained heavily for LDH while proximal muscle, nerve and connective tissue cells of both stump and wound stained lightly.

Seven days after amputation the characteristic histolysis of wound tissues that precedes blastema formation was already noticeable but scar tissue was still present. The stump muscle was heavily stained for SDH although the more distal dedifferentiating portions were noticeably lighter. The linear orientation of dye deposits characteristic of proximal stump muscle was seen distally in various stages of disarray. Connective tissues of both the stump and wound site stained lightly and nerve showed no changes in staining. Both stump and wound epidermis were heavily stained although that of the wound was more active. As for LDH, distal muscle reacted more intensely than the more proximal portions; there was no notable change in the activity in the nerve; connective tissue cells of the scar were somewhat more heavily stained than those of the 5-day animals. Stump and wound epidermis were heavily reactive with no sharp distinction between the two.

By 10 days after amputation, histolysis is far advanced; scar tissue is largely dissolved as are muscle and connective tissue of the stump near the wound; accumulation of mesenchymatous cells of regeneration forming the blastema under the epidermis is obvious at this time. The regenerate cells showed little or no SDH activity but pronounced LDH activity. The wound epidermis now appeared less intensely stained for SDH than that of the stump but no changes in intensity were seen for LDH. Connective tissue cells of the stump reacted weakly for SDH and somewhat more strongly for LDH. Proximal muscle stained more heavily than distal muscle for SDH while just the opposite was true for LDH.

Moderate early regenerate bud stages of 10-12 days in which the blastemal accumulation is definite but not voluminous showed considerable SDH activity in the stump muscle. Stump connective tissue and nerve showed no changes in reaction; and the epidermis was similar to the preceding stage. Blastemal cells distal to the amputation line showed no SDH activity at all while morphologically similar cells proximal to the line of amputation had some small scattered formazan deposits. There was very little LDH activity in the proximal muscle at this time. More distal portions showed some activity while nerve and connective tissue were only slightly reactive. Stump and wound epidermis were as active as in previous stages. The blastema, however, was heavily stained while mesenchymatous cells proximal to the level

of amputation appeared somewhat less active.

The pattern of SDH in the early bud of about 12 to 14 days resembled that of the moderate early, except for a decline in activity of the wound epidermis. The pattern of LDH activity was unchanged. This level of SDH activity persisted until the enlarged and rapidly growing medium bud stage when a slight amount of activity confined to a few cells was detected in the blastema of some animals. Lactic dehydrogenase showed no changes in activity from that of earlier stages.

The late bud, 18 to 24 days after amputation, when histogenesis first appears, showed a positive reaction for SDH in the early differentiating cartilage. The reaction declined radially from the core of cartilage. In the case of LDH the intensity of staining faded noticeably in most blastemal cells although the core of precartilage was still quite active.

The palette stage 26 to 30 days after amputation, during which histogenesis is in full swing, was marked by a further increase in SDH activity in the central area of differentiating cartilage. There was some activity in adjacent surrounding cells where muscle was about to differentiate although the activity was still slight in relation to adult stump tissues. There was further decrease in LDH except in precartilage.

Controls for both the succinic and lactic dehydrogenase series were negative.

Conclusions and discussion. At 5 and 7 days after amputation, the wound epidermis exhibited high SDH and LDH activity. Providing there are no other blocks in the respiratory pathways, the epidermis is probably actively respiring as is proximal muscle. However, in the case of distal sarcolysing muscle a decrease in SDH paralleled by an increase in LDH appeared, indicating a decreased Krebs cycle metabolism and perhaps an increased reduction of pyruvic acid. The changes in LDH and SDH in the connective tissue of the wound were similar to the sarcolysing portions of the muscle and therefore their metabolism may be interpreted similarly.

By 10 days, the mesenchymatous accumulation of the blastema had no detectable SDH activity but LDH was very active. Morphologically similar cells proximal to the amputation line did show some SDH activity; perhaps, they are dedifferentiating cells in a transitional state. Thus, the blastemal cells appear to receive their energy from sources other than the Krebs cycle while morphologically similar cells more proximally are respiring at a low level. Furthermore, the high level of LDH in the blastema may indicate an increased lactic acid production due to pyruvate reduction.

This pattern, suggesting high glycolysis and energy sources other than from the Krebs cycle, persisted among blastemal cells until the time of first histogenesis in the medium or late bud stages. The change was not sudden, but gradually increased in intensity, suggesting that metabolism *via* the Krebs cycle increases with differentiation. An exception was that LDH activity remained high in the precartilage cells, perhaps due to the fact that cartilage lacks a blood supply and chondrocytes may metabolize glycolytically. In contrast to this cycle of change in the blastemal cells, proximal muscle not undergoing sarcolysis and epidermis appeared to respire relatively normally.

The main pathway of carbohydrate metabolism and energy production in most normal tissues is that of glycolysis and the Krebs cycle. Glycolysis can occur either anaerobically, in which glucose is broken down to pyruvic acid which is then reduced to lactic acid, or it can occur aerobically, in which pyruvate is oxidatively decarboxylated to acetyl CoA which is fed into the Krebs cycle. Although aerobic glycolysis as described occurs in most normal cells, the term is currently used to describe increased glycolytic metabolism in systems with decreased respiratory metabolism but in which oxygen is available. Anaerobic glycolysis refers to glycolytic metabolism due to a lack of oxygen. In general, respiratory metabolism refers to metabolism *via* the Krebs cycle and electron transport system and to any catabolic pathway dependent on the electron transport system and oxygen for its functioning.

The functioning of the Krebs cycle de-

pends on the supplies of pyruvate, oxygen, ADP and phosphate and on the presence and activity of the enzymes and co-factors of the Krebs cycle and electron transport system. A block at any step in the Krebs cycle or the electron transport system due to the lack or inactivity of any of the above would result in the accumulation of products of earlier reactions in the pathway. Since the system tends to go to equilibrium, a block would shut down the system and pyruvic acid would accumulate even in the presence of oxygen. Furthermore, if pyruvate were not shunted into other pathways, glycolysis would be similarly blocked. One alternative to the Krebs cycle in the utilization of pyruvate is its reduction to lactic acid in the presence of lactic dehydrogenase. If the block were other than the lack of oxygen, for example the apparent absence of succinic dehydrogenase in the undifferentiated blastema, aerobic glycolysis would be the result. If the block were due to lack of oxygen, glycolysis would then be anaerobic.

Carbohydrates can also be broken down by the pentose phosphate pathway which bypasses many glycolytic steps. The sugars necessary for nucleic acid synthesis may be produced *via* this system and one would expect that during regeneration there must be a continuous synthesis of nucleic acids and that therefore the pentose phosphate pathway would be operative. In fact, Wolfe and Cohen(7), and Schmidt (see footnote †) reported increased levels of glucose-6-phosphate dehydrogenase, one of the enzymes of this pathway, during those stages of regeneration when succinic dehydrogenase and other oxidative enzymes appear to be absent. This may indicate an increased role of this pathway during regeneration.

The presence of high lactic dehydrogenase activity in the blastema at the same time that succinic dehydrogenase is apparently absent may indicate an increase in the amount of pyruvate being converted to lactic acid and perhaps a relative increase in glycolysis. However, the pentose phosphate pathway produces 3-phosphoglyceraldehyde. This compound is also produced by glycolysis and through a series of reactions is converted to pyruvate. It is possible that excess 3-phosphoglyceraldehyde produced by the pentose pathway is converted to pyruvate and hence to lactic acid. This appears even more likely in light of the finding by Schmidt of a decrease in the activity of blastemal aldolase. This glycolytic enzyme is necessary for splitting fructose-1,6-diphosphate to 3-phosphoglyceraldehyde and dihydroxyacetone phosphate. Its absence would block the glycolytic production of 3-phosphoglyceraldehyde, and therefore the glycolytic production of pyruvate and lactic acid. The reverse reaction is also catalyzed by this enzyme and is important in completing the pentose pathway cycle. Normally 3-phosphoglyceraldehyde would not accumulate as a product of the pentose pathway. Isomerization of 3-phosphoglyceraldehyde to dihydroxyacetone would be followed by condensation of the 2 trioses in the presence of aldolase, to form fructose 1,6-diphosphate. Its hydrolyzation by fructose-1,6-diphosphate to give fructose-6-phosphate would complete the cycle. However, the absence of aldolase, although not blocking the pentose pathway in general, would block this particular step, accounting for the accumulation of 3-phosphoglyceraldehyde. This could then be converted to pyruvate and, if the Krebs cycle were not operating, to lactic acid.

While glycolysis is not dependent on the presence of oxygen, due to the cycling of DPN and DPNH, the pentose pathway probably depends on the electron transport system since 2 TPNH are formed in the cycle while none are used. It should be noted that TPNH diaphorase was reported to increase in the blastema(7). If oxidative metabolism in general were shut down, one might expect to see changes in the cellular mitochondrial pattern since the mitochondria are the site of the electron transport system. However, if the pentose pathway is predominantly involved, one would not expect mitochondrial changes. While some changes have been reported for larval Amblystoma(13), no unusual or obvious ultrastructural differences have been seen in the mitochondria of the adult newt's regenerate that could not be due to fixation(14).

Summary. Succinic (SDH) and lactic (LDH) dehydrogenase activity was studied histochemically in the regenerating forelimb of adult Triturus. During the stages of wound healing and of blastemal cell accumulation, SDH activity decreased in the tissues underlying the wound epidermis while LDH increased. During stages of rapid growth of the regenerate, SDH was not histochemically demonstrable in the mesenchymatous tissue; but LDH activity was high. As the tissue differentiated in later stages of regeneration, SDH activity increased while LDH in general declined. The wound epithelium showed both LDH and SDH activity throughout regeneration with variations in intensity less striking than in underlying parts. The results of our work and of others indicate that in the stages of appearance, accumulation and proliferation of the blastema, metabolism *via* the Krebs cycle is replaced by glycolytic and/or the pentose pathway metabolism.

1. Okuneff, N., *Biochem. Z.*, 1933, v257, 242.
2. Vladimirova, E. A., *Akad. Nauk SSSR Compt. Rend.*, 1934, v3, 479.
3. Okuneff, N., *Biochem. Z.*, 1928, v195, 421.
4. Ryvkina, D. E., *Akad. Nauk SSSR Compt. Rend.*, 1945, v49, 457.
5. Gezsik, R., Wolsky, A., *Anat. Rec.*, 1959, v134, 568.
6. Niwelinski, J., *Folia Biol.*, 1960, v8, 1.
7. Wolfe, H. J., Cohen, R. B., *Dev. Biol.*, 1963, v8, 48.
8. Singer, M., *Quart. Rev. Biol.*, 1952, v27, 169.
9. Nachlas, M. M., Tsou, K., DeSouza, E., Seligman, A. M., *J. Histochem. and Cytochem.*, 1957, v5, 420.
10. Nachlas, M. M., Walker, D. G., Seligman, A. M., *J. Biophys. Biochem. Cytol.*, 1958, v4, 29.
11. Hess, R., Scarpelli, D. G., Pearse, A. G. E., *ibid.*, 1958, v4, 753.
12. Pearse, A. G. E., *Histochemistry, Theoretical and Applied,* Little Brown & Co., Boston, 1960.
13. Hay, E. D., *J. Biophys. Biochem. Cytol.*, 1958, v4, 583.
14. Salpeter, M., Singer, M., *Dev. Biol.*, 1960, v2, 516.

Received February 24, 1964. P.S.E.B.M., 1964, v117.

Changes of Tissue Catecholamines in Thiazide-Fed Rats. (29489)

P. A. SERRANO AND L. B. CHÁVEZ (Introduced by W. Raab)
Department of Endocrinology, Instituto Nacional de Cardiologia, Mexico City

The hypotensive action of thiazides has been previously established both in the experimental and therapeutic fields. No satisfactory explanation has been found to account for the prolonged hypotensive mechanism of action of these compounds(1).

During the initial period of treatment with thiazides, the decrease in blood pressure can be explained on the basis of the known change in electrolytes(1-4), as well as on the observed diminution of plasma volume and cardiac output caused by these substances(5); the hypotensive effect after long-term treatment cannot be explained on this basis, as the lowering of blood pressure persists even after the electrolyte changes, the decrease of cardiac output and the decrease of plasma volume have disappeared or have been compensated(5-9).

A possible connection between the hypotensive effect of these drugs and the action of amines has been suspected. It has been shown that administration of the thiazides decreases the hypertensive response to infusion of epinephrine(10-11). Hollander and co-workers(12), found that the diminished reactivity to intravenous administration of noradrenaline disappeared after 20 days treatment with these drugs. Previous communications from our department(13-14) reported a diminution of urinary catecholamine excretion in a group of hypertensive patients who had been treated with thiazides. Diazoxide, a compound with hypotensive effect, which on several trials displayed the ability to retain sodium and water(15-17), clearly shows the dissociation between natriuretic and hypotensive effects of thiazides.