CHAPTER 11

STUDIES ON FAT, GLYCOGEN AND ENZYMES LIKE LIPASE, ESTERASE, β-HYDROXY BUTYRATE DEHYDROGENASE, LACTATE DEHYDROGENASE AND SUCCINATE DEHYDROGENASE DURING WOUND HEALING AND REPAIR IN THE PIGEON LIVER

Metabolic activities during the processes of wound healing and repair in the visceral organs have so far attracted little attention. It was shown that the processes of wound healing and repair in visceral organs like liver of pigeon, differ from those occurring in the peripheral regions. Major difference is in the formation of a wound covering which is accomplished by the participation of lymphocytes which transform into macrophages and fibroblasts and part take in the formation of connective tissue (see Chapter 6). The repair process which includes the proliferation of normal cells at the wound site, was found to be initiated only when a large number of injured cells are present at the wound site (as discussed in Chapter 6). Since these processes require energy, it was deemed worthwhile to study the metabolic activities at the wound site. Hence, the pattern of distribution of metabolites like fat and glycogen and some enzymes viz., lipase, esterase,

β-hydroxy butyrate dehydrogenase (BDH), succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) which greatly figure in utilization of the metabolites stated above through preferential pathways, were studied histochemically.

MATERIAL AND METHODS

For initiating the process of repair and wound healing in the pigeon liver, the procedure followed was the same as described earlier (Chapter 6). Forty-five adult pigeons (Columba livia) reared in laboratory conditions were selected and operated upon to remove a part of the liver and subjecting the cut edge to a high pressure. Of these operated pigeons, three birds at a time were sacrificed at certain intervals viz., hrs 2, 4, 6, 8, 12, and days 1, 2, 3, 4, 6, 10, 15, 20, 25, and 30 after the operation. The liver was removed quickly and the injured part along with adjacent normal region was fixed on the chuck of a microtome mounted in a cryostat for taking fresh frozen sections for the histochemical studies of the enzymes referred above.

The oxidative enzymes like SDH, LDH and BDH were studied histochemically using the methods described by Ogata and Mori (1964). The substrates used were sodium

succinate, sodium lactate and sodium β-hydroxy butyrate for SDH, LDH and BDH respectively.

For the histochemical localization of lipase activity, the method described by Moskowitz and Moskowitz (1965) was employed. In this method, the endogenous fat, present in the tissue itself, formed the substrate. The fresh frozen liver sections were fixed in 4% cold formalin for 10 to 20 minutes. After washing thoroughly in water, sections were transfered to medium containing 0.1 M tris buffer (pH 8.6) and 0.2 M calcium chloride and were incubated overnight. After the incubation, the sections were transfered to 2% lead nitrate and then treated with 1% ammonium sulphide. Thereafter, the sections were washed thoroughly in distilled water and mounted in glycerine jelly. Apart from this method the "Tween" method of Gomori (Pearse, 1960) was also employed using "Tween 85" with modifications suggested by George and Scaria (1958).

The esterase activity was demonstrated using the azo dye coupling method of Burstone (1962) using Napthol-AS-D-Acetate as the substrate and Fast Blue B as the diazonium salt.

For the histochemical studies of all the enzymes suitable controls were also prepared to eleminate artifacts.

The distribution of fat, in the tissue sections of

liver fixed in Baker's calcium formalin, was studied using various dyes such as Sudan Black B, Fettrot 7B, Oil Red O as well as acid haematin method as described by Pearse (1960). Glycogen was demonstrated in paraffin sections of the liver pieces fixed in cold alcoholic picroformol, together with suitable controls, using PAS technique as described by Pearse (1960). For these also suitable controls were prepared.

Quantitative estimation of glycogen was carried out by the anthrone method of Seifter et al. (1950).

RESULTS

Fat:

The type of fat present in abundance in the parenchymal cells of the liver as well as at the site of the wound was neutral fat which appeared as fine droplets in the sections stained with Fettrot and Oil red O.

By about 4 hrs after inflicting injury the injured area became completely devoid of fat. But later, after about 8 to 12 hrs, fine droplets of neutral fat appeared in the region termed as 'zone' separating the normal and injured areas, and its concentration gradually increased to a fairly high level by about 24 hrs after operation (Fig. 1). At this time similar fine droplets of fat were

also seen in the cells of lymphocytopoietic nodules present in the intact regions of the liver (Fig. 2). Accumulation of neutral fat in the 'zone' as well as in the lymphocytopoietic nodules showed progressive increase upto 96 hrs after inflicting injury (Figs. 3 & 4). But later the fat from the cells of lymphocytopoietic nodules totally disappeared, while that in the 'zone' region, remained more or less in same high concentration even after 6th day. Soon after this period a gradual reduction started and it completely disappeared by 10th day.

The parenchymal cells near the wound area did not show any change from the normal state in the amount of neutral fat even at about 24 hrs after the infliction of injury (Fig. 1). However, a progressive decrease in the total lipid amount was observed thereafter (Fig. 4) and by about 10th day (a period of active proliferation of hepatocytes at the wound area) the intact parenchymal cells subadjacent to the 'zone' showed very little fat. Again by 15th day the newly formed parenchymal cells began to accumulate appreciable amount of neutral fat (Fig. 5) and by 20th day its concentration reached the preoperative level (Fig. 6) that was noted in the normal hepatocytes.

Glycogen:

Histochemically noticeable amount of glycogen

comparable to that in the normal liver was present in the intact region near the injured area up to 72 hrs after the infliction of injury (Fig. 7). But, thereafter, its depletion from many of the lobules situated subjacent to the injured area was noticed, especially by 4th day (Fig. 8). Soon after the concentration of glycogen gradually increased again and finally reached the preoperative level (Fig. 9).

Quantitative changes in the glycogen level:

Maximum reduction of glycogen content in the wound and near by area of liver was between 4th and 6th day after the infliction of the injury (Table I).

TABLE I

Percentage reduction of glycogen content at the wound site compared to the healthy areas of the liver

hrs					days					
12	24	48	72	96	6	10	15	20	25	30
7.5	18.3	18.8	19.1	32.0	31.8	17.8	5.4	4.6	2.3	2.5

Lipase:

The localization of lipase was similar with both methods employed in this investigation, though the intensity was more with "Tween" method of Gomori, which may be probably due to esterase activity. Hence, the observations made were from the histochemical preparations using the method of Moskowitz and Moskowitz (1965). An appreciable increase in the lipase activity was seen in the area adjacent to injured part at about 48 hrs after the infliction of injury (Fig. 10) which continued to increase in the subsequent periods, and reached a maximum level by about 10th day (Fig. 11). However, in the 'zone' region maximum lipase activity was seen between 3rd and 6th day after inflicting the injury. Thereafter, in the wound site (area covered by newly formed connective tissue) the enzyme activity decreased to the preoperative level.

Esterase:

Like lipase, esterase also was found to be only moderately active in the normal pigeon liver cells. After inflicting injury, the esterase activity remained without change till about 24 hrs (Fig. 12). However, by about 48 hrs a significant increase in its activity was noticed in the uninjured, healthy region of the liver situated adjacent to the injured area (Fig. 13). The increased

activity of the enzyme persisted in this region till 72 hrs after the infliction of the injury. Beyond this region, (uninjured part closely adjacent to injured area) the other normal areas of the liver showed relatively undisturbed normal esterase activity pointing to the fact that the changes in the enzyme intensity ~~was~~ were confined only to the adjacent area around the injured one. Thereafter, by about 96 hrs a considerable drop occurred in the intensity of the esterase activity in the area adjacent to the injured one (Fig. 14); and this decreased state remained up to 10th day. But after this period the enzyme activity gradually increased and reached the preoperative level.

β-Hydroxy butyrate dehydrogenase (BDH):

A moderate BDH activity was found in the normal liver cells of pigeon. Following injury to the liver there was a slight decrease in the intensity of the enzyme at the wound site (Fig.15) which progressively decreased further till 96 hrs. At this time cells of the 'zone' region showed a slight increase in the enzyme activity (Fig. 16). Thereafter, in the parenchymal cells in the normal healthy region near the 'zone', the BDH activity began to increase till it reached the normal by about 15th day after inflicting the injury (Fig. 7).

Succinate dehydrogenase (SDH):

Normally, the SDH activity was found to be very high in the healthy liver cells. After the infliction of injury, the SDH activity showed no change till 48 hrs at the wound site (Figs. 18 & 19). However, in the cells of the 'zone' the enzyme activity declined retaining only a weak reactivity (Fig. 19) which also disappeared later. Likewise, in the intact healthy region near the 'zone' a slight decrease in the enzyme activity was perceived between 3rd and 6th day after operation (Fig. 20) which returned to the normal level by 10th day (Fig. 21).

Lactate dehydrogenase (LDH):

The LDH in the normal and healthy liver cells was less active compared to SDH. About 24 hrs following injury, there was a considerable increase in LDH activity in the healthy hepatocytes in the intact region near the 'zone' (Fig. 22). This increased level of the enzyme remained so till 6th day (Fig. 23). The 'zone' area, which was devoid of LDH activity showed fairly detectable activity by 4th day and the maximal level of its activity was noticed by about 6th day (Fig. 23). Soon after 6th day the enzyme level began to decrease and by 15th day its activity came down to the same level as that of normal cells of the liver (Fig. 24).

(Chapter 11: Figs. 1 to 6. Photomicrographs of the liver of pigeon showing the histochemical localizations of neutral fat stained with Fettrot 7B, during wound healing and repair)

Fig. 1. 24 hrs after the infliction of the injury. Note the accumulation of fine droplets of neutral fat (arrows) between the normal intact area (NA) and the injured area (IA).

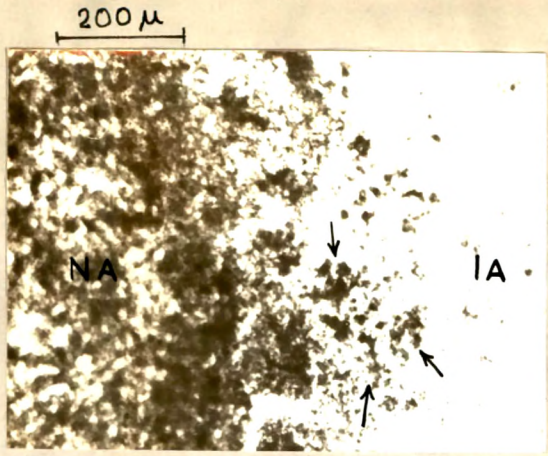
Fig. 2. 24 hrs after the infliction of the injury. Note the fat droplets in the lympocytopoietic nodule (N). Fat (arrows) is also seen in the 'zone' (Z).

Fig. 3. 48 hrs after the infliction of the injury. The accumulation of fat droplets increased in the 'zone' (Z) situated between normal intact area (NA) and the injured area (IA).

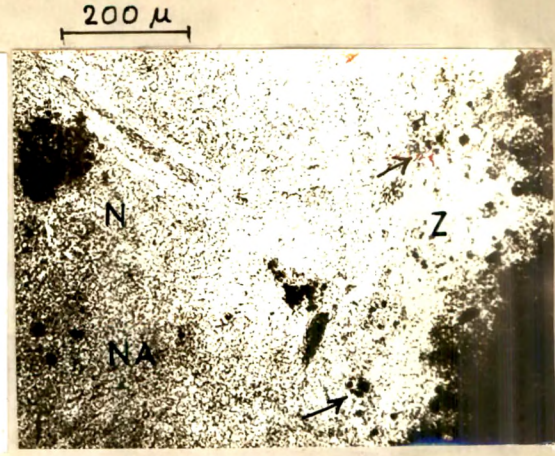
Fig. 4. 96 hrs after the infliction of the injury. Maximum concentration of neutral fat in the 'zone' (Z) is seen at this stage. While in normal intact area (NA) the fat content decreased considerably.

Fig. 5. 15 days after the infliction of the injury. The newly formed hepatic cells (HC) have began to acquire more neutral fat.

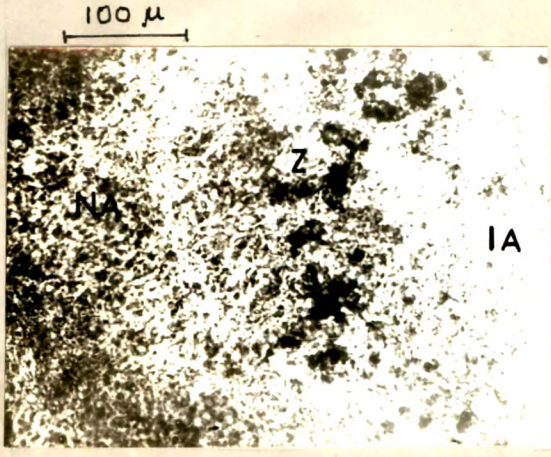
Fig. 6. 20 days after the infliction of the injury. The fat content has reached the preoperative level in the normal intact area (NA) as well as in the newly formed hepatic cells (HC).



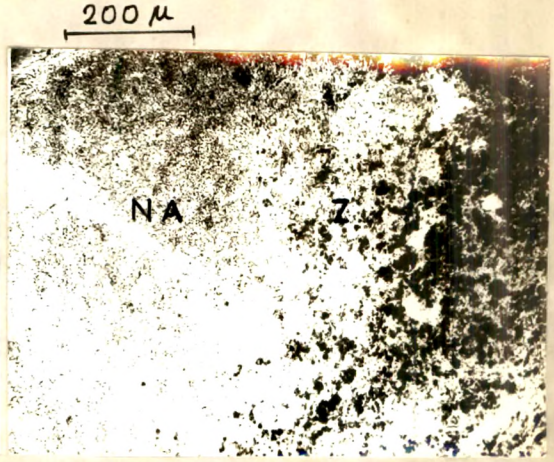
1



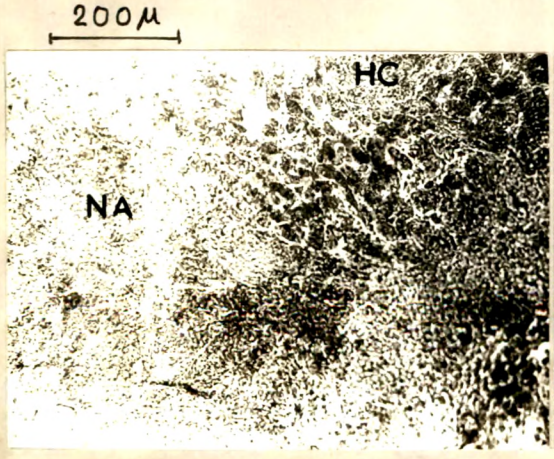
2



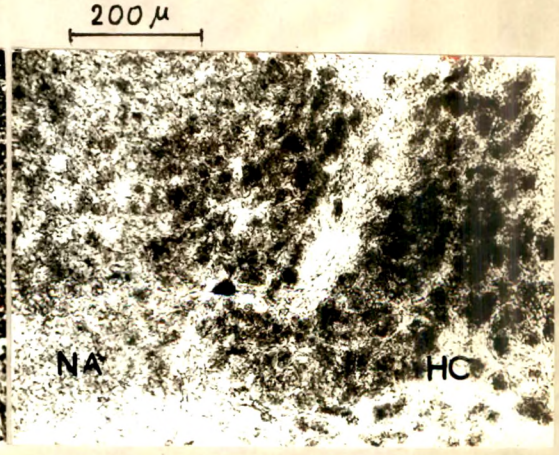
3



4



5



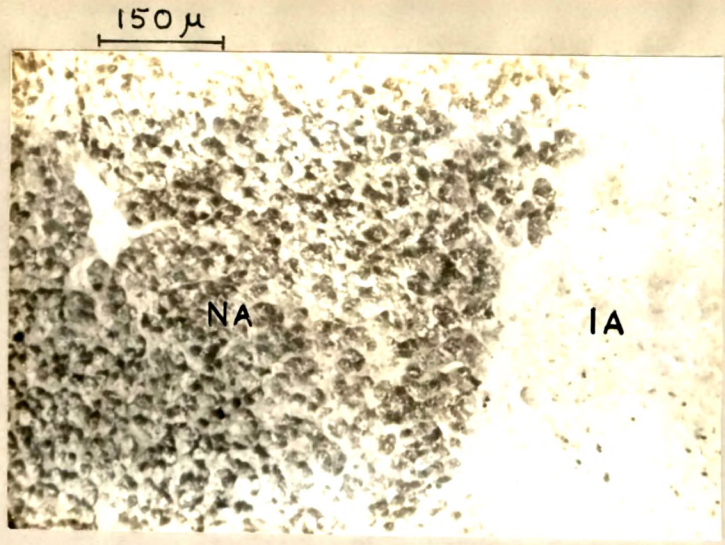
6

(Chapter 11: Figs. 7 to 9. Photomicrographs of the pigeon liver showing the histochemical distribution of glycogen during wound healing and repair . PAS technique)

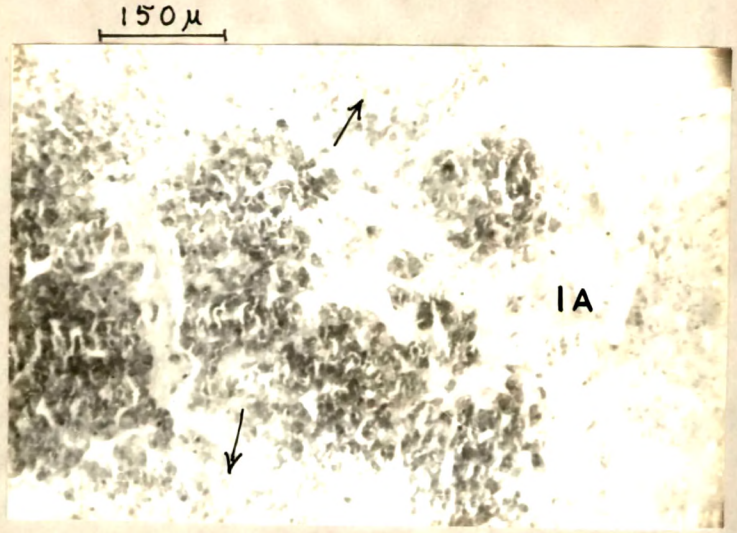
Fig. 7. 24 hrs after the infliction of the injury. The intact normal region (NA) shows not much change in the concentration of glycogen in contrast to the injured area (IA) where the glycogen has completely disappeared.

Fig. 8. 96 hrs after the infliction of the injury. Reduction in the concentration of glycogen could be seen in many of the lobules (arrows) situated near the injured area (IA).

Fig. 9. 10 days after the infliction of the injury. The glycogen concentration has increased to a more or less preoperative level.



7



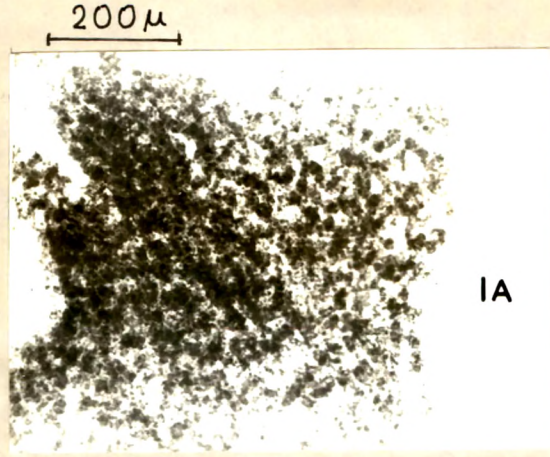
8

9

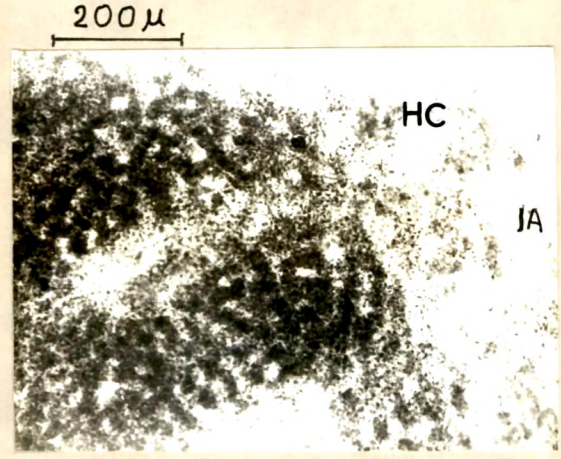
(Chapter 11: Figs. 10 and 11. Photomicrographs of the liver of pigeon showing the lipase activity at the injured area)

Fig. 10. 48 hrs after the infliction of the injury. Note the high concentration of the enzyme in the area near the injured region (IA).

Fig. 11. 10 days after the infliction of the injury, The concentration of lipase reached a peak level at this period. The newly formed area (HC) of the liver near the injured area (IA) also shows fairly good concentration of the enzyme activity.



10



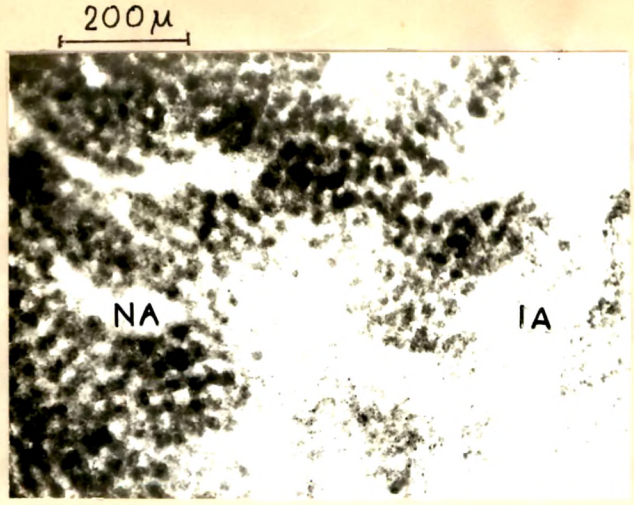
11

(Chapter 11: Figs. 12 to 14. Photomicrographs of the liver of pigeon showing the histochemical localizations of esterase during wound healing and repair. Burstone's method)

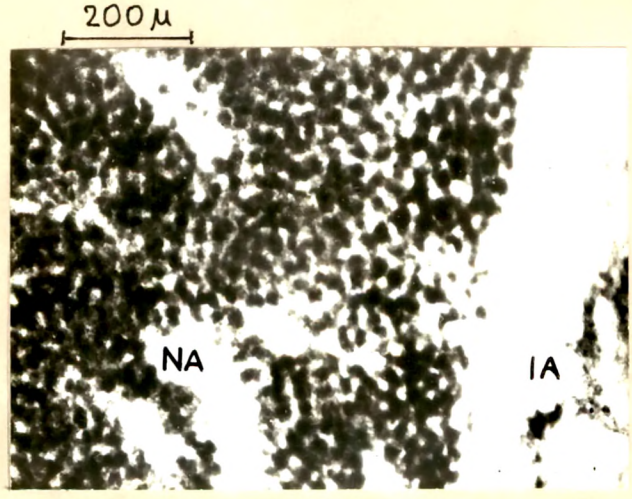
Fig. 12. 24 hrs after the infliction of the injury. The activity of the enzyme is more or less same in the intact area (NA) as that of a normal liver. The injured area (IA) is devoid of enzyme activity.

Fig. 13. 48 hrs after the infliction of the injury. Esterase activity increased to a maximum level at this period in the normal intact area (NA) adjacent to the injured area (IA).

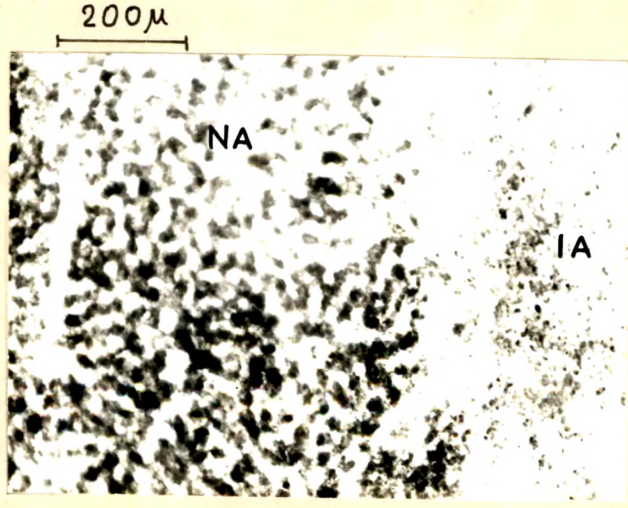
Fig. 14. 96 hrs after the infliction of the injury. Esterase activity in the normal area (NA) situated near the injured area (IA) decreased considerably.



12



13



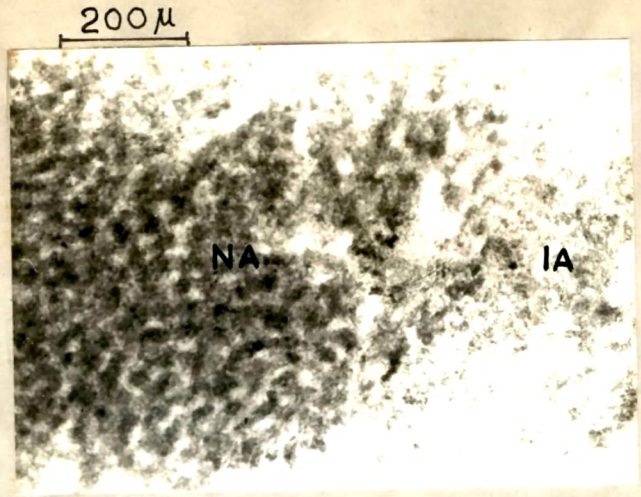
14

(Chapter 11: Figs. 15 to 17. Photomicrographs of the pigeon liver showing the distribution of β-hydroxy butyrate dehydrogenase at the injured area)

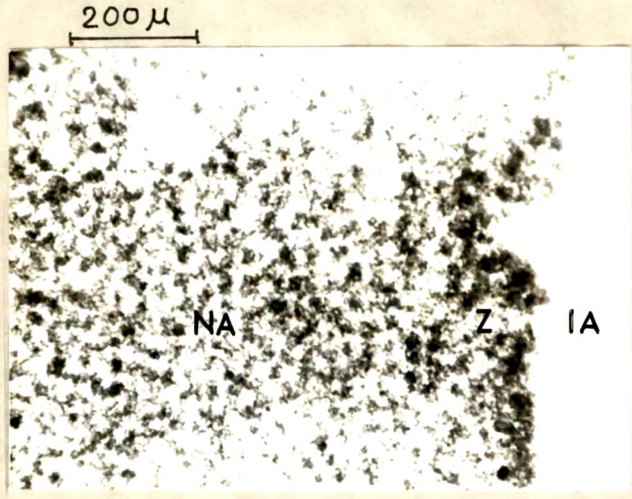
Fig. 15. 24 hrs after the infliction of the injury. The enzyme activity decreased slightly in the non injured region (NA) adjacent to the injured area (IA).

Fig. 16. 96 hrs after the infliction of the injury. A further decrease is noticed in the normal region (NA) while the enzyme activity increased in the 'zone' (Z) near the injured area (IA).

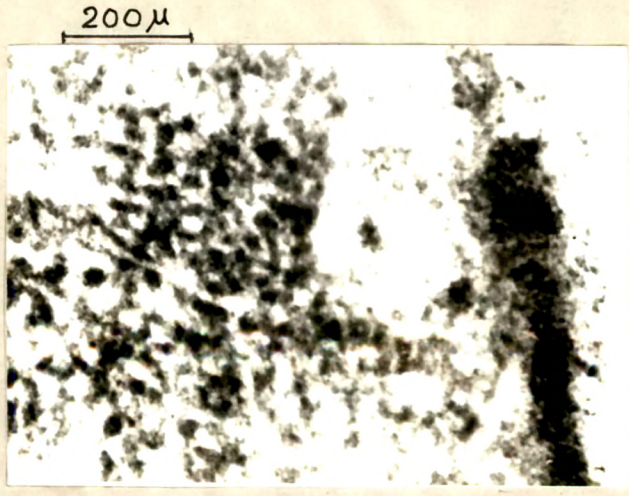
Fig. 17. 15 days after the infliction of the injury. The activity has increased in the normal area (NA) to reach the preoperative level.



15



16



17

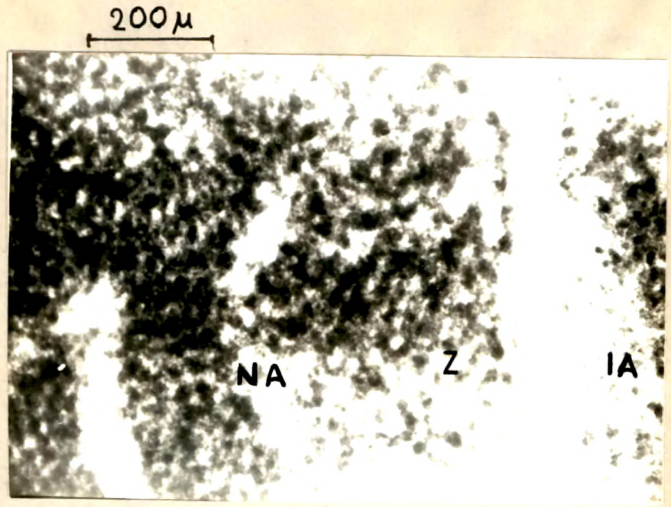
(Chapter 11: Figs. 18 to 21. Photomicrographs of the pigeon liver showing the histochemical localizations of succinate dehydrogenase in the injured area)

Fig. 18. 24 hrs after the infliction of the injury. No perceivable change occured in the normal region (NA) as well as in the 'zone' (Z) near the injured one (IA).

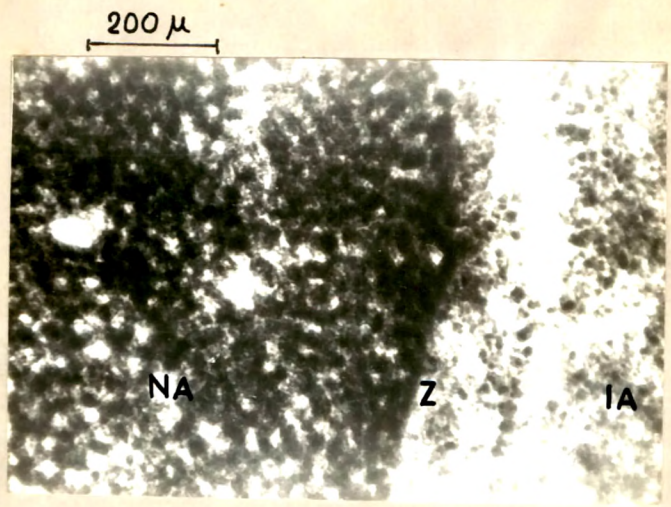
Fig. 19. 48 hrs after the infliction of the injury. Even at this stage the enzyme activity showed no change in the intact area (NA) while in the 'zone' (Z) the activity decreased considerably. IA - injured area.

Fig. 20. 96 hrs after the infliction of the injury. While the activity of the enzyme disappeared from the 'zone' (Z) the normal intact area (NA) near the 'zone' showed a slight decrease.

Fig. 21. 10 days after the infliction of the injury. The activity of the enzyme has returned to the normal preoperative level in the normal area (NA). IA - injured area.

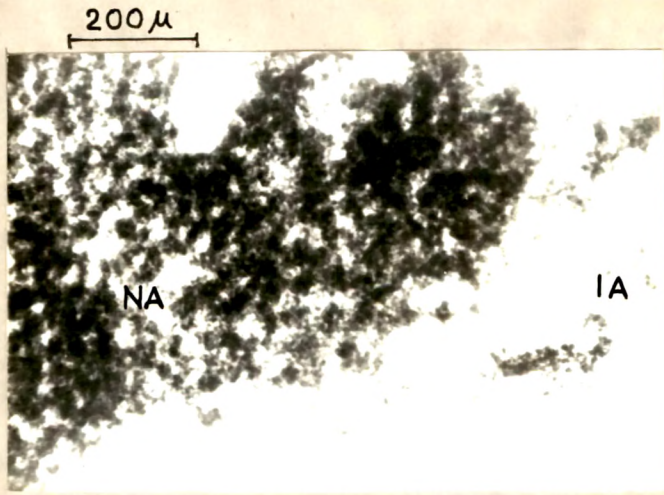


18



19

20



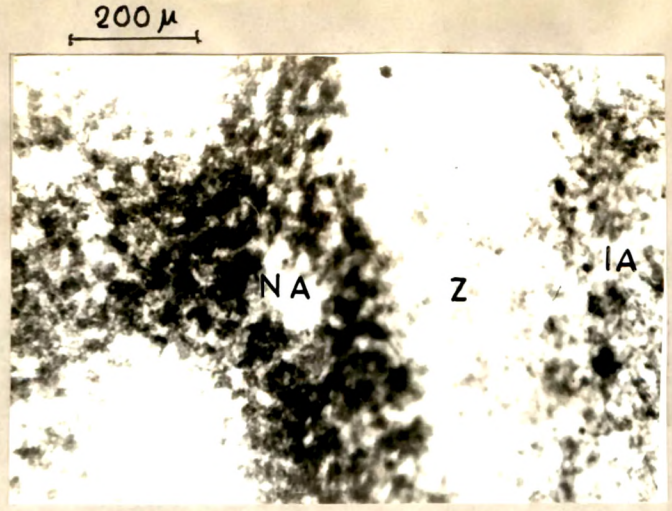
21

(Chapter 11: Figs. 22 to 24. Photomicrographs of the pigeon liver showing the localizations of the lactate dehydrogenase during wound healing and repair)

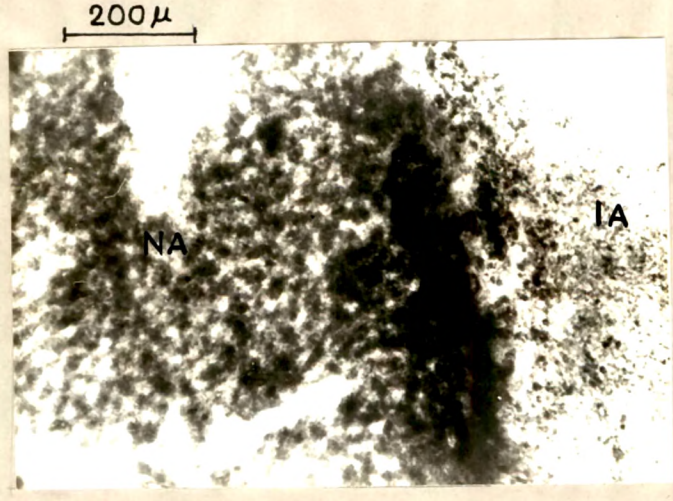
Fig. 22. 24 hrs after the infliction of the injury. The enzyme activity increased in the normal intact area (NA) while the 'zone' (Z) and injured area (IA) showed no activity.

Fig. 23. 6 days after the infliction of the injury. The enzyme activity increased greatly in the normal area (NA) as well as in the 'zone' (Z) while injured area (IA) was devoid of the enzyme.

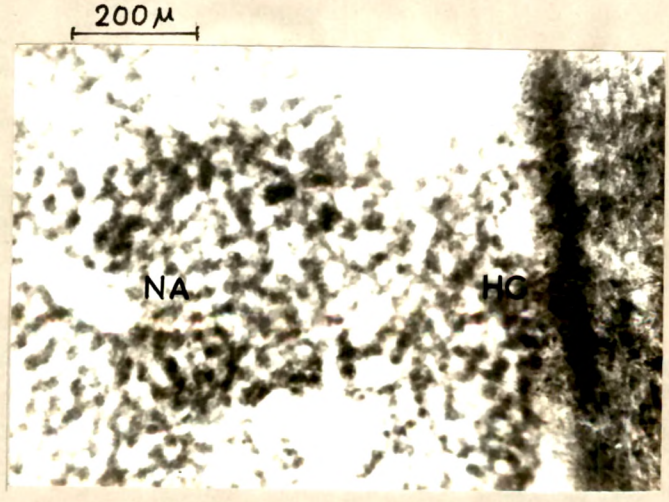
Fig. 24. 15 days after the infliction of the injury. The enzyme activity began to decrease in the normal area (NA) as well as in the newly formed (HC) region.



22



23



24

Fig. 25. Graphic representation of changes in the metabolites and various enzymes in the intact region near the 'zone' in the pigeon liver.

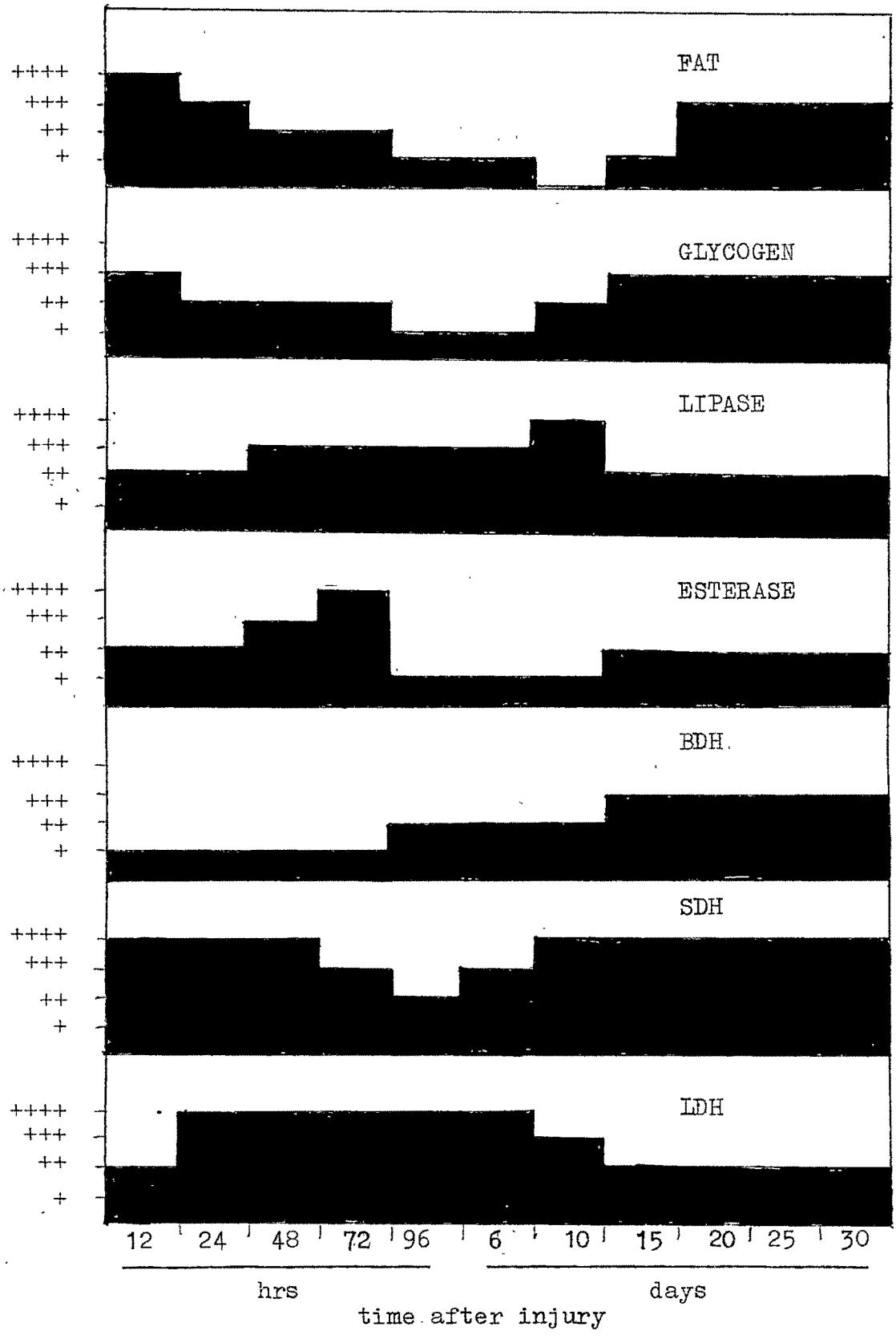
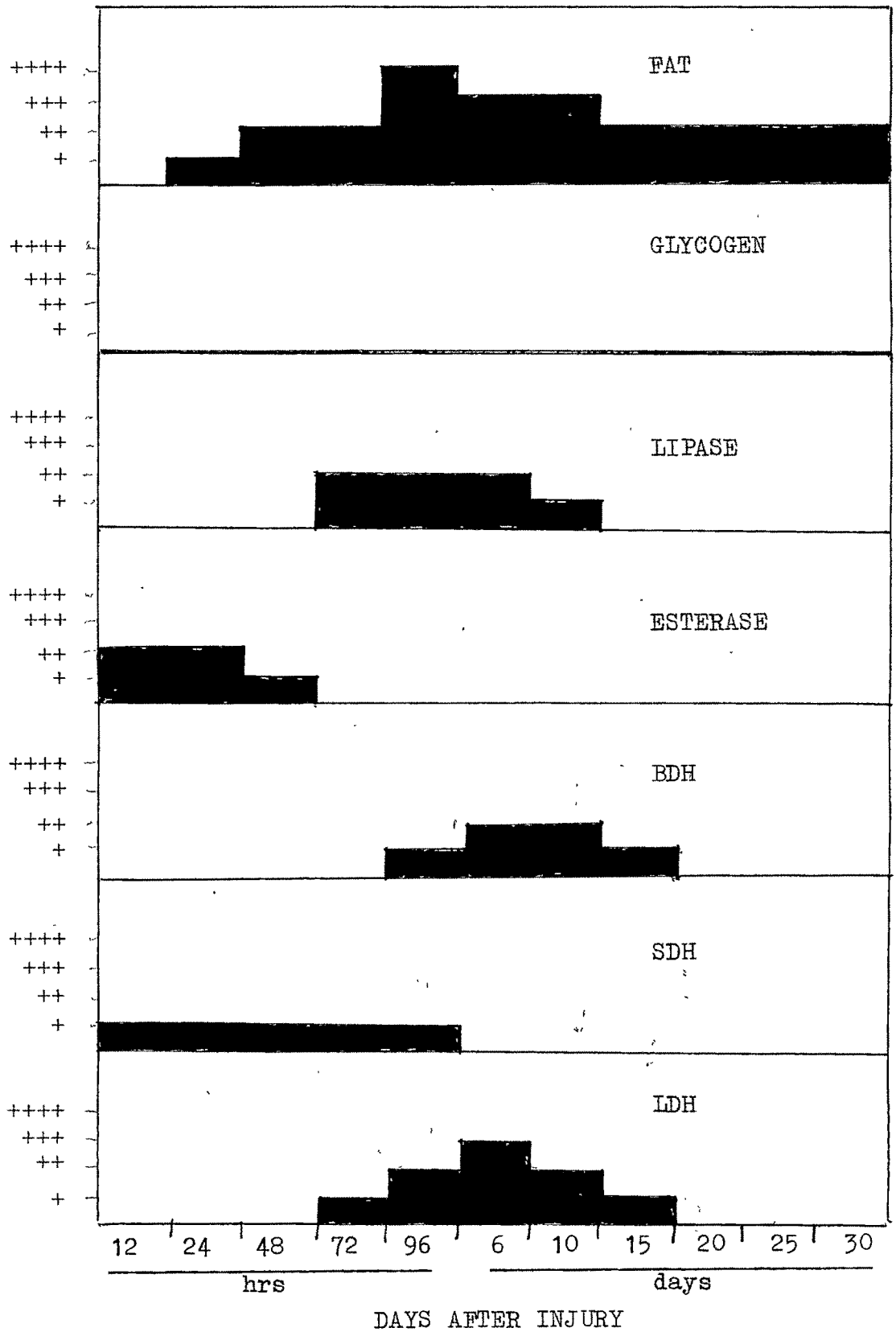


Fig. 26. Graphic representation of changes in the metabolites and various enzyme in the 'zone' near the injured area in the pigeon liver.



A comparative account of the concentrations of glycogen, neutral fat and the enzymes observed in the present study is presented diagramatically in (Figs. 25 & 26).

DISCUSSION

From the observations, it is clear that neutral fat accumulation takes place in the 'zone' (the region which demarcates the intact normal tissue from the injured one and has the composition of both normal and injured liver cells (Chapter 6). Most of the neutral fat accumulation was in the lymphocytes present in the 'zone'. Perhaps this neutral fat was either transported to the zone from healthy region of liver or it may be getting transported from the 'zone' to other regions. Possibility of fat transport by lymphocytes from lymphocytopoietic nodules of the pigeon liver has been suggested by Pilo, (1970).

Though it is clear that neutral fat gets accumulated in the 'zone' during the earlier part of wound healing, its significance is difficult to understand. Since it is known that degenerative activity is usually accompanied by fat accumulation (Rouiller, 1964), it could be reasoned that the accumulated fat in the 'zone'

is due to the degenerating process that takes place there. This fat may retard the wound healing process that is destined to take place there and hence is removed by the lymphocytes and macrophages. To aid the speedy removal of fat, lipase as well as esterase are active. It is interesting to note that an increased esterase activity has been reported in the cirrhotic mammalian liver by Wachstein (1963).

While degenerative activity is proceeding in the 'zone', an entirely different type of activity takes place in the intact region subjacent to the 'zone'. In this region also a progressive fat depletion was observed (Fig. 25). Normally, it could be reasoned that this decrease in fat content in this region is due to increased utilization or oxidation to produce energy. But it is unlikely that oxidative metabolism is stepped up at this time. This becomes evident from the fact that activities of neither BDH nor SDH were significantly increased during this period. Thus, the increased activity of lipase at the time (10th day) when fat was depleted maximally, could be to breakdown long chain fatty acids which are invariably removed from the site, perhaps as a preparation for the mitotic activities that were found to take place at this period. It is well known that the mitotic activities depend on the anaerobic metabolism rather than an

aerobic one. Hence, the reduction of stored fat and a concomitant reduction of oxidative metabolism could be a prerequisite for the initiation of mitotic activities. It is interesting to note that the activity of SDH decreased significantly between 4th and 6th day, a period when the initiation of mitotic activity took place (Chapter 6).

The decrease in the amount of glycogen was observed from 24 hrs after the infliction of injury (Table I and Fig. 25) which may be due to shock caused by injury. The maximum depletion of glycogen from healthy cells nearer to the 'zone', however, was observed between 4th and 6th day a period coincided with the mitotic activity of the parenchymal cells after the injury. Thus, the energy required for the wound repair, and especially for the division of hepatic cells situated subjacent to 'zone', evidently came from the breakdown of glycogen. The fact, that there was an increased anaerobic glycolysis during the time when mitotic activities of parenchymal cells took place, is also supported by the observation that LDH was very active during this period. These observations are in conformity with the oxygen consumption studies on the healing tissues by Szanto et al. (1963) which revealed that during early period of wound healing phase, anaerobic metabolism was effective.

Needham (1952) also reported similar changes in the metabolic pattern during wound healing in epithelial tissues.