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Intracellular enzymes and protein synthesis in rabbit skin after thermal injury

G. P. LEWIS, JUDY PETERS AND A. M. WHITE

CIBA Laboratories, Horsham, Sussex

Summary

1. The concentrations of several intracellular enzymes in rabbit skin have been measured 5 min, 2, 6 and 24 h after thermal injury.

2. At 5 min and 2 h after a burn (60° C for 1 min) there was a significant fall in the enzyme activities whereas at 6 h their activities were higher than control.

3. It appears that the increase in enzyme concentrations in the lymph during the first few hours after thermal injury is associated with a fall in the enzyme concentrations in the tissues and therefore might be the result of leakage of enzyme from storage sites in the injured cells.

4. The second increase in enzyme concentrations in the lymph which has been observed 6–18 h after thermal injury occurs at a time when there is also an increase in the enzyme concentrations in the tissue.

5. It seems unlikely that these increased activities are due to new synthesis since there was no apparent correlation between tissue enzyme concentrations and protein synthetic activity, and the changes still occurred after administration of cycloheximide.

6. There was a change in the LDH isoenzyme pattern after injury towards a predominance of LDH-1. This change did not occur immediately after the burn, but was present at 2 and 6 h, and returned to normal 24 h later.

Introduction

Lewis, Lowe, White & Worthington (1970) found that thermal injury to the hind limb of cats resulted in changes in the activity of several enzymes in the skin of the injured limb. When the injury was by heating at 80° C total acid phosphatase and β-glucuronidase activities fell significantly 4 h after injury whereas creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) activities were slightly, but not significantly, increased. However, all of these changes became significant when the limb was injured by freezing in dry ice. Accompanying the change in enzyme activities there was a marked reduction in overall protein synthetic activity of the skin, a fall in the concentration of glycogen and an increase in the proportion of LDH present as LDH-1.

This study in rabbits was principally designed to examine changes at earlier times after injury, and to compare the results with the changes found in hind limb lymph over this period. It had been shown earlier (Lewis, 1969) that after a mild thermal injury in rabbits there was an increase in the concentrations of cytoplasmic and mitochondrial enzymes in lymph and that the increase occurred in two phases. The

first phase of enzyme output reached a maximum 2–6 h after the burn and the second 10–16 h afterwards. It was of interest to know to what extent these changes in the lymph draining the hind limb reflected tissue changes. The role of protein synthesis was also examined and changes in glycogen and LDH-1 concentrations were measured.

Methods

Radioactive materials

L-[(U-^{14}C)] leucine (specific activity 311 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, Bucks) as a sterile aqueous solution containing 2% ethanol.

Drugs

Pentobarbitone sodium (Nembutal veterinary) was obtained as a sterile solution containing 60 mg/ml from Abbott Laboratories Ltd., Agro. Vet. Division, Queensborough, Kent. Cycloheximide (3[2(3,5-dimethyl-2-oxocyclohexl)-2-hydroxyethyl] glutarimide) was obtained from Sigma London Chemical Co. Ltd., London SW6.

Anaesthesia

New Zealand white rabbits weighing approximately 3 kg were anaesthetized with intravenous pentobarbitone sodium (40 mg/kg). The dose of anaesthetic was adjusted according to the depth of respiration and corneal and other reflexes. In experiments where skin was obtained 2 h and 6 h after burning, anaesthesia was maintained over this period. In experiments where skin was obtained 24 h after burning, the animal was allowed to recover after the initial dose of anaesthetic required during injury.

An attempt had been made to maintain the rabbits under anaesthesia for 24 h with urethane (1·5 g/kg) but this procedure itself reduced the protein synthetic activity of the skin to 69% of control. In a group of rats it was found that when anaesthesia was maintained for 7 h or longer, even with pentobarbitone sodium, protein synthesis on isolated ribosomes was reduced to 89%. Therefore, in these experiments anaesthesia was not prolonged for more than 6 hours.

Injury

A burn at the skin surface of 60° C was induced by immersing one limb of the rabbit in water at 61°–62° C for 1 minute. The contralateral limb was used as control.

Incorporation of amino-acids by isolated ribosomes

Skin ribosomes were prepared by the method of White & Worthington (1968). The final supernatant was layered over medium containing 1·5 M sucrose. The incubation procedures, method for protein isolation and counting and RNA estimation were as described by Bullock, White & Worthington (1968). Sucrose density gradients (20 ml) were prepared from 15% (w/v) to 50% (w/v) sucrose in medium containing 9 mM $MgCl_2$, 75 mM KCl, 35 mM tris-KCl buffer pH 7·4 at 37° C, by the method of Britten & Roberts (1960). Ribosomal suspensions (1·0 ml) were layered

on the gradients which were then centrifuged for 1·5 h at 128,000 *g* in a 3 × 23 ml MSE swing-out rotor.

A close-fitting Teflon cap equipped with two, thin stainless steel tubes was pushed into the centrifuge tube, and the gradient was pumped out from the bottom with 60% sucrose over 40 min by using a slow-infusion apparatus (C. F. Palmer Ltd., London, S.W.2). The E260 of the suspension was directly recorded by means of a Uvicord (LKB Instruments Ltd., Selsdon, Surrey) connected to a potentiometric recorder.

Incorporation of amino-acids into whole skin in vitro

Finely chopped skin was washed in Krebs-Henseleit medium to remove extraneous protein probably originating in the plasma. Chopped skin (200 mg) was incubated with L-[(U-^{14}C)] leucine and Krebs-Henseleit medium for 1 and 2 h at 37° C in an atmosphere of 95% O_2:5% CO_2. Incorporation of the label was terminated by precipitation of the protein with hot 10% (w/v) trichloracetic acid. The tissue was then homogenized with an Ultra Turrax homogenizer, run at 50% of the maximum speed for 1 minute. The proteinaceous pellet was washed twice in 3:1 (v/v) ethanol:ether, washed 3 times with hot 5% (w/v) trichloracetic acid, washed twice in absolute ethanol, and dissolved in 2 N NaOH, and counted in a Packard Tri-Carb liquid scintillation counter in glass vials with a scintillation fluid (Bray, 1960), containing Cab-O-Sil (4·5%). The alkaline protein solution was diluted 40-fold for estimation of protein by the method of Lowry, Roseborough, Farr & Randall (1951).

Glycogen

Glycogen from 100 mg skin was isolated and estimated by the anthrone reagent according to the method of Hassid & Abraham (1957).

Correction for water content of skin

As the skin became oedematous after burning, the wet weights of control and burned tissues were not comparable. In five experiments ribosomal RNA was isolated in the same proportion as the dry weight of the tissues. Thus, all calculations are corrected for a discrepancy in wet weight by a factor corresponding to the relative amounts of ribosomal RNA isolated from the tissue.

Preparation of tissue for enzyme determinations

Tissue homogenates for enzyme estimations were prepared in a medium containing sucrose (0·15 M), tris buffer, pH 7·4 at 37° C (0·1 M), KCl (0·185 M), and $MgCl_2$ (9 mM) (Leon, Arrhenius & Hultin, 1962). The tissue was maintained at less than 3° C throughout. Skin (4 g), previously shaved of fur, was finely chopped with scissors and homogenized in the medium with an Ultra Turrax homogenizer run at 50% of the maximum speed for 1 minute. The homogenate was then centrifuged at 1,000 *g* for 10 min to obtain a clear supernatant.

Enzyme analysis

Descriptions of the analysis and calculation of units of the following enzymes were given previously by Lewis *et al.* (1970): lactate dehydrogenase (LDH) (L-

lactate:NAD oxidoreductase, E.C.1.1.1.27); acid cathepsin (cathepsin D. E.C. 3.4.4.23); β-glucuronidase (β-D-glucuronide glucuronohydrolase, E.C. 3.2.1.31); acid phosphatase (orthophosphoric monester phosphohydrolase, E.C.3.1.3.2.); creatine phosphokinase (CPK) (ATP: creatine phosphotransferase, E.C.2.7.3.2.). Lactate dehydrogenase iosenzyme LDH-1 was measured after adsorption on DEAE Sephadex A-50 according to the Biochemica Test combination of C. F. Boehringer & Soehne GmbH.

Results

Intracellular enzymes in the skin

Table 1 shows the mean enzyme concentrations of homogenates prepared from burned and control skin. It may be seen that 5 min after burning at 60° C there were significant decreases in the activity of all the enzymes examined. Two hours after burning there was still a reduction in the activities of all the enzymes. The activities of β-glucuronidase and acid phosphatase were of the same order as those 5 min after injury. The activities of the cytoplasmic enzymes creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) although still reduced, had returned towards the normal activity; the level of CPK rose from 22% of control to 60% and the level of LDH rose from 62% of control to 77%. However, the decreases in activity of these two enzymes were still significant. The behaviour of the proteolytic enzyme cathepsin D differed from either of these patterns in that there was a further decrease of its activity from that at 5 min, from 43% of control to 25%.

At 6 h after the injury there was an increase in the activities of all lysosomal and cytoplasmic enzymes with the exception of cathepsin. All the activities were sig-

TABLE 1. *Enzyme concentrations in skin homogenates after burning at 60° C*

Time after burn		Cathepsin (μg/mg)	β-Glucuronidase (μg/mg)	Acid phosphatase (μg/g)	Creatine phosphokinase (μg/mg)	Lactate dehydrogenase (μg/g)
5 min	Mean B	0·37	18·9	208·6	1·22	4·35
	Mean C	0·85	23·5	286·7	5·48	6·97
	Mean difference	−0·48	−4·6	−78·1	−4·26	−2·62
	S.E.	±0·12	±0·95	±24·8	±0·62	±0·46
2 h	Mean B	0·31	28·5	320·9	2·98	8·23
	Mean C	1·25	36·3	445·6	5·01	10·71
	Mean difference	−0·94	−7·8	−124·7	−2·02	−2·48
	S.E.	±0·15	±2·86	±36·5	±0·51	±0·84
6 h	Mean B	0·32	40·3	427·1	6·09	20·39
	Mean C	1·91	32·5	353·1	3·45	15·41
	Mean difference	−1·59	+7·8	+74·0	+2·65	+4·99
	S.E.	±0·27	±1·91	±8·19	±0·43	±1·67
24 h	Mean B	0·38	57·1	520·2	8·06	14·86
	Mean C	0·75	34·2	507·2	7·14	12·78
	Mean difference	−0·37	+22·9	+13·0	+0·92	+2·08
	S.E.	±0·11	±8·02	±9·32	±0·26	±0·31

Enzyme estimations were made on a 1,000 *g* supernatant prepared from skin homogenates as described in **Methods**. One limb was burned at 60° C for 1 min and after the times indicated the animal was killed and the skin removed for analysis. The mean values for burned (B) and control (C) limbs are given. The mean differences represent a mean of six differences (burned from control limbs), together with the standard error (S.E.) of each difference from the mean. The differences were significant ($P<0·05$) in all cases.

nificantly increased above control values. On the contrary the activity of cathepsin was further reduced from 25 to 17% of control.

Twenty-four hours after injury the concentrations of all the enzymes except cathepsin were still significantly increased although the activity of LDH and CPK had fallen from 177 and 131% of control at 6 h, to 112 and 116%, respectively, at 24 h. There was a reduction in the concentration of acid phosphatase from 121% of control at 6 h to 104% at 24 hours. Cathepsin, too, tended to return towards normal since there was an increase in its concentration from 17% of control at 6 h to 50% at 24 hours.

The control values in Table 1 show considerable variation, possibly this is the result of an effect of the injury on the control limb mediated through some systemic change. In order to determine whether such an effect was occurring, experiments were designed in which control sample biopsies of skin were removed from both limbs before injury, and then further skin samples were taken from both limbs after injury to one limb. Table 2 shows the results obtained and comparisons of enzyme activities in samples from different limbs, and from the same limb before and after injury. No significant difference in activity was observed between samples taken from the two limbs before injury, or between samples taken from the control limb before and after injury. Significant differences were seen, however, in samples from the burned limb before and after injury, and in samples from both limbs after injury to one.

The injury thus has no effect on the control limb, so that the technique used to obtain the results in Table 1 is valid.

TABLE 2. *Enzyme concentrations in skin homogenates taken from the same limbs before and 6 h after burning at 60° C*

Enzyme		Time after injury (h) 0	Time after injury (h) 6	Mean difference ± S.E.	
Lactate dehydrogenase (μg/g)	Mean B	8·73	11·51	+2·77±0·73	$P'<0·02$
	Mean C	8·83	8·94	+0·11±0·58	N.S.
	Mean difference ± S.E.	+0·10±0·69 N.S.	+2·57±0·46 $P<0·01$		
Lactate dehydrogenase isoenzyme (% total LDH)	Mean B	27·2	33·1	+5·9±1·77	$P'<0·01$
	Mean C	26·4	26·4	+0·01±1·74	N.S.
	Mean difference ± S.E.	+0·77±5·09 N.S.	+6·73±2·57 $P<0·05$		
Creatine phosphokinase (μg/mg)	Mean B	4·08	5·47	+1·39±1·63	N.S.
	Mean C	2·70	1·37	+1·33±0·72	N.S.
	Mean difference ± S.E.	−1·38±0·92 N.S.	+4·10±1·52 $P<0·05$		
β-Glucuronidase (μg/mg)	Mean B	38·6	45·6	+6·9±3·88	$P'<0·05$
	Mean C	37·1	36·7	−0·4±3·17	N.S.
	Mean difference ± S.E.	+1·4±0·79 N.S.	+8·9±1·8 $P<0·01$		

Enzyme estimations were made on a 1,000 *g* supernatant prepared from skin homogenates as described in **Methods.** Skin samples (0·5 g) were taken from both hind limbs and then one limb was burned at 60° C for 1 minute. Further skin samples were taken from burned (B) and control (C) limbs 6 h later. The mean differences represent a mean of six differences. *P* is the significance between samples from different limbs taken before and after injury. *P'* is the significance between samples taken from the same limb before and after injury. N.S. = no significant difference ($P>0·05$).

Isoenzyme lactate dehydrogenase-1

Table 3 shows that 5 min after the injury the percentages of LDH-1 in burned and control skin were identical. At 2 h, however, the percentage of LDH-1 in burned skin was nearly twice that of control skin. At 6 h, it was approximately 1·5 times that of control skin, while 24 h after the burn the isoenzyme pattern had returned to that of the control.

Protein synthesis

The effect of burning on the activity of ribosomes isolated from skin is shown in Table 4. The results which are means of five experiments show that 5 min and 2 h after the burn, incorporation of amino-acids into isolated ribosomes was reduced by 38% and 40% respectively. However, 6 h after, the lower synthesis was reduced by only 14%, which was no longer a significant reduction. After 24 h it was increased by 67% and single experiments (which are not included in the table), in which activity was measured at 16, 27, 48, 60 and 72 h after injury, showed that this increase in synthetic activity is maintained for at least 3 days after the injury. This effect might well be associated with a regenerative phase of the injured skin.

The reduction in protein synthetic activity 5 min and 2 h after injury was not due to the presence of a greater ribonuclease activity in the burned skin than in the control. This was shown by an experiment in which a postnuclear supernatant,

TABLE 3. *Effect of burning at 60° C on the percentage of skin LDH present as LDH-1*

Time after burn		Per cent LDH-1	Mean difference ±S.E.
5 min	Mean B (5)	27	0
	Mean C	27	
2 h	Mean B (5)	61	+26±3·6
	Mean C	35	$P<0·01$
6 h	Mean B (4)	55	+20±3·01
	Mean C	35	$P<0·01$
24 h	Mean B (4)	32	+2±3·04
	Mean C	34	N.S.

Experiments, enzyme estimations and statistical calculations were carried out as in Table 1. The number of animals in each experiment is shown in brackets.

TABLE 4. *Effect of a burn at 60° C on the amino-acid incorporating ability of skin ribosomes*

	Incorporation in d.p.m./μg ribosomal RNA			
	Time after burn			
	5 min	2 h	6 h	24 h
Mean B	37·36	27·69	33·76	64·08
Mean C	60·16	45·81	39·45	38·34
Mean diff.	−22·80	−18·12	−5·69	+25·74
S.E.	±2·39	±2·44	±3·69	±8·23
Significance	$P<0·001$	$P<0·01$	N.S.	$P<0·05$

Ribosomes were isolated and incubated as described in **Methods.** Only control (C) and test ribosomes (B) for each of the different time intervals were prepared and incubated at the same time and under the same conditions, making comparisons between groups taken at different times impossible. There were five animals in each group. N.S.=not significant.

treated with 1% sodium desoxycholate, was incubated at 37° C for 5 and 10 min before incubation with ribosomes. The results in Fig. 1 show that the incorporating ability of ribosomes isolated from both control and injured skin was not reduced after 5 min of incubation, and after 10 min the small reductions in activity were similar in control and test samples. Since all operations were carried out in the cold after killing the animals, ribosomal activity in both burned and control skin would appear to be completely stable at the temperatures and over the time periods involved.

In order to characterize the fraction active in protein synthesis, sucrose density gradient centrifugation studies of the ribosomal fraction were undertaken. Such gradients showed a large peak which corresponded in sedimentation value to monomeric and dimeric ribosomes, with only a small shoulder of polysomal units. This pattern was present in both burned and control skin. After fractionation of the density gradient it was shown that incorporation of amino-acids was mainly associated with the monomeric ribosomes. This result is in agreement with the findings of Freedberg (1970) that incorporation was mainly associated with monomeric ribosomes isolated from hair roots, and with Freedberg, Fine & Cordelle (1967) who observed a similar association in cell-free ribosomes in mammalian skin.

In these studies the ribosomes were supported by an artificial medium of enzymes and energy producing systems since they were incubated with liver cell sap. Therefore, any effect of the injury on the system of enzymes supporting protein synthesis in the skin would not be evident. In an attempt to overcome this deficiency three experiments were designed in which whole pieces of skin were incubated with a labelled radioactive precursor. Skin was removed from the limbs 5 min, 2 and 6 h after the injury in order to reproduce the conditions under which ribosomal activity had been investigated. The result was a reduction of incorporating ability of whole skin by 34% and 50% of control 5 min and 2 h after the burn. This result agreed with the reduced incorporation observed with ribosomes at the same intervals after the burn (Table 4) and supports the view that dysfunctional ribosomes are directly responsible for the reduced protein synthetic activity of injured skin.

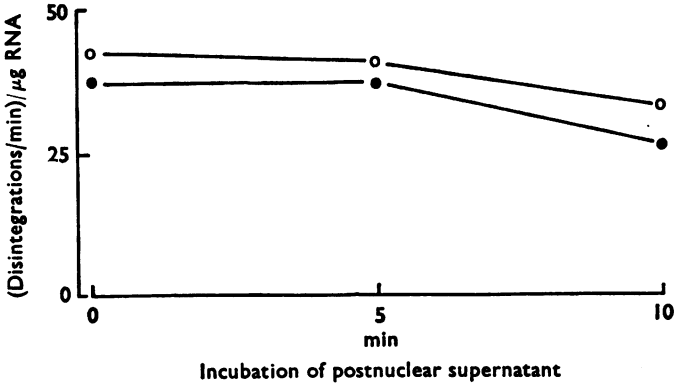

FIG. 1. Amino-acid incorporating ability of ribosomes isolated from the skin of a control limb (○——○) and a limb burned at 60° C (●——●). The ribosomes were prepared from a postnuclear supernatant, which, having been treated with desoxycholate was incubated at 37° C for 5 min and 10 minutes.

Effect of cycloheximide

In cats Lewis *et al.* (1970) showed that increases in the activity of several intracellular enzymes in the skin of the hind limb and the lymph draining the limb 4 h after a thermal injury were not influenced by prior administration of an inhibitor of protein synthesis, cycloheximide.

The results of Table 5 show a similar finding in rabbits. It may be seen that the increases in the activities of the enzymes β-glucuronidase, acid phosphatase, LDH and CPK after a 60° C burn, remained unaffected by inhibition of protein synthesis. In all experiments, the control concentrations of all the enzymes became depressed with cycloheximide and thus the increase in activity resulting from burning was exaggerated when expressed as a percentage of control.

In three experiments which were carried out as described by Lewis (1969) the increases of cytoplasmic enzymes LDH and CPK in the lymph following a 60° C burn to the hind limb still occurred after administration of cycloheximide.

Glycogen

The results of Table 6 show the effect of burning on the glycogen concentrations in skin. Five minutes after the burn the glycogen remained at control concentration. However, 2 h after the burn the glycogen concentration fell significantly to 72% of control, and 6 h after the burn it remained low (77% of control), but the reduction was not then significant. Twenty-four hours after the injury the glycogen concentration had not only returned to control concentration but had increased to 48% above control.

TABLE 5. *Enzyme concentrations in skin homogenates after a 60° C burn and treatment with cycloheximide*

	Cathepsin (μg/mg)	β-Glucuronidase (μg/mg)	Acid phosphatase (μg/g)	Creatine phosphokinase (μg/mg)	Lactate dehydrogenase (μg/g)
Mean B	0·72	42·79	291·91	33·17	37·12
Mean C	1·00	20·02	165·74	1·73	5·05
Mean difference	−0·28	+22·77	+126·17	+31·44	+32·07
S.E.	±0·06	±2·22	±15·06	±6·19	±7·34
Significance	$P<0·02$	$P<0·01$	$P<0·01$	$P<0·01$	$P<0·02$

Enzyme estimations were made on a 1,000 *g* supernatant of skin 6 h after injury as described in **Methods.** Cycloheximide (30 mg/kg) was administered intravenously immediately before injury.

TABLE 6. *Effect of burning at 60° C on glycogen concentrations in skin*

	Glycogen μg/mg skin (wet weight)			
	Time after burn			
	5 min	2 h	6 h	24 h
Mean burned	1·13	0·71	0·75	0·99
Mean control	1·11	0·99	0·98	0·67
Mean difference	+0·02	−0·28	−0·22	+0·32
S.E.	±0·18	±0·04	±0·17	±0·14
Significance	N.S.	$P<0·01$	N.S.	$P<0·05$
% of control	102	72	77	148
Number of animals	5	4	3	5

N.S. = not significant.

Discussion

Our results in rabbits are in general agreement with previous findings in cats (Lewis, *et al.*, 1970). In cats the various parameters were measured only 4 h after the thermal injury. It is now clear that certain features of the changes may have been missed. Our findings show that almost immediately after injury the activities of all the enzymes examined in the skin were significantly reduced. However, at 6 h the situation was different and the enzyme activities were higher than the control concentrations. Thus in the experiments with cats when the tissues were examined 4 h after the burn the concentrations of LDH and CPK had already started to increase, whilst those of β-glucuronidase, acid phosphatase and cathepsin were still reduced. It appears that cathepsin takes the longest time to recover from the injury since, although it had started to increase, the concentration in the tissues had not reached control concentration even at 24 h after injury.

The change in the proportion of the isoenzyme LDH-1 after thermal injury was observed in these experiments just as it was earlier in cats. However, in these experiments the change had not occurred at 5 min after injury, which means that it is not the direct result of the increase in temperature during the burn, and in addition the isoenzyme pattern was already starting to revert to normal at 6 h and had become normal at 24 h after the burn.

Our findings are also consistent with the changes observed in the lymph draining the burned limb (Lewis, 1969). It has been shown that the concentrations of cytoplasmic enzymes in the hind limb lymph also increase immediately after injury, reaching a maximum at 2–4 hours. Further, a second increase in enzyme activities in lymph occurred, reaching a maximum between 10 and 16 hours. It appears, therefore, that the first increase in the lymph corresponded to a reduction of enzyme activity in the tissues, while the second peak occurred at a time when there was also an increase of enzyme concentrations in the tissue.

In earlier investigations in cats (Lewis et al., 1970), it was concluded that the increase in the activity of lymph enzymes during the first 4 h after injury was not the result of leakage since tissues taken at 4 h already showed increased enzyme activities. It is possible therefore that this interpretation was incorrect since our experiments in rabbits are consistent with the view that enzyme protein leaves the tissue and passes to the lymph during the first few hours after injury.

It seemed possible that the increase in the activities of the enzymes in the tissues which occurred 6 h after injury and their appearance in the lymph during the subsequent hours might be the result of enzyme synthesis. This possibility arose because although protein synthesis was reduced after the burn it was recovering at 6 h and had increased over the normal level at 24 h after injury. However, using the inhibitor of protein synthesis, cycloheximide, the changes in both tissues and lymph still occurred. The nature of the second increase is therefore still not evident.

Little can be said about the overall metabolic state of the skin immediately after this type of injury but some aspects should be mentioned. The net loss of glycogen in the tissue suggests, if glucose uptake and utilization are not impaired, a move towards anaerobic glycolysis. At the same time, however, the increased concentration of LDH-1, the isoenzyme which is inhibited by pyruvate, suggests a higher rate or aerobic metabolism. This paradox might be explained if the skin mitochondria become uncoupled during the process of injury. In this case increased respiration

would not lead to the production of ATP at the optimum rate, therefore, breakdown of glycogen might ensue in an attempt to make up the deficit.

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