REFERENCES

- Avigad, G. (1959). *Biochem. J.* **73**, 587.
 Bailey, R. W. (1959). *Biochem. J.* **72**, 42.
 Bailey, R. W., Barker, S. A., Bourne, E. J. & Stacey, M. (1957). *J. chem. Soc.* p. 3536.
 Bailey, R. W. & Bourne, E. J. (1959). *Nature, Lond.*, **184**, 904.
 Bailey, R. W. & Oxford, A. E. (1958*a*). *Nature, Lond.*, **182**, 185.
 Bailey, R. W. & Oxford, A. E. (1958*b*). *J. gen. Microbiol.* **19**, 130.
 Bourne, E. J., Hutson, D. H. & Weigel, H. (1959). *Chem. & Ind.* p. 1047.
 Bourne, E. J., Hutson, D. H. & Weigel, H. (1960). *Chem. & Ind.* p. 1111.
 Bragg, P. D. & Hough, L. (1957). *J. chem. Soc.* p. 4347.
 Dain, J. A., Neal, A. L. & Seely, H. W. (1956). *J. Bact.* **72**, 209.
 Feingold, D. S., Avigad, G. & Hestrin, S. (1956). *Biochem. J.* **64**, 351.
 Foster, A. B. (1953). *J. chem. Soc.* p. 982.
 Hehre, E. J. (1951). *Advanc. Enzymol.* **11**, 297.
 Koepsell, H. J., Tsuchiya, H. M., Hellman, N. N., Kazenko, A., Hoffmann, C. A., Sharpe, E. S. & Jackson, R. W. (1953). *J. biol. Chem.* **200**, 793.
 Schwimmer, S. & Bevenue, A. (1956). *Science*, **123**, 543.
 Shaffer, P. A. & Hartmann, A. F. (1921). *J. biol. Chem.* **124**, 425.
 Sharpe, E. S., Stodola, F. H. & Koepsell, H. J. (1954). *Abstr. Pap. Amer. Chem. Soc.* p. 5-D.
 Sharpe, E. S., Stodola, F. H. & Koepsell, H. J. (1960). *J. org. Chem.* **25**, 1062.
 Stodola, F. H., Sharpe, E. S. & Koepsell, H. J. (1956). *J. Amer. chem. Soc.* **78**, 2514.
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
 Tsuchiya, H. M., Koepsell, H. J., Carman, J., Bryant, G., Bogard, M. O., Ferger, V. H. & Jackson, R. W. (1952). *J. Bact.* **64**, 521.
 Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.
 Wise, C. S., Dimler, R. J., Davis, H. A. & Rist, C. E. (1955). *Analyt. Chem.* **27**, 33.
 Yemm, E. W. & Willis, A. J. (1954). *Biochem. J.* **57**, 508.

Biochem. J. (1961) **79**, 553

The Effect of Hind-Limb Ischaemia on the Products and Intermediates of Glycolysis in Rat Tissues

By C. J. THRELFALL AND H. B. STONER

Toxicology Research Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey

(Received 22 August 1960)

In small mammals the survival period after fatal limb ischaemia can be divided into two parts. In the first phase, under ordinary environmental conditions (air temperature 18–22°), the temperatures of the main heat-producing organs in the rat, the liver and brain, fall before their oxygenation is impaired (Stoner, 1958*a*). This has been interpreted as an effect of injury on the control of heat production (see Stoner & Threlfall, 1960; Stoner, 1960). In the second phase, when the animal is dying, the physiological and biochemical changes are dominated by the gradually increasing hypoxia of the tissues. Our interest has been focused on the first stage and attempts to elucidate the mechanism of its changes have so far yielded the following information.

The injured rat's condition cannot be explained by depletion of the stores of energy-rich phosphates (Goranson, Hamilton & Haist, 1948; Stoner & Threlfall, 1954; Threlfall & Stoner, 1957). After injury the catabolism of glucose is inhibited in the early stages of its breakdown and in the oxidation of the pyruvate formed from it (Stoner, Heath & Collins, 1960). At the same time it would appear

that ischaemic limb injury does not damage liver mitochondria, in so far as this can be judged from their behaviour *in vitro* after isolation (Aldridge & Stoner, 1960).

We have now investigated the concentrations of glycolytic products and intermediates in the tissues during the first part of the survival period, determining them chromatographically (except for lactate) since the methods used previously (LePage, 1946; Threlfall & Stoner, 1954) were inadequate. The effect of hind-limb ischaemia has been studied under different environmental and endocrinological conditions to determine the effect of varying the intensity of the response to injury. Some of the results have been mentioned in reviews (Stoner & Threlfall, 1960; Stoner, 1960).

METHODS

Animal techniques. Male albino rats (242 ± 36 g. body wt.) of the Porton strain were used. M.R.C. diet 41B (Bruce & Parkes, 1956) was available until the start of the experiment except when 'fasting' rats were used, when it was removed from the cages on the day before the experiment.

The methods for producing limb ischaemia, cold-acclimatization, adrenalectomy and adrenal-medullectomy and altering environmental conditions have been described previously (Stoner, 1958*a, b*). The cold-acclimatized rats were given supplementary ascorbic acid (150 mg. by mouth two or three times/week) and used after they had been at 3° for 58–77 days. Adrenalectomized rats, given 1% (w/v) NaCl to drink, were used 3–4 days after operation. Adrenal-medullectomized rats were used 50–103 days after operation. Liver samples were obtained from guillotined rats or rats anaesthetized with sodium pentobarbitone [5 mg. of Veterinary Nembutal (Abbott)/100 g. body wt., intraperitoneally]. Muscle samples were taken under sodium pentobarbitone anaesthesia. In all cases the samples were frozen immediately in liquid N₂.

Analytical methods. The phosphorylated glycolytic intermediates were estimated as previously described (Threlfall, 1957), the equivalent of 0.5 g. of each tissue being used for chromatographic separation. Glucose 6-phosphate and fructose 6-phosphate were not adequately separated by the solvent systems used and were pooled. This mixture is described in the text as hexose 6-phosphate. Lactate was determined by the method of Barker & Summerson (1941). Pyruvate and α -oxoglutarate were determined chromatographically with the solvent system of El Hawary & Thompson (1953) on extracts prepared in a Nelco 10 homo-

genizer by the method of Cavallini & Frontali (1954). The control values were similar to those obtained by these workers and by Neish (1953).

Results have been expressed as μ moles of compound/g. wet wt. of tissue and where possible are shown as mean $\pm$ standard deviation. The changes found in muscle could not be accounted for by alterations in its water content (Threlfall & Stoner, 1957). The size and water content of the liver were unaffected by limb ischaemia. In 12 intact control rats the liver weight was $3.4 \pm 0.69/100$ g. body wt. and its mean water content $71.2 \pm 0.5\%$. In 12 intact rats 3 hr. after a 4 hr. period of bilateral hind-limb ischaemia the corresponding values were $3.2 \pm 0.43/100$ g. body wt. and $71.2 \pm 0.8\%$. When possible statistical comparison of the means has been carried out according to Student's *t* test as modified by Fisher (1934) for small samples.

RESULTS

The physiological disturbance in the rat after bilateral hind-limb ischaemia for 4 hr. has been described previously (Threlfall & Stoner, 1954, 1957; Stoner, 1958*a, b*; Stoner & Threlfall, 1960). In fed rats at an air temperature of 18–22° this injury has an 85% mortality rate with a survival

Table 1. *Phosphorylated glycolytic intermediates in injured muscle*

Effect of a 4 hr. period of bilateral hind-limb ischaemia in fed rats on the phosphorylated glycolytic intermediates in the injured hind-limb muscle and uninjured fore-limb muscle. Samples were obtained under sodium pentobarbitone anaesthesia. Number of rats is shown in parentheses. Environmental temperature 18–22°.

Compound	Controls	End of hind-limb ischaemia	3 hr. after hind-limb ischaemia
		Injured muscle	
Glucose 1-phosphate	0.20 $\pm$ 0.11 (7)	0.41 $\pm$ 0.48 (5)	0.18 $\pm$ 0.11 (6)
Hexose 6-phosphate	1.12 $\pm$ 0.37 (8)	2.05 $\pm$ 0.81* (5)	0.60 $\pm$ 0.21† (6)
Fructose 1:6-diphosphate	0.22 $\pm$ 0.06 (8)	0.08 $\pm$ 0.03* (5)	0.13 $\pm$ 0.08 (6)
Phosphoglycerate	0.13 $\pm$ 0.04 (6)	0.19 (2)	0.17 $\pm$ 0.07 (5)
α -Glycerophosphate	0.51 $\pm$ 0.18 (6)	1.59 $\pm$ 0.39* (5)	0.51 $\pm$ 0.21 (5)
		Uninjured muscle	
Glucose 1-phosphate	0.15 $\pm$ 0.04 (7)	0.23 $\pm$ 0.22 (4)	0.25 $\pm$ 0.13 (7)
Hexose 6-phosphate	0.99 $\pm$ 0.46 (8)	1.72 $\pm$ 0.89 (4)	1.46 $\pm$ 0.43 (7)
Fructose 1:6-diphosphate	0.25 $\pm$ 0.07 (8)	0.27 $\pm$ 0.13 (4)	0.35 $\pm$ 0.08† (7)
Phosphoglycerate	0.16 $\pm$ 0.03 (5)	0.23 (2)	0.18 $\pm$ 0.11 (6)
α -Glycerophosphate	0.53 $\pm$ 0.14 (5)	0.75 $\pm$ 0.40 (3)	0.62 $\pm$ 0.22 (5)

* Difference significant at $P < 0.001$.

† Difference significant at $P < 0.05$.

time of about 13 hr. Previous fasting, adrenal-medullectomy, adrenalectomy and raising the environmental temperature all increase the mortality rate to 100% and shorten the survival time, whereas after cold-acclimatization the mortality rate, at an air temperature of 18–22°, is reduced to 23% and the time to death is greatly prolonged in those which die.

Muscle

In the controls there was no difference between the results on fore- and hind-limb samples (Table 1) or on samples taken at different times of the day. The hexose monophosphate concentrations in the control samples were of the same order as those reported by Cori & Cori (1931) and Frunder, Richter, König & Börnig (1959), but lower than those given by Conway & Hingerty (1946), LePage (1948) and Derache, Trémolières, Griffaton & Lowy (1956).

At the end of bilateral hind-limb ischaemia for 4 hr. there were significant increases in the hexose 6-phosphate and α -glycerophosphate concentra-

tions in the injured hind-limb muscle and a fall in its fructose 1:6-diphosphate concentration. There were no significant changes in the fore-limb muscle at this time (Table 1). Three hours after the tourniquets had been removed the only significant changes were a fall in the hexose 6-phosphate concentration in the injured muscle and an increase in that of fructose 1:6-diphosphate in the uninjured muscle (Table 1). The increases in the concentrations of glucose 1-phosphate and glucose 6-phosphate did not quite reach significance at $P < 0.05$. In four medullectomized rats the concentrations of glucose 1-phosphate, hexose 6-phosphate and fructose 1:6-diphosphate in the uninjured muscle 3 hr. after removal of the tourniquets were 0.14 ± 0.08 , 0.99 ± 0.49 and 0.22 ± 0.11 μ mole/g. wet wt. respectively (medullectomized control values were within the range for intact rats).

The concentrations of the products of glycolysis, together with α -oxoglutarate, in the uninjured fore-limb muscle of intact and medullectomized rats, are shown in Table 2. The pyruvate concentration was higher and the lactate/pyruvate ratio lower than

Table 2. *Concentration of lactate, pyruvate and α -oxoglutarate in rat muscle*

The muscle was removed from the fore-limbs of control intact and medullectomized rats, and from intact rats 3 hr. and medullectomized rats 2.5 hr. after a 4 hr. period of bilateral hind-limb ischaemia. Samples were removed under sodium pentobarbitone anaesthesia. Environmental temperature 19°.

Treatment	Pyruvate	Lactate	α -Oxoglutarate	Lactate/pyruvate ratio
	[μ moles/g. wet wt. (mean $\pm$ s.d.)]			(mean $\pm$ s.d.)
Control	0.113 ± 0.034 (5)	4.71 ± 1.47 (6)	0.007 ± 0.004 (5)	47 ± 14 (5)
Experimental	$0.244 \pm 0.088^*$ (6)	6.42 ± 2.81 (6)	0.007 ± 0.002 (6)	$27 \pm 6^*$ (6)
Control†	0.083 (2)	2.83 (2)	0.010 (2)	34.4 (2)
Experimental†	0.059 ± 0.014 (4)	2.39 ± 0.55 (4)	0.007 ± 0.001 (4)	41 ± 10 (4)

* Significantly different from corresponding control at $P < 0.05$.

† Medullectomized.

Table 3. *Phosphorylated glycolytic intermediates in rat liver*

Liver samples were taken from intact rats under sodium pentobarbitone anaesthesia; rats were given access to food up to the start of the experiment. All rats except those killed at 10.0 a.m. were given an ether anaesthetic lasting 3 min. at 10.0 a.m. on the day of sampling. Environmental temperature 18–22°.

Time	Duration of fast (hr.)	No. of rats	Glucose 1-phosphate	Hexose 6-phosphate	Fructose 1:6-diphosphate
			[μ mole/g. wet wt. (mean $\pm$ s.d.)]		
10.0 a.m.	0	6	0.38 ± 0.13	0.55 ± 0.17	0.23 ± 0.10
2.0 p.m.	4	6	0.47 ± 0.19	$0.83 \pm 0.25^*$	0.29 ± 0.06
5.0 p.m.	7	5	0.53 ± 0.15	0.60 ± 0.12	0.27 ± 0.05
3.0 a.m.	17	2	Not detected	0.33	0.33
10.0 a.m.	24	5	$0.12 \pm 0.01^\dagger$	0.68 ± 0.31	$0.38 \pm 0.09^*$
2.0 p.m.	28	4	$0.12 \pm 0.12^*$	0.46 ± 0.14	$0.38 \pm 0.02^*$
5.0 p.m.	31	3	Trace	0.45 ± 0.11	0.33 ± 0.13

* Difference significant at $P < 0.05$.

† Difference significant at $P < 0.01$.

Table 4. *Phosphorylated glycolytic intermediates in rat liver*

Effect of 4 hr. bilateral hind-limb ischaemia (10.0 a.m.-2.0 p.m.) on the phosphorylated glycolytic intermediates in the livers of intact rats given access to food until the beginning of the experiment (fed rats) or fasted for 24 hr. before the experiment (fasted rats). Environmental temperature 18-22°. All results are expressed as $\mu\text{moles/g. wet wt. (mean} \pm \text{s.d.)}$.

Results in injured rats are compared with corresponding controls and results in guillotined controls are compared with those in the corresponding controls sampled under sodium pentobarbitone.

Time after removal of tourniquets (hr.)	Treatment	Method of sampling	No. of rats	Fed rats					
				Glucose 1-phosphate	Hexose 6-phosphate	Fructose 1:6-diphosphate	Phospho-glycerate	α -Glycero-phosphate	β -Glycero-phosphate
—	Control (2.0 p.m.)	Pentobarbitone	6	0.47 $\pm$ 0.19	0.83 $\pm$ 0.25	0.29 $\pm$ 0.06	0.18 $\pm$ 0.07	1.91 $\pm$ 0.25	0.81 $\pm$ 0.32
0	Limb ischaemia	Pentobarbitone	5	0.54 $\pm$ 0.19	0.71 $\pm$ 0.32	0.25 $\pm$ 0.07	0.22 $\pm$ 0.07	1.76 $\pm$ 0.37	0.72 $\pm$ 0.25
—	Control (5.0 p.m.)	Pentobarbitone	5	0.53 $\pm$ 0.15	0.60 $\pm$ 0.12	0.27 $\pm$ 0.05	0.18 $\pm$ 0.09	2.10 $\pm$ 1.07	0.62 $\pm$ 0.22
3	Limb ischaemia	Pentobarbitone	6	0.53 $\pm$ 0.17	0.56 $\pm$ 0.23	0.39 $\pm$ 0.06†	0.23 $\pm$ 0.08	2.27 $\pm$ 1.01	0.99 $\pm$ 0.60
—	Control (5.0 p.m.)	Guillotine	6	0.46 $\pm$ 0.16	0.95 $\pm$ 0.19†	0.16 $\pm$ 0.04†	0.21 $\pm$ 0.08	0.50 $\pm$ 0.24*	0.24 $\pm$ 0.17*
3	Limb ischaemia	Guillotine	6	0.41 $\pm$ 0.07	0.81 $\pm$ 0.42	0.34 $\pm$ 0.09†	0.89 $\pm$ 0.57†	0.61 $\pm$ 0.40	0.41 $\pm$ 0.27
—	Control† (5.0 p.m.)	Pentobarbitone	8	0.48 $\pm$ 0.16	1.15 $\pm$ 0.33	0.44 $\pm$ 0.14	0.25 $\pm$ 0.07	1.61 $\pm$ 0.39	0.84 $\pm$ 0.19
3	Limb ischaemia†	Pentobarbitone	9	0.23 $\pm$ 0.10†	0.84 $\pm$ 0.35	0.46 $\pm$ 0.07	0.20 $\pm$ 0.07	2.06 $\pm$ 0.55	0.98 $\pm$ 0.11
Fasted rats									
—	Control (2.0 p.m.)	Pentobarbitone	4	0.12 $\pm$ 0.12	0.46 $\pm$ 0.14	0.38 $\pm$ 0.02	0.14 $\pm$ 0.03	1.82 $\pm$ 0.08	0.65 $\pm$ 0.06
0	Limb ischaemia	Pentobarbitone	5	0.18 $\pm$ 0.05	0.74 $\pm$ 0.18*	0.43 $\pm$ 0.07*	0.19 $\pm$ 0.03*	2.35 $\pm$ 0.24†	1.05 $\pm$ 0.22*
—	Control (5.0 p.m.)	Pentobarbitone	3	Trace	0.45 $\pm$ 0.11	0.33 $\pm$ 0.13	0.10 $\pm$ 0.06	1.68 $\pm$ 0.34	0.68 $\pm$ 0.31
3	Limb ischaemia	Pentobarbitone	6	Trace	0.88 $\pm$ 0.38	0.57 $\pm$ 0.13*	0.22 $\pm$ 0.07	1.64 $\pm$ 0.79	0.54 $\pm$ 0.52

* Difference significant at $P < 0.05$.

† Difference significant at $P < 0.01$.

‡ Cold-acclimatized rats.

in the controls after removal of the tourniquets in the intact but not in the medullectomized rats.

Liver

Method of sampling. Most of the liver samples were obtained under light surgical anaesthesia produced by sodium pentobarbitone but some were quickly excised after guillotining. The results for the control samples obtained by these two methods differed (Tables 4 and 7). The concentrations of fructose 1:6-diphosphate, α -glycerophosphate, β -glycerophosphate and lactate were higher and that of hexose 6-phosphate was lower in the samples taken under anaesthesia. The reason for the change in hexose 6-phosphate is obscure but the other changes would be explained if barbiturate anaesthesia altered the equilibrium between the reduced and oxidized forms of diphosphopyridine nucleotide in favour of the reduced form (Gilmore, 1958), provided that the anaesthesia did not significantly alter the rate of glucose utilization (Stoner *et al.* 1960). Since rats can be guillotined with very little disturbance the values obtained by that method may approximate more closely to those *in vivo*. The concentrations of the phosphorylated glycolytic intermediates in the controls were lower than those reported by LePage (1948) and Derache *et al.* (1956). They agreed more closely with those obtained by ion-exchange chromatography on rabbit liver by Sacks, Kuroda & Hurley (1955).

The concentrations of hexose 6-phosphate, glucose 1-phosphate and fructose 1:6-diphosphate varied with the rat's nutritional state (Table 3), so that the results in injured rats were always compared with those in controls killed at the same time of the day.

Phosphorylated glycolytic intermediates. In fed intact rats the main change was in the fructose 1:6-diphosphate fraction, in which an increase was found with both methods of sampling after the tourniquets had been removed (Table 4). In the samples from guillotined rats there was also an

increase in the phosphoglycerate concentration possibly related to the increase in the amount of pyruvate present in these samples (see below). In fasted rats there were more widespread changes at the end of the period of limb ischaemia but 3 hr. later the increase in the fructose 1:6-diphosphate concentration was the only significant change (Table 4).

In control-fed medullectomized rats the concentrations of phosphorylated intermediates were within the range for intact rats. Liver samples, taken under sodium pentobarbitone anaesthesia from four fed medullectomized rats 3 hr. after the 4 hr. period of bilateral hind-limb ischaemia, showed high values for hexose 6-phosphate (1.12 ± 0.38 μ moles/g. wet wt.) and fructose 1:6-diphosphate (0.66 ± 0.15 μ mole/g. wet wt.).

Table 5. *Phosphorylated glycolytic intermediates in liver of fed adrenalectomized rats*

Samples were taken under sodium pentobarbitone anaesthesia from control rats between 2.20 and 3.20 p.m., and from rats after a 4 hr. period (10.0 a.m.–2.0 p.m.) of bilateral hind-limb ischaemia at intervals after removal of the tourniquets. Control values are shown as means $\pm$ s.d. with number of rats shown in parentheses. Environmental temperature 18–22°.

Treatment	Time after removal of tourniquets (min.)	Fructose 1:6-diphosphate (μ mole/g. wet wt.)	Phosphoglycerate (μ mole/g. wet wt.)
Controls	—	0.36 ± 0.12 (11)	0.07 ± 0.05 (10)
Limb ischaemia	10	0.29	0.16
	10	0.39	0.15
	15	0.40	0.12
	40	0.24	—
	50	0.41	0.23
	52	0.43	0.15
	65	0.41	0.16
	67	0.59	0.12
	70	0.79	0.10
	72	0.29	0.09
	74	0.39	0.07
	77	0.52	0.10
	80	0.33	0.12

Table 6. *Concentration of phosphorylated glycolytic intermediates in the liver of fed rats at an environmental temperature of 30°*

Experimental rats were subjected to bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. and were killed under sodium pentobarbitone anaesthesia 15–80 min. later. Number of rats is shown in parentheses.

Treatment	Glucose 1-phosphate	Hexose 6-phosphate	Fructose 1:6-diphosphate	Phosphoglycerate	α -Glycerophosphate	β -Glycerophosphate
			[μ moles/g. wet wt. (mean $\pm$ s.d.)]			
Controls	0.46 ± 0.16 (5)	1.67 ± 0.39 (5)	0.32 ± 0.07 (5)	0.16 ± 0.04 (5)	1.61 ± 0.32 (5)	0.86 ± 0.44 (5)
Limb ischaemia	$0.29 \pm 0.14^*$ (10)	$1.19 \pm 0.38^*$ (10)	0.33 ± 0.08 (10)	$0.13 \pm 0.05^\dagger$ (9)	$2.21 \pm 0.37^\dagger$ (10)	0.82 ± 0.48 (8)

* Difference significant at $P < 0.05$.

† Difference significant at $P < 0.01$.

Table 7. *Lactate, pyruvate and α -oxoglutarate concentrations in rat liver*

Samples were taken from control rats at the times shown and from rats after a 4 hr. period (10.0 a.m.–2.0 p.m.) of bilateral hind-limb ischaemia. Number of rats is shown in parentheses. Environmental temperature 18–20°. Statistical comparison is made between fed controls and between the experimental rats and their corresponding controls.

Treatment	Method of sampling	Pyruvate	Lactate [μ moles/g. wet wt. (mean $\pm$ s.d.)]	α -Oxoglutarate	Lactate/ pyruvate ratio
Fed rats					
Control (2.0–5.0 p.m.)	Guillotine	0.033 $\pm$ 0.018 (18)	1.06 $\pm$ 0.43 (11)	0.015 $\pm$ 0.006 (18)	39.6 $\pm$ 19.7 (11)
Control (2.0–5.0 p.m.)	Pentobarbitone	0.047 $\pm$ 0.019 (8)	3.01 $\pm$ 0.88† (5)	0.017 $\pm$ 0.008 (8)	—
End of limb ischaemia	Guillotine	0.031 $\pm$ 0.011 (5)	1.71 $\pm$ 0.56* (6)	0.020 $\pm$ 0.006 (5)	58.6 $\pm$ 10.3 (4)
3 hr. after	Guillotine	0.202 $\pm$ 0.120† (12)	1.43 $\pm$ 0.43 (6)	0.014 $\pm$ 0.007 (12)	10.0 $\pm$ 7.1† (6)
3 hr. after	Pentobarbitone	0.031 $\pm$ 0.013 (10)	2.64 $\pm$ 0.55 (7)	0.014 $\pm$ 0.01 (10)	—
Fasted rats					
Control (4.0 p.m.)	Guillotine	0.026 $\pm$ 0.009 (3)	0.55 $\pm$ 0.31 (3)	0.010 $\pm$ 0.002 (3)	22.0 $\pm$ 12.0 (3)
2 hr. after	Guillotine	0.154 $\pm$ 0.032† (3)	1.72 $\pm$ 1.27 (3)	0.015 $\pm$ 0.005 (3)	10.8 $\pm$ 6.9 (3)
Fed medullectomized rats					
Control (4.30 p.m.)	Guillotine	0.022 (2)	0.82 (2)	0.008 (2)	36.7 (2)
2.5 hr. after	Guillotine	0.070 $\pm$ 0.047 (4)	0.92 $\pm$ 0.21 (4)	0.010 $\pm$ 0.003 (4)	16.1 $\pm$ 6.5 (4)
Fed adrenalectomized rats					
Control (3.0 p.m.)	Guillotine	0.027 $\pm$ 0.011 (6)	0.96 $\pm$ 0.32 (6)	0.014 $\pm$ 0.003 (6)	39.7 $\pm$ 17.8 (6)
1 hr. after	Guillotine	0.074 $\pm$ 0.042* (6)	1.11 $\pm$ 0.45 (6)	0.015 $\pm$ 0.002 (6)	18.7 $\pm$ 11.1* (6)

* Difference significant at $P < 0.05$.

† Difference significant at $P < 0.001$.

† Difference significant at $P < 0.01$.

Table 8. *Lactate, pyruvate and α -oxoglutarate concentrations in the liver of fed rats at an environmental temperature of 30°*

Samples from control rats were taken at 3.0 p.m. and from rats injured by a 4 hr. period (10.0 a.m.–2.0 p.m.) of bilateral hind-limb ischaemia at intervals after removal of the tourniquets. All rats were guillotined. Control values are shown as means $\pm$ s.d. with the number of rats in parentheses.

Treatment	Time after removal of tourniquets (min.)	Pyruvate	Lactate (μ moles/g. wet wt.)	α -Oxoglutarate	Lactate/pyruvate ratio
Control	—	0.029 $\pm$ 0.016 (7)	1.38 $\pm$ 0.31 (7)	0.018 $\pm$ 0.011 (7)	54.3 $\pm$ 18.8 (7)
Limb ischaemia	32	0.297	2.05	0.018	6.9
	35	0.131	5.12	0.013	39.0
	59	0.160	2.24	0.014	14.0
	59	0.037	4.70	0.010	126.0
	63	0.075	3.62	0.014	48.3
	70	0.047	4.67	0.009	99.2
	73	0.047	2.15	0.012	46.1
	75	0.042	7.16	0.008	170.0
	77	0.073	3.19	0.014	44.0
	93	0.023	5.27	0.005	231.0
	104	0.028	2.37	0.015	83.7

In cold-acclimatized rats (Table 4) the fructose 1:6-diphosphate concentration was high in the livers of the controls but did not rise any further 3 hr. after the injury.

In adrenalectomized rats samples were taken at different times after removal of the tourniquets (Table 5). The only constant change was an increase, during the early and middle parts of the survival period, in the concentration of phosphoglycerate, which was very low in the controls. Some injured adrenalectomized rats showed high concentrations of fructose 1:6-diphosphate in the liver but the majority did not.

At an environmental temperature of 30° uniform values were obtained between 15 and 80 min. after removal of the tourniquets (Table 6). At no time during this period was there any increase in the concentration of fructose 1:6-diphosphate. Instead the concentrations of glucose 1-phosphate and of hexose 6-phosphate fell and that of α -glycerophosphate rose. The high environmental temperature alone led to a rise in the hexose 6-phosphate concentration in the controls ($P < 0.01$) and even when the level had fallen after injury it was still above that in controls at an air temperature of 18–22°.

Pyruvate and lactate. At the end of the period of limb ischaemia the concentration of lactate in the liver was high in fed intact rats but 3 hr. later the significant changes were a very high pyruvate concentration and a fall in the lactate/pyruvate ratio (Table 7). These changes were found only in samples from guillotined animals. This pattern of a high pyruvate concentration with a low lactate/pyruvate ratio was also found in the liver after limb ischaemia in small groups of medullectomized, adrenalectomized and fasted intact rats (Table 7).

In rats subjected to limb ischaemia at an air temperature of 30° the concentrations of pyruvate and lactate were both high shortly after removal of the tourniquets (Table 8). The change in the pyruvate concentration was more marked and the lactate/pyruvate ratio was low. Later, the pyruvate concentration fell and the lactate/pyruvate ratio rose, reaching very high values when there was a further increase in the pyruvate concentration.

α -Oxoglutarate. The concentration of this compound in both liver and muscle remained unaffected by the experimental procedures (Tables 2, 7 and 8).

DISCUSSION

Changes in injured muscle

The striking changes in the injured hind-limb muscle at the end of the 4 hr. period of ischaemia can be attributed to anaerobic glycolysis. However, glycolysis did not go to completion, for large amounts of hexose monophosphate remained, and

the fructose 1:6-diphosphate concentration was very low. This may be due to lack of adenosine triphosphate in the injured muscle (Threlfall & Stoner, 1957). A similar picture was seen after glycolysis in excised muscle by Bailey & Marsh (1952). The large increase in the α -glycerophosphate content of the injured muscle may also be due to the anoxia.

When the tourniquets are removed the muscle is reoxygenated and some adenosine triphosphate can be generated in it (Threlfall & Stoner, 1957), permitting further glycolysis to occur. Some glycogen is also formed in the hind limbs at this time (Stoner, 1958*b*) and the combination of these processes could account for the situation in the damaged muscle 3 hr. later.

Changes in uninjured muscle

The main change in the phosphorylated glycolytic intermediates in the uninjured fore-limb muscle was the increase in the concentration of fructose 1:6-diphosphate after the tourniquets had been removed. The statistically insignificant increases in the hexose monophosphate concentrations probably reflect the active glycogenolysis going on in this muscle before and after removal of the tourniquets (Stoner, 1958*b*). Adrenal-medullectomy prevents this breakdown of muscle glycogen (Stoner, 1958*b*) and then the hexose monophosphate concentrations are well within the control range. Adrenal-medullectomy also prevented the rise in the concentration of fructose 1:6-diphosphate. Cori & Cori (1931) have shown, and we have confirmed (unpublished results), that when muscle glycogenolysis is produced by the injection of adrenaline the concentration of hexose 6-phosphate increases whereas that of fructose 1:6-diphosphate does not. As the formation of the latter will also be increased under these conditions it would appear that an increase in fructose 1:6-diphosphate formation alone does not lead to an observable rise in its concentration. This suggests that the change after injury was due to the partial inhibition of fructose 1:6-diphosphate utilization so that an increase in its concentration was seen only when its rate of formation was also increased.

The absence of an increase in the products of glycolysis in the fore-limb muscle after hind-limb ischaemia in the medullectomized rat suggests that the main reason for the raised muscle-pyruvate concentration in the injured intact rat was its increased rate of formation from glycogen, in the adrenaline-mediated glycogenolysis in the fore-limb muscle after hind-limb ischaemia (Stoner, 1958*b*). If the increase in glycolytic products was due to anoxaemia the main change should have been in the lactate fraction with a rise in the lactate/pyruvate ratio. The absence of a rise in this

ratio is in accord with our previous view that the fore-limb muscle is adequately oxygenated at this time (Threlfall & Stoner, 1957).

Effect of limb ischaemia on the liver

Phosphorylated glycolytic intermediates. The outstanding change in these intermediates was the increase in the concentration of fructose 1:6-diphosphate which occurred in the majority of the groups of injured rats. Since, compared with the change in the intact fed rats, the increase was greater in the medullectomized and fasted rats, which were more sensitive to the injury, and minimal or absent in resistant cold-acclimatized rats, it seemed that the change might be related to the severity of the clinical state. However, this hypothesis of a simple inverse relationship between survival time and the rise in the fructose 1:6-diphosphate concentration could not be sustained because of the findings in the experiments at an environmental temperature of 30°.

Although raising the environmental temperature shortens the survival time after the injury from about 13 hr. to 2.22 ± 0.32 hr. (Stoner & Threlfall, 1960), the behaviour of these rats after injury is so different that it was hardly surprising that they also differed chemically from the other injured rats. The main differences between the behaviour of the injured rats at an air temperature of 30° and those at an air temperature of 18–22° are a rise instead of a fall in liver temperature (Stoner, 1958*a*), a rapid fall in hepatic blood flow instead of a slow fall after a period of some hours during which the flow is within normal limits (Stoner, 1958*a*), a fall in the adenosine triphosphate content of the liver (Stoner & Threlfall, 1954) and more rapid hepatic glycogenolysis (unpublished results). The changes in the phosphorylated glycolytic intermediates found at the higher environmental temperature could well be a reflexion of these events.

The increase in the fructose 1:6-diphosphate concentration found during the first part of the survival period only occurred when the injury caused a fall in liver temperature, i.e. in fed and fasted intact rats and in fed medullectomized rats but not in cold-acclimatized rats, in which there is only a slight fall in liver temperature after limb ischaemia (Stoner, 1958*a*). If the effect is causally related to the temperature change the variable behaviour of the injured adrenalectomized rats requires an explanation, since the temperature regularly falls in these animals.

In the adrenalectomized rat the survival time after 4 hr. bilateral hind-limb ischaemia is only 1.86 ± 0.67 hr. (Stoner & Threlfall, 1960). The contraction of the survival period is largely at the expense of its first part (Stoner, 1960), so that it is difficult to choose a point for comparison with the

intact rat. The variability in the short survival time also makes it difficult to know the stage reached by an individual rat. To try to circumvent this difficulty samples were taken at different times after removal of the tourniquets. In some the concentration of fructose 1:6-diphosphate in the liver was high but in the majority it was not. It is not known whether this means that an excess of fructose 1:6-diphosphate is present only for a short time in an individual rat so that its detection is a matter of chance or whether the increase occurs only in rats with accessory adrenal tissue. To minimize the latter possibility the fat around the adrenal was always removed with the gland and there has been no previous indication that such tissue is common in our rats. Other factors may be concerned. Although the results do not exclude a relationship between the fall in temperature and the rise in the fructose 1:6-diphosphate concentration, more work is necessary to elucidate the possible role of the adrenal cortex in this response.

Pyruvate and lactate concentrations. A further significant change in the liver after limb ischaemia was an increase in its pyruvate concentration with a fall in the lactate/pyruvate ratio. When the sampling times were such that the rats were in a comparable state, the picture was common to intact, medullectomized and adrenalectomized rats (Table 7) but was not found when the liver samples were removed under barbiturate anaesthesia. If the excess of pyruvate had been converted into lactate under the influence of sodium pentobarbitone (see above) the change would not have been detected. Reasons have already been given for considering that the results in the guillotined rats represent conditions *in vivo*.

The changes in rats subjected to limb ischaemia at an air temperature of 30° resembled those in other groups more closely than did the changes in the phosphorylated glycolytic intermediates. Some of the difficulties in assessing changes in injured rats at an air temperature of 30° have already been mentioned. In general, the longer the rat has remained alive after the limb ischaemia the shorter its expectation of life, but the survival time is very variable, and, as there is no progressive visible change (unlike in injured rats at the lower environmental temperature), it is extremely difficult to decide an individual rat's proximity to death. The best guide in these rats is the fall in liver blood flow (Stoner, 1958*a*). In the light of our previous work our interpretation of the results in Table 8 is that for a short time after limb ischaemia pyruvate accumulates in the liver as in other injured rats but this process is cut short by hypoxia due to the failing liver blood flow, so that ultimately there is an accumulation of lactate. On this interpretation the rats with high lactate/pyruvate ratios

would be those nearest to death. The large amount of lactate present at all times can be attributed to the enormous breakdown of liver glycogen which occurs under these conditions.

Unlike muscle, the liver contains a relatively large amount of blood, about 0.1 ml./g. wet wt. (Caster, Simon & Armstrong, 1955). From previous studies (Stoner, Threlfall & Green, 1952; Stoner *et al.* 1960) it would seem that in the controls most of the pyruvate could be in the blood of the liver and not in its parenchyma. The majority of the lactate and phosphorylated intermediates, on the other hand, would be in the liver cells (Stoner *et al.* 1952; Bartlett, 1959). Although the blood concentrations of lactate and pyruvate are raised for a time after removal of the tourniquets (McShan, Potter, Goldman, Shipley & Meyer, 1945; Stoner *et al.* 1952, 1960), the levels at the time of sampling could not have accounted for the changes in the liver. This means that the percentage increase in intracellular pyruvate was greater than would be apparent from the values in Tables 7 and 8.

The low lactate/pyruvate ratio in the injured rats is consistent with the view that there is a period of adequate hepatic oxygenation after removal of the tourniquets, although it may be quite short, as in those at an air temperature of 30°.

α -Oxoglutarate concentration. The liver contained more α -oxoglutarate than did muscle. The concentration of this metabolite remained constant throughout the various groups of control and injured rats. Although this result is of interest as liver mitochondria isolated from rats after limb ischaemia behaved in the same way *in vitro* as those from normal rats (Aldridge & Stoner, 1960), the work of Cavallini & Frontali (1954) suggests that most of the α -oxoglutarate could be accounted for by the blood in the liver.

Conclusions

The most significant changes during the early part of the survival period after limb ischaemia in the intact rat were an increase in the concentrations of fructose 1:6-diphosphate and pyruvate in the uninjured muscle and an increase in the concentration of pyruvate in the liver and also of fructose 1:6-diphosphate when its temperature fell. A rise in the concentration of a tissue constituent can be due to its increased formation or decreased utilization or both. In the uninjured muscle, even if the utilization of fructose 1:6-diphosphate and pyruvate was inhibited, the rise in their concentrations was found only when there was over-production by increased glycogenolysis.

The situation in the liver is not so clear. Both fructose 1:6-diphosphate and pyruvate can be formed and utilized by a number of alternative re-

actions. Previous work with [14 C]glucose and [2-^{14} C]pyruvate (Stoner *et al.* 1960) showed that during the early part of the survival period the metabolism of glucose was inhibited in the early stages of its catabolism and also in the steps subsequent to the formation of pyruvate, the oxidation of which was diminished. Those changes were thought to be causally related to the fall in liver temperature. There is agreement between the two sets of experiments in that the present ones also show carbohydrate breakdown to be altered at two such points. Furthermore it would seem that the two changes can arise independently, as in the rats subjected to limb ischaemia at an air temperature of 30° where there was a rise in the pyruvate concentration but not in that of fructose 1:6-diphosphate. The latter increase was only seen when the liver temperature fell. Although the mechanism of these changes cannot yet be described some possibilities can be eliminated.

Increased formation of fructose 1:6-diphosphate and pyruvate could occur by: (1) an increase in glucose breakdown; (2) an increase in glycogen breakdown in the liver; (3) increased gluconeogenesis from the extra amounts of pyruvate and lactate being brought to the liver after the increase in muscle glycogenolysis; (4) gluconeogenesis from non-carbohydrate sources.

The first of these possibilities can be excluded (Stoner *et al.* 1960). Neither (2) nor (3) is likely to be a major factor because the enormous differences in the amounts of liver and muscle glycogen broken down in the different experimental groups (Stoner, 1958*b*) were not reflected in the results. The influence of (4) is difficult to assess, but in accounting for the alterations in the distribution of glucose and glycogen after limb ischaemia (Stoner, 1958*b*) it was not necessary to postulate any increase in gluconeogenesis from non-carbohydrate sources above the amount in comparable controls.

An acceptable explanation for the increased concentrations of fructose 1:6-diphosphate and pyruvate in the liver would be a decrease in their utilization. In the breakdown of fructose 1:6-diphosphate towards pyruvate the most important step would probably be the triose phosphate-dehydrogenase reaction. Because of the ready reversibility of the aldolase reaction, inhibition of the triose phosphate-dehydrogenase reaction leads to fructose 1:6-diphosphate accumulation (see Krebs & Kornberg, 1957). Similarly, if the oxidation of pyruvate is decreased its concentration will rise unless there is a compensatory increase in the other reactions utilizing pyruvate.

The most important feature of these results is the way in which they confirm, by quite different methods, the conclusion of the previous paper (Stoner *et al.* 1960) that the carbohydrate

metabolism of these injured rats is altered at two points, and the help they give in planning further experiments to elucidate the altered mechanisms.

SUMMARY

1. The early effects of bilateral hind-limb ischaemia on the concentrations of phosphorylated glycolytic intermediates, pyruvate, lactate and α -oxoglutarate, in muscle and liver have been investigated in intact, intact cold-acclimatized, adrenal-medullectomized and adrenalectomized rats at air temperatures between 18° and 22° and also in intact rats at an air temperature of 30°.

2. The changes in the phosphorylated glycolytic intermediates in the injured muscle during ischaemia were the same as those reported for anoxic muscle *in vitro*.

3. In the uninjured fore-limb muscle the fructose 1:6-diphosphate and pyruvate concentrations rose after hind-limb ischaemia. These changes were attributed to increased glycogenolysis and to decreased utilization of fructose 1:6-diphosphate and possibly also of pyruvate.

4. The most constant change in the liver after limb ischaemia was an increase in the pyruvate concentration and also in that of fructose 1:6-diphosphate when conditions were such that the liver temperature fell. These changes are attributed to a decrease in the utilization of fructose 1:6-diphosphate and in the oxidation of pyruvate.

Our thanks are due to Miss M. O'Donnell and Mr R. Manston for their technical assistance.

REFERENCES

- Aldridge, W. N. & Stoner, H. B. (1960). *Biochem. J.* **74**, 148.
 Bailey, K. & Marsh, B. B. (1952). *Biochim. biophys. Acta*, **9**, 133.
 Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
 Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 449.
 Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.
 Caster, W. O., Simon, A. B. & Armstrong, W. D. (1955). *Amer. J. Physiol.* **183**, 317.
 Cavallini, D. & Frontali, N. (1954). *Biochim. biophys. Acta*, **13**, 439.
 Conway, E. J. & Hingerty, D. (1946). *Biochem. J.* **40**, 561.
 Cori, C. F. & Cori, G. T. (1931). *J. biol. Chem.* **94**, 581.
 Derache, R., Trémolières, J., Griffaton, G. & Lowy, R. (1956). *Bull. Soc. Chim. biol., Paris*, **38**, 1457.
 El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
 Fisher, R. A. (1934). *Statistical Methods for Research Workers*, 5th ed. London: Oliver and Boyd Ltd.
 Frunder, H., Richter, G., König, W. & Börnig, H. (1959). *Hoppe-Seyl. Z.* **314**, 188.
 Gilmore, J. P. (1958). *Amer. J. Physiol.* **195**, 465.
 Goranson, E. S., Hamilton, J. E. & Haist, R. E. (1948). *J. biol. Chem.* **174**, 1.
 Krebs, H. A. & Kornberg, H. L. (1957). *Ergebn. Physiol.* **49**, 212.
 LePage, G. A. (1946). *Amer. J. Physiol.* **146**, 267.
 LePage, G. A. (1948). *Cancer Res.* **8**, 193.
 McShan, W. H., Potter, V. R., Goldman, A., Shipley, E. G. & Meyer, R. H. (1945). *Amer. J. Physiol.* **145**, 93.
 Neish, W. J. P. (1953). *Rec. Trav. chim. Pays-Bas*, **72**, 105.
 Sacks, J., Kuroda, L. M. & Hurley, P. D. (1955). *Biochem. J.* **61**, 244.
 Stoner, H. B. (1958a). *Brit. J. exp. Path.* **39**, 251.
 Stoner, H. B. (1958b). *Brit. J. exp. Path.* **39**, 635.
 Stoner, H. B. (1960). *Lect. sci. Basis Med.* (in the Press).
 Stoner, H. B., Heath, D. F. & Collins, O. M. (1960). *Biochem. J.* **76**, 135.
 Stoner, H. B. & Threlfall, C. J. (1954). *Biochem. J.* **58**, 115.
 Stoner, H. B. & Threlfall, C. J. (1960). *The Biochemical Response to Injury*, p. 105. Oxford: Blackwell Scientific Publications Ltd.
 Stoner, H. B., Threlfall, C. J. & Green, H. N. (1952). *Brit. J. exp. Path.* **33**, 131.
 Threlfall, C. J. (1957). *Biochem. J.* **65**, 694.
 Threlfall, C. J. & Stoner, H. B. (1954). *Quart. J. exp. Physiol.* **39**, 1.
 Threlfall, C. J. & Stoner, H. B. (1957). *Brit. J. exp. Path.* **38**, 339.

Biochem. J. (1961) **79**, 562

The Degradation of Cotton Cellulose by the Extracellular Cellulase of *Myrothecium verrucaria*

By K. SELBY

Shirley Institute, B.C.I.R.A., Didsbury, Manchester 20

(Received 15 August 1960)

Many papers on the action of extracts from cellulolytic fungi on various cellulosic substrates have been published over the last two decades. It is now well established that any pretreatment of cellulose involving either swelling or dissolution increases its accessibility both to attack by cellulase and to other reagents. This aspect of the subject has been

adequately reviewed elsewhere (Pigman, 1951; Cumberbirch, 1955). It is still uncertain, however, whether the attack of the enzyme on the accessible glucoside linkages in the cellulose is random or endwise, as it is known to be with certain other glucosidases. An answer can only be obtained by examining the relation between such factors as the