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Comparison of some Metabolic Parameters in the Perfused and the Incubated Rat Diaphragm Muscle with Diaphragm Muscle *in vivo*

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1. A method is described for perfusing the rat diaphragm muscle. 2. The following parameters were compared in both perfused and non-perfused incubated preparations: water content, sorbitol space, rate of lactate production, and the concentrations of tissue glucose, pyruvate, lactate, hexose phosphate intermediates, ATP and AMP. No significant differences were found. 3. Significant differences, however, were found on comparison of the tissue kept *in vitro* with the tissue *in vivo*. Immediately after removal of the tissue from the animal, the concentrations of the hexose phosphates and ATP were found to be much higher than after incubation or perfusion, and the concentrations of free glucose and of AMP were much lower, possibly indicating that the capacity for oxidative phosphorylation of glucose is impaired *in vitro* because of hypoxia.

The incubated rat diaphragm preparation has been used extensively for metabolic studies since its introduction by Meyerhof & Himwich (1924), especially since Gemmill (1941) demonstrated an effect of insulin *in vitro* on glucose uptake and glycogen synthesis.

It was decided to compare quantitatively some metabolic parameters determined in this incubated preparation with the same parameters measured in the perfused rat diaphragm. The parameters chosen for comparison were the amounts of some glucose metabolites, and of ATP and AMP, together with the water content and the sorbitol space of the diaphragm.

EXPERIMENTAL

Animals. Fed male Sprague–Dawley rats (280–320g) bred in the department were used.

Materials. All chemicals were obtained from either BDH Chemicals Ltd., Poole, Dorset, U.K., Hopkin and Williams, Chadwell Heath, Essex, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K. Enzymes were obtained from Boehringer Ltd., London W.5, U.K. [^{3}H]Sorbitol (TRA 288) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Incubation of diaphragm muscle. The incubation procedure was identical with that previously described (Beloff-Chain & Rookledge, 1968) except that a bicarbonate buffer (Krebs & Henseleit, 1932) was used instead of a phosphate buffer and the medium was gassed with O_2+CO_2 (95:5).

Perfusion procedure. The procedure was based on the method of Brownlee & Straughan (1957). It is essentially a retrograde perfusion via the vena cava in the thoracic region.

Rats were maintained under ether anaesthesia and the skin was removed from the ventral surface. The pectoral muscles were removed, the abdominal cavity was opened and the vena cava, exposed between the liver and diaphragm, was ligated. The thorax was then opened and the posterior vena cava dissected free of any adhering fat and of the vagus nerve. A cannula of stainless steel through which bicarbonate buffer was pumped at 2ml/min was inserted through a cut in the vein, and secured with ligatures (Fig. 1, L). Thus the blood in the veins was only static for a few seconds before perfusion began.

The vein supplying the left portion of the diaphragm was ligated close to its inception with the vena cava. The two branches of the right vein, ventral and dorsal, were also ligated some 8–10mm along their length and only the lateral branches remained open.

A T-shaped piece of platinum wire was tied to the diaphragm (Fig. 1, N) by means of several ligatures at its junction with the ribs. The diaphragm section was cut free by taking a fan-shaped piece of muscle including the cannulated vena cava. Adhering liver and connective tissue was removed and the preparation was suspended in the perfusion vessel by means of the platinum wire. The lower part was secured to a stainless-steel support by means of the ligature below the diaphragm (Fig. 1, M) and the cannula was also secured to its support. During these operations the perfusate was drawn from a reservoir kept at 0°C and on completion of the preparation the perfusate was supplied from a reservoir maintained at 37°C. The rate of perfusion was maintained at 2ml/min. The

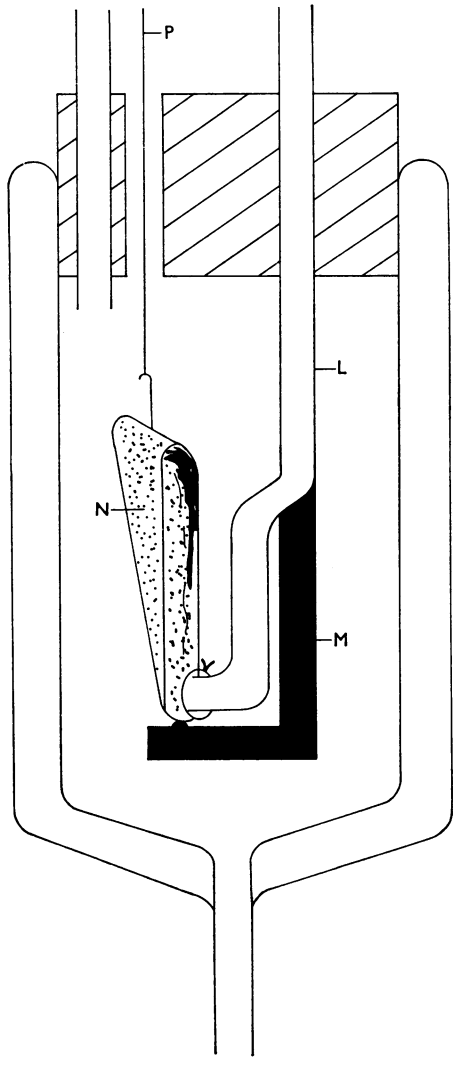

Fig. 1. Diagram of perfusion vessel. N, diaphragm muscle; M, support joined to cannula; L, stainless-steel cannula; P, platinum wire holding diaphragm.

perfusion medium was identical with that used in the incubation studies, consisting of Krebs–Henseleit (1932) bicarbonate buffer containing 5.55mM-glucose.

Extraction procedure. In all cases the tissue was first frozen in liquid N_2 either directly after removal (initial values) or after incubation or perfusion, then weighed and powdered in a percussion mortar cooled with solid CO_2. The time required to remove the diaphragm to obtain initial values was less than 1 min. The powder was transferred to a tube containing 2.0ml of cold 60% (w/v) $HClO_4$–5mM-EDTA–acetone (6:19:25, by vol.). The powder was briefly homogenized and centrifuged at 0°C. The residue after centrifugation was washed twice with 0.5ml of the above mixture. The contents of the tube were kept cooled on ice throughout the procedure.

The pH of the supernatants was adjusted to 7.0 with 30% (w/v) KOH saturated with KCl and 0.2M-tris buffer (6:4, v/v), the precipitate being removed by centrifugation.

Analytical methods. Glycogen was determined by the method of Walaas & Walaas (1950), and glucose by a modification of the method of Crowne & Mansford (1962), and also on the auto-analyser by the method of Marks & Lloyd (1963). All enzymic analyses of intermediates were performed by methods described by Bergmeyer (1963). Analyses of pyruvate, ATP and AMP and fructose diphosphate were performed on the same day; all the remaining analyses were performed within 3 days and the samples were meanwhile kept frozen at −20°C.

Sorbitol space. This was determined essentially as described by Morgan, Henderson, Regen & Park (1961). Tissue was incubated or perfused for 90min at 37°C in Krebs–Henseleit buffer containing 5.55mM-glucose and 5.55mM-sorbitol together with about 5μCi of [^{3}H]sorbitol.

After incubation or perfusion the tissue was washed in saline (0.9% NaCl), blotted dry, weighed and homogenized in 6% (w/v) $HClO_4$. The protein precipitate after centrifugation was washed twice with 6% $HClO_4$ and then the combined supernatants were neutralized to pH 7.0 and counted for radioactivity as described by Beloff-Chain & Rookledge (1968).

Water content. Water content of the tissue was obtained by weighing the tissue before and after drying at 105°C for 16h.

RESULTS AND DISCUSSION

There was little difference between the values of any of the various parameters determined in the perfused and incubated tissue (Tables 1, 2 and 3).

The values for the water content (Table 1) of the perfused and incubated rat diaphragm were not significantly different and were close to those found in the uncut rat diaphragm (Kipnis & Cori, 1957), in the rat heart after perfusion (Morgan *et al.* 1961) and the extensor digitorum muscle after incubation (Manchester & Pain, 1970). The values for sorbitol space (Table 1) were also not significantly different in the perfused and incubated rat diaphragm; in the former they did not change even after prolonged perfusion. They agree in general with those found by Mansford (1968) and Morgan *et al.* (1961) in the perfused rat heart.

The rate of lactate production (Table 2) was constant throughout the period of perfusion and was similar to that found in the incubated rat diaphragm. Similar values have been reported by Hollanders (1968) and Rowlands (1969), who also used the perfused rat diaphragm, and the rate of production in the Langendorff-perfused rat heart was also very similar (Chain, Mansford & Opie, 1969).

The values for the parameters listed in Table 3

Table 1. *Tissue water content and sorbitol space in the incubated and perfused rat diaphragm*

Results are expressed as means ±S.E.M., with the number of determinations in parentheses. No significant differences were found.

Time of incubation or perfusion (min)	Tissue water content (mg/100 mg wet wt.)		Tissue sorbitol space (μl/100 mg wet wt.)	
	Incubated diaphragm	Perfused diaphragm	Incubated diaphragm	Perfused diaphragm
0	79.0±0.6 (8)	—	—	—
15	—	—	—	58.2±2.3 (5)
90	80.0±0.7 (8)	80.0±0.6 (6)	56.4±1.5 (19)	60.6±2.0 (5)

Table 2. *Rate of lactate production by the perfused and incubated rat diaphragm*

The results are expressed as means ±S.E.M., with the number of determinations in parentheses. No significant differences were found.

Diaphragm preparation	Lactate produced (nmol/min per 100mg wet wt.)				
Time period after commencement (min) ...	30–45	45–60	60–75	75–90	0–90
Perfused	27.9±1.5 (20)	26.0±1.7 (20)	27.8±2.0 (18)	26.5±1.9 (18)	—
Incubated	—	—	—	—	23.7±1.4 (24)

Table 3. *Effect of incubation and perfusion on concentrations of lactate, pyruvate, hexose phosphates, adenine nucleotides and glycogen in the rat diaphragm muscle*

Values are expressed as means ±S.E.M. with the number of determinations in parentheses. The tissue was perfused with or incubated in Krebs–Henseleit bicarbonate buffer pH 7.4, containing 5.55 mM-glucose, for 90 min at 37°C.

Metabolite	Concentration (nmol/100 mg wet wt.)		
	Initial value (*in vivo*)	After incubation	After perfusion
Lactate	311±42 (7)	861±41* (7)	517±100† (8)
Pyruvate	6.4±0.6 (7)	8.1±1.3 (7)	8.5±1.1 (8)
Glucose 6-phosphate	141±18.7 (7)	31.0±2.7* (7)	23.8±4.4* (8)
Fructose 6-phosphate	27.7±2.3 (6)	14.3±2.3* (6)	8.5±1.9* (8)
Fructose 1,6-diphosphate	8.2±0.7 (7)	2.9±0.5* (7)	5.9±1.1† (8)
Glucose	136±10 (7)	434±62* (7)	439±41* (8)
ATP	435±16 (7)	213±22* (7)	231±19* (8)
AMP	7.1±0.9 (7)	18.4±4.7* (6)	9.6±1.7 (7)
Ratio ATP/AMP	67.5±9.8 (7)	18.9±5.4* (6)	25.6±4.8* (7)
Glycogen (as glucose)	2517±135 (10)	1653±143 (10)	—
	1883±71 (16)	—	877±78* (19)

* Significantly different from initial value; $P < 0.05$.
† Significantly different from values after incubation; $P < 0.05$.

for the perfused and incubated rat diaphragm showed only minor differences.

It is noteworthy, however, that as shown in Table 3 the values for the hexose phosphates and ATP, when determined immediately after removal of the tissue from the animal, were much higher than after incubation or perfusion, but glucose and AMP concentrations were much lower before incubation or perfusion, possibly indicating that the glucose-phosphorylating activity of the tissue is impaired *in vitro*, because of hypoxia.

A hypoxic condition might be expected, as although the flow rate of the perfusate through the tissue approximates to that of blood, the oxygen-carrying capacity will only be about one-seventh because of the absence of haemoglobin. However,

measurements of the oxygen concentration of the perfusate indicated that there was still oxygen left in the effluent perfusate.

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REFERENCES

Beloff-Chain, A. & Rookledge, K. A. (1968). *Biochem. J.* **110**, 529.

Bergmeyer, H. U. (Ed.) (1963). *Methods of Enzymatic Analysis*. Weinheim: Verlag Chemie; New York and London: Academic Press.

Brownlee, G. & Straughan, D. W. (1957). *J. Physiol., Lond.*, **136**, 6*P*.

Chain, E. B., Mansford, K. R. L. & Opie, L. H. (1969). *Biochem. J.* **115**, 537.

Crowne, R. S. & Mansford, K. R. L. (1962). *Analyst, Lond.*, **87**, 294.

Gemmill, C. L. (1941). *Bull. Johns Hopkins Hosp.* **68**, 329.

Hollanders, F. D. (1968). *Comp. Biochem. Physiol.* **26**, 907.

Kipnis, D. M. & Cori, C. F. (1957). *J. biol. Chem.* **224**, 681.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyler's Z. physiol. Chem.* **210**, 33.

Manchester, K. L. & Pain, V. M. (1970). *Biochem. J.* **118**, 209.

Mansford, K. R. L. (1968). Ph.D. Thesis: University of London.

Marks, W. & Lloyd, K. (1963). *Proc. Ass. clin. Biochem.* **2**, 176.

Meyerhof, D. & Himwich, H. E. (1924). *Pflügers Arch. ges. Physiol.* **205**, 415.

Morgan, H. E., Henderson, M. J., Regen, D. M. & Park, C. R. (1961). *J. biol. Chem.* **236**, 253.

Rowlands, S. D. (1969). *Comp. Biochem. Physiol.* **29**, 1215.

Walaas, O. & Walaas, E. (1950). *J. biol. Chem.* **187**, 769.