Biochem. J. (1971) **124**, 639–651
Printed in Great Britain

Evaluation of the Isolated Perfused Rat Hindquarter for the Study of Muscle Metabolism

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(*Received* 13 *April* 1971)

1. The metabolic integrity of a new isolated rat hindquarter preparation was studied. The hindquarter was perfused with a semi-synthetic medium containing aged human erythrocytes. More than 95% of the oxidative metabolism of the preparation was due to muscle, the remainder being due to bone, adipose tissue and, where present, skin. 2. Consumption of O_2, glucose utilization, glycerol release and lactate production were similar in the presence and in the absence of the skin, indicating that the latter contributed little to the overall metabolism of the preparation. 3. After 40min of perfusion, tissue concentrations of creatine phosphate, ATP and ADP were similar to those found in muscle taken directly from intact animals. The muscle also appeared normal under the electron microscope. 4. The hindquarter did not lose K^+ to the medium during a 30min perfusion. In the presence of insulin it had a net K^+ uptake. 5. Insulin caused a sixfold increase in glucose uptake, stimulated O_2 consumption by nearly 40% and depressed glycerol release to less than half the control value. 6. Bilateral sciatic-nerve stimulation caused severalfold increases in O_2 consumption and lactate production. In the absence of insulin nerve stimulation also enhanced glucose uptake; in the presence of insulin it did not further increase the already high rate of glucose uptake. 7. Rates of lactate production and O_2 consumption of the rat hindquarter *in vivo* and the isolated perfused hindquarter were very similar. 8. Ketone bodies were a major oxidative fuel *in vivo* of the hindquarter of a rat starved for 2 days. If the acetoacetate and 3-hydroxybutyrate removed by the tissue were completely oxidized, they would have accounted for 77% of the O_2 consumption. 9. Acetoacetate accounted for 84% of the ketone bodies removed by the hindquarter *in vivo* even though its arterial concentration was half that of 3-hydroxybutyrate. 10. Similar rates of acetoacetate and 3-hydroxybutyrate utilization were observed in the perfused hindquarter. 11. Acetoacetate utilization by the perfused hindquarter was not diminished by the addition of either oleate or insulin to the perfusate. 12. Oxidation of glucose to CO_2 accounted for less than 4% of the O_2 consumed by the perfused hindquarter in both the presence and the absence of insulin. 13. The results indicate that the isolated perfused hindquarter is a useful tool for studying muscle metabolism. They also suggest that ketone bodies, if present in sufficient concentration, are the preferred oxidative fuel of resting muscle.

The present paper is concerned with a systematic investigation of the metabolic performance of the isolated perfused hindquarter of the rat, a preparation consisting mainly of muscle tissue. It was prompted by the experience that isolated perfused organs are very useful for the study of control mechanisms of metabolism. Hindquarters of larger animals such as dogs and cats have often been used in the past (Burn & Dale, 1926; Best, 1926; Blixenkrone-Møller, 1938). Experiments on the hindquarter of the rat have been reported by several investigators (Robinson & Harris, 1959; Forsander, Räihä & Suomalainen, 1960; Mortimore, Tietze & Stetten, 1959; Mahler, Szabo & Penhos, 1968; Szabo, Mahler & Szabo, 1969), but it appears that no systematic effort has been made to compare the activities of the isolated preparation with those of the hindquarter *in situ*. In the present work O_2

consumption, utilization of glucose and ketone bodies, lactate production, efflux of K^+, tissue concentrations of creatine phosphate and the adenine nucleotides, appearance under the electron microscope and the response to insulin and sciatic-nerve stimulation were investigated in the isolated preparation and compared with the metabolic responses of the hindquarter *in vivo*. The results indicate that the behaviour of the perfused hindquarter very closely resembles that of the hindquarter of the intact rat. They also suggest that ketone bodies are the main oxidative fuel of resting muscle when they are present in sufficient concentration. Part of this work has appeared in preliminary reports (Houghton, Ruderman & Hems, 1970; Houghton & Ruderman, 1970; Ruderman, Houghton & Hems, 1970).

MATERIALS AND METHODS

Animals. Female Wistar rats weighing 170–230g were used. The animals were starved for 48h before the experiment unless otherwise stated.

Reagents. [U-^{14}C]Glucose and ^{131}I-labelled polyvinylpyrrolidone were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Solutions of pentobarbitone (20mg/ml) were freshly prepared from pentobarbitone sodium powder (Abbott Laboratories, Queenborough, Kent, U.K.). Bovine serum albumin (Cohn fraction V) was obtained from Pentex Corp. [Miles Seravac (Pty.) Ltd., Maidenhead, Berks., U.K.]. It was dissolved in Krebs–Henseleit bicarbonate saline (Krebs & Henseleit, 1932) and dialysed twice against 2 litres of the bicarbonate saline for 24h. If the albumin solution was turbid, it was centrifuged at 20000*g* for 20min in an MSE High-Speed 18 refrigerated centrifuge to remove particulate debris.

Oleic acid (99% pure) was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Ethyl acetoacetate and DL-3-hydroxybutyrate were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Acetoacetate was prepared from its ethyl ester as described by Krebs & Eggleston (1945).

Perfusion medium. The standard perfusate was composed of Krebs–Henseleit bicarbonate buffer, containing 4g of bovine serum albumin/100ml, aged washed human erythrocytes (7–8g of haemoglobin/100ml) (Hems, Ross, Berry & Krebs, 1966), 5.5mM-glucose and 0.15mM-sodium pyruvate. Pyruvate was added to give an initial [lactate]/[pyruvate] ratio of about 15. The initial lactate concentration in the medium was 1.5–2.5mM; the lactate originated from the erythrocytes.

Surgical preparation of the isolated hindquarter. The operative procedure involved a pelvic evisceration, followed by ligation of some of the major abdominal branches of the great vessels and finally cannulation of the aorta and vena cava. In some of the experiments the skin over the lower half of the animal was also removed. An electrocautery (model 708SP 65; Holborn Surgical Instrument Co. Ltd., London E.C.1, U.K.) was used for dissection wherever possible to minimize blood loss. The details of the operative procedure are as follows.

Rats were anaesthetized by intraperitoneal injection of aqueous pentobarbital (4.5mg/100g body wt.). After a midline abdominal incision, the skin was reflected and the superficial epigastric vessels were ligated (for anatomical nomenclature see Greene, 1959). The abdominal wall was then incised from the pubic symphysis to the xiphoid process by using an electrocautery. After this (see Fig. 1), the uterine (1), ovarian (2) and inferior mesenteric (3) arteries were ligated and the upper half of the uterus, the ovaries and part of the descending colon (4) were excised, together with contiguous adipose tissue. Next, branches of the hypogastric (5) and pudic-epigastric trunks supplying pelvic viscera and adipose tissue were ligated. Ligatures were also placed around the neck of the bladder and the residual portions of the uterus (6) and descending colon. Adipose tissue in the perineal and retroperitoneal regions was then removed by the cautery.

Next, two pairs of loose ligatures were placed around the aorta and vena cava, one (7, 8) just above the origin of the right iliolumbar vessels and the other (9, 10) above the origin of the renal vessels. The inferior epigastric (11), iliolumbar (12) and renal (13) arteries and veins were then ligated as were the coeliac axis (14) and portal vein. A ligature was also placed around the tail (15). The ligatures previously placed around the vena cava and aorta above the origin of the renal vessels were then tied. The aorta was incised between the left renal and iliolumbar vessels (X) by using iridectomy scissors and a no. 18 polyethylene catheter (Intracatheter; Bard–Davol Ltd., Clacton-on-Sea, Essex, U.K.) filled with 0.85% NaCl containing 200 units of heparin/ml was introduced and passed to a point midway between the iliolumbar vessels and the aortic bifurcation (▽). About 0.2ml of the heparin–NaCl solution was injected from a syringe to determine whether the catheter was properly placed and to diminish the risk of intravascular coagulation. The catheter was then tied in place.

The vena cava was cannulated with a no. 16 Frankis–Evans needle (trocar and cannula, Luer fitting), which was passed through the vessel wall. It was positioned so that its tip (▽) was at the same level as the aortic catheter and then secured. After cannulation, the needle was connected by a Luer adaptor to a piece of transparent vinyl tubing (NT/6; Portex Ltd., Hythe, Kent, U.K.), which passed through a hole in the centre of the operating platform. The preparation was then transferred to the perfusion apparatus, where the aortic cannula was attached to the tubing containing the oxygenated medium. Some 2–3min elapsed from the time the great vessels were ligated and the circulation was re-established. The entire operative procedure required 20–25min.

In some experiments the skin covering the lower half of the animal was removed. The object was twofold: to determine the contribution of the skin to the metabolism of the hindquarter and to facilitate muscle biopsies. The skin was detached from the carcass except at the ankles and in the perineum before intra-abdominal surgery, but was left in place so as to envelop the carcass and thus help maintain tissue warmth during most of the operative procedure. The remaining attachments were severed and the whole skin was removed immediately before the ligation of the great vessels. Warm saline soaks were then used to prevent surface evaporation. The removal of the skin by this procedure required 5–10min.

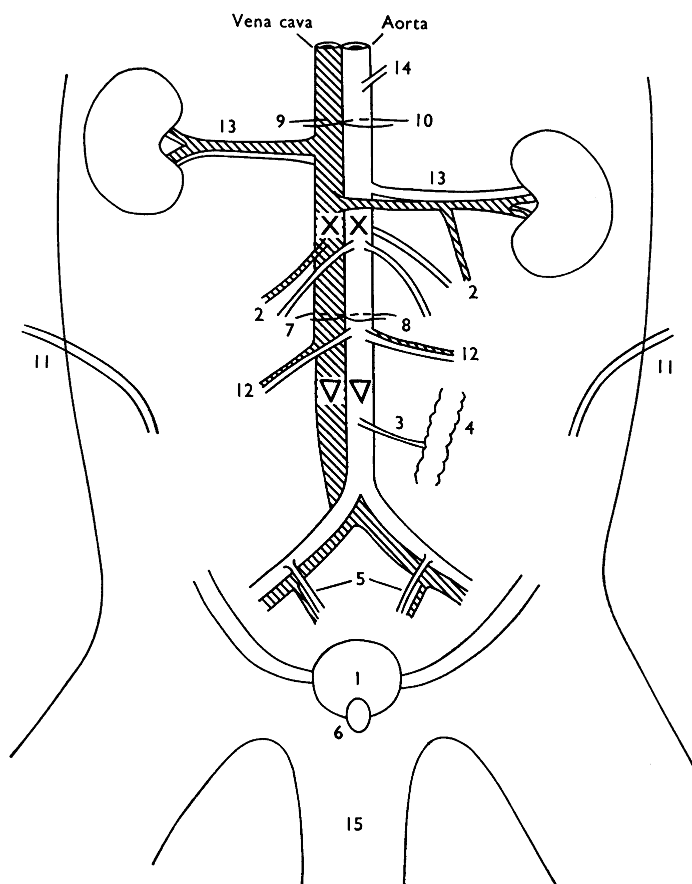

Fig. 1. Vessels ligated during operative preparation of isolated rat hindquarter. Names and numbers of the vessels are given in the text.

Perfusion apparatus. The perfusion apparatus and glassware were a modification of that described by Hems *et al.* (1966) for liver perfusion (Fig. 2). The perfusion medium was pumped from the reservoir by a type MHRE roller pump supplied by Watson–Marlow Ltd., Falmouth, Cornwall, U.K. It then passed through a plastic mesh filter taken from a disposable blood-transfusion set and entered a multibulb oxygenator. Here it was gassed with O_2+CO_2 (95:5) that had been humidified by passage through water. A gas flow of 300 ml/min was sufficient to maintain a constant pressure of O_2 and CO_2 in the arterial blood. After leaving the oxygenator, the medium was propelled into the arterial circulation of the animal by either a second roller pump or by the first pump fitted with an adaptor for holding two lines of rubber tubing with different diameters. The blood leaving the animal was then returned to the reservoir, from which it was recycled.

Arterial pressure was adjusted by means of a needle valve (Hone Ltd., London S.E.25, U.K.), which connected the tubing leading to the aortic cannula to the reservoir (see Fig. 2). The valve was set to produce a flow rate of 8–12 ml/min. This usually required a pressure of 80–110 mmHg in the arterial tubing. The pressure in the aorta itself was not measured, but was undoubtedly lower because of the fall in pressure across the aortic cannula. Pressure was measured by a mercury or aneroid manometer connected by a side arm to the arterial tubing. The vinyl tubing leading into the aorta and out of the vena cava was replaced in one region by a length of gum rubber obtained from a disposable blood-transfusion set. This allowed arterial and venous samples to be withdrawn through a hypodermic needle.

Unless otherwise stated the initial volume of perfusing medium was 200 ml. The first 70 ml of perfusate that passed through the preparation was discarded and from then onwards the perfusate was recycled. Then 5 min later a sample was collected for the determination of 'zero' values. The 'wash-out period' allowed some equilibration of perfusate and tissue before the perfusion medium was recycled. As a result, changes in the composition of the perfusate before the start of the

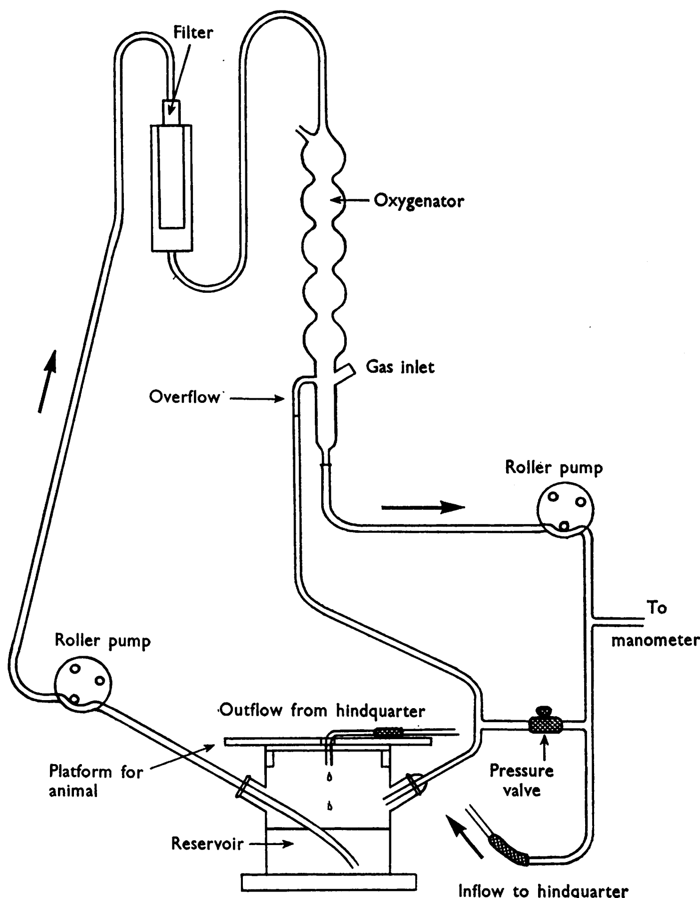

Fig. 2. Perfusion apparatus used for isolated rat hindquarter.

experimental period were minimal. When insulin was added to the perfusate the 'wash-out period' allowed sufficient time (13–15 min) for the full effect of the hormone to become evident before the experimental period began (see Mahler *et al.* 1968).

Electrical stimulation. Electrical stimulation was applied by means of Dastre's electrodes (Holborn Surgical Instrument Co. Ltd.) on a Students stimulator [C. F. Palmer (London) Ltd., London S.W.2, U.K.] at a rate of five/s, long pulse (1.5 ms) at strength 3. The electrodes were attached to the sciatic nerves in their gluteal course during the operative procedure; care was taken not to stretch or squeeze the nerves during dissection.

Measurement of metabolism of the hindquarter in vivo. To compare the performance of the isolated hindquarter with the hindquarter *in vivo*, female rats (170–230 g) were starved for 48 h, then anaesthetized with intraperitoneal pentobarbital (4.5 mg/100 g body wt.), and the abdomen was opened as described above. A blood sample (0.5 ml) was withdrawn, first from the inferior vena cava near its bifurcation, and then from the aorta. Plastic tuberculin syringes with a no. 25 needle were used for this purpose. Bleeding was avoided if the syringe used to draw blood from the cava was not removed until the arterial specimen had been taken. When metabolites other than O_2 were to be determined, the blood was added to 10% (w/v) $HClO_4$ and processed as described below. To obtain a larger volume for analyses, O_2 was determined in a separate series of experiments. The O_2 content of arterial and venous blood was determined by the method of van Slyke & Neill (1924). Creatine phosphate and adenine nucleotide contents of muscle were determined in specimens taken from animals anaesthetized with pentobarbital but not undergoing abdominal surgery. The skin was removed from a leg immediately before freeze-clamping *in situ*.

Analytical methods. Rat blood and perfusate were deproteinized in iced 10% (w/v) $HClO_4$. The extract was neutralized with KOH and the precipitated $KClO_4$ was removed by centrifugation. Glucose was determined by the method of Huggett & Nixon (1957) as modified by Krebs, Dierks & Gascoyne (1964). Lactate was determined enzymically as described by Hohorst (1963) and glycerol as described by Garland & Randle (1962).

Acetoacetate and 3-hydroxybutyrate were determined by the method of Williamson, Mellanby & Krebs (1962).

For the analysis of tissue samples, a portion of muscle, mainly gastrocnemius and posteroinferior thigh muscles, was rapidly frozen *in situ* with small aluminium clamps cooled in liquid N_2, as described by Wollenberger, Ristau & Schoffa (1960). The clamps were specially designed to fit the regional anatomy of the hindlimb. The frozen tissue was ground in a cooled percussion mortar, weighed and then homogenized in 10vol. of 10% (w/v) $HClO_4$. The sample was neutralized and $KClO_4$ removed as described above. ATP was determined by the method of Lamprecht & Trautschold (1963) and ADP by the method of Adam (1963). Creatine phosphate was determined as described by Lamprecht & Stein (1963).

Potassium was determined by flame photometry of samples of perfusate supernatant. O_2 consumption was calculated from (arteriovenous differences in O_2 content) × (flow rate). The flow rate was calculated from the number of drops of venous perfusate falling into the reservoir/unit time: the volume of 50 drops was 2.5ml when the vinyl tubing described above was used. $^{14}CO_2$ was collected as described by Ruderman, Toews, Lowy, Vreeland & Shafrir (1970). The samples were placed in a solution containing (per litre): 600ml of toluene, 400ml of methoxyethanol, 80g of naphthalene and 6g of 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole scintillator (Ciba Ltd., Duxford, Cambridge, U.K.) and counted for radioactivity in a Beckman model 200S liquid-scintillation counter.

Electron microscopy. Tissue was excised from the gastrocnemius or adductor muscles of the perfused hindquarter. The muscle was cut into 1mm cubes and then fixed in 3% glutaraldehyde buffered with 0.17M-phosphate (NaH_2PO_4–K_2HPO_4) buffer, pH 7.3 (Sabatini, Miller & Barnett, 1964). After this it was dehydrated with ethanol and epoxypropane and then embedded in Araldite. Sections were cut with a Huxley–Cambridge microtome (Cambridge Scientific Instruments Ltd., Cambridge, U.K.). They were stained with 2% (w/v) lead citrate and 5% (w/v) uranyl acetate and examined in a Phillips 100 or a Siemens Elmiskop 101 electron microscope.

RESULTS

Composition of perfused tissue. In a series of nine perfusions, Evans Blue (E. Gurr Ltd., London S.W.14, U.K.) was injected into the arterial circulation after approx. 40min to delineate the tissues that had been perfused. The stained tissue, which was confined to the hindquarter, was then severed from the remainder of the carcass and weighed. It was also weighed after removal of the skin and after dissolution of the soft tissue with 30% (w/v) potassium hydroxide. The latter measurement provided an estimate of bone weight. The relative percentages of skin, bone and soft tissue (muscle+adipose tissue) determined by this method are listed in Table 1.

Soft tissue comprised nearly 80% of the mass of the hindquarter. Most of this was muscle. There was relatively little visible adipose tissue. Further, only 4–5% of the net weight of muscle samples was extractable by chloroform–methanol. Since adipose tissue has a low rate of oxidative metabolism, its contribution to the total O_2 consumption of the perfused soft tissue must have been negligible. Therefore it is possible to estimate the percentages of total hindquarter O_2 consumption by bone, skin and muscle by using standard values for Q_{O_2} (O_2 consumption/h per mg dry wt.). The calculations (Table 1) indicate that muscle accounts for about 95% of the O_2 consumption of the perfused tissue.

Weight of muscle perfused. Since adipose tissue comprised a small proportion of the soft tissue mass, the latter will be referred to as 'muscle' throughout the rest of the paper. The absolute quantity of 'muscle' perfused was proportional to the weight of the rat when the animals weighed 170–240g. It was therefore possible to calculate the approximate quantity of 'muscle' perfused on the basis of body weight. The ratio of body weight to 'muscle' perfused was 6.03±0.10 (mean±S.E.M. for nine observations). All values are expressed per 30g of 'muscle', i.e. for an animal weighing 181g.

Loss of perfusate into the systemic circulation. The rat possesses a vertebral venous plexus that is not excluded by the operative procedure. Since the vertebral veins eventually join the systemic circulation, a certain amount of blood would escape the perfusion system. Indeed, both Mortimore *et al.* (1959) and Mahler *et al.* (1968) hemisected

Table 1. *Composition of perfused tissue rat hindquarter*

Evans Blue was injected into the arterial circulation and the perfused tissue was dissected as described in the text. Values for percentage of total weight are the means of nine studies. The proportion of fat is discussed in the text. It was assumed that red marrow comprised 20% of bone weight. The Q_{O_2} values are taken from Kratzing (1961).

	Percentage of total wt.±S.E.M.	Q_{O_2} (μl/h per mg dry wt.)	Estimated percentage of total O_2 consumption
Bone	10 ±0	4 (red marrow)	2–3
Skin	12.3±0.8	0.5	2–3
Muscle (+fat)	77.7±0.8	3	95

their animals to obviate this difficulty. To evaluate the significance of such blood loss, ^{131}I-labelled polyvinylpyrrolidone was added to the perfusate, and the radioactivities present in the perfusate and in right atrial blood were compared at the end of the perfusion. In seven experiments the radioactivity (c.p.m./ml) of the right atrial blood averaged 30.8% (range 5.3–54.5) of that of the perfusate. Since the heart and the great vessels were not obviously engorged, the blood volume in the systemic circulation was in all likelihood no more than 6ml. Therefore an average of 1.8ml (range 0.3–3.3ml) would have escaped the hindquarter circulation. Since a loss of this magnitude would not have had a significant effect on the results, the animal was not hemisected.

Appearance of the preparation. The routine operative procedure involved a period of anoxia of 2–3min during the cannulation of the aorta and vena cava. During this time the feet of the animal became pale and somewhat cyanotic. Within 2–3min after re-establishment of the circulation, the feet had usually regained the same colour that they had *in vivo*. Animals whose skin had been removed required up to 15min to regain the colour that they had *in vivo* and to achieve maximal flow rates.

Criteria of tissue integrity. The appearance of the preparation to the naked eye and under the electron microscope, the flow rate, the rates of O_2 consumption and lactate production, the maintenance of tissue concentrations of creatine phosphate, ATP and ADP, and the shedding of K^+, served as criteria of tissue integrity. A gross indicator of inadequate perfusion is the persistence of cyanotic discoloration of the feet. An arterial flow rate under 6ml/min is an even more sensitive indicator. When facility with the operative technique was gained these were very infrequent occurrences.

On the basis of rates of lactate production and tissue concentrations of creatine phosphate, ATP and ADP after 40–50min of perfusion, experiments in which the perfusion appeared to be grossly adequate could be placed into two groups. In group I (Table 2) the values for tissue concentrations of the organic phosphates were close to those found in fresh muscle biopsy material. Group II was smaller and the preparations had both a lower concentration of creatine phosphate and higher rates of lactate production, suggesting that these perfusions may have been inadequate because of ischaemia (Bollman & Flock, 1944) or muscle spasms. Since the increment in lactate production seemed to correlate with the decrease in creatine phosphate, a persistently high rate of lactate production (>4μmol/min per 30g of muscle) was used as a routine index of the inadequacy of the perfusion. About 10% of the resting control muscle preparations were eliminated for this reason. It remains to be determined whether this criterion can be applied to hindquarters treated in other ways.

Metabolism of the perfused hindquarter. When the hindquarter was perfused with a medium not containing insulin, O_2 consumption tended to remain constant during the 30min experimental period. Glycerol release was usually about 25% higher during the initial 15min, as was lactate production. When the skin was not removed, glucose uptake tended to be higher during the second 15min of perfusion (1.7±0.5μmol/min per 30g of muscle). The results for the first 15min of perfusion are given in Table 3.

In the absence of insulin, the mean rate of glucose uptake was 0.5–1.7μmol/min per 30g of muscle. If all of this glucose were oxidized it could have accounted for 30–100% of hindquarter O_2 consumption. On the other hand if it were converted

Table 2. *Concentrations of creatine phosphate and adenine nucleotides in perfused rat hindquarter and rat hindquarter* in vivo

Results are the means±S.E.M. with the numbers of observations in parentheses. Lactate production and O_2 consumption are expressed as μmol/min per 30g of 'muscle' perfused. Lactate production was determined during the first (*a*) and second (*b*) 15min of perfusion, O_2 consumption only in the first period. Results are corrected for lactate produced by erythrocytes in the absence of tissue. Tissue metabolites in the perfused hindquarter were determined after 40–50min of perfusion. Tissue metabolites in intact rats were determined in animals anaesthetized with pentobarbital.

	Lactate production	O_2 consumption	Creatine phosphate (μmol/g)	ATP (μmol/g)	ADP (μmol/g)
Hindquarter *in vivo* (5)	—	—	15.6±0.8	5.0±0.2	0.75±0.01
Perfused hindquarter					
Group I (9) (*a*)	2.7±0.6	10.8±0.8	15.1±0.6	5.2±0.3	0.95±0.09
(*b*)	2.1±0.2				
Group II (3) (*a*)	4.6±0.1	10.2±0.5	11.5±0.2	4.8±0.2	0.94±0.03
(*b*)	5.9±0.5				

Table 3. *Fuel metabolism of isolated perfused rat hindquarter*

Hindquarters of rats starved for 48h were perfused with standard medium containing 5.5mM-glucose as described in the text. O_2 consumption was determined after 10–15min of perfusion. Other results were obtained during the first 15min of the 30min experimental period. Results are expressed as μmol/min per 30g of 'muscle' tissue perfused (means±S.E.M. with the numbers of observations in parentheses).

	Glucose uptake	Lactate production	O_2 consumption	Glycerol release
Skin on				
Control	0.5±0.3 (6)	2.4±0.3 (8)	9.0±1.2 (7)	0.25±0.02 (6)
+Insulin (12.5munits/ml)	7.3±0.6 (7)	2.1±0.3 (7)	12.5±0.8 (7)	0.09±0.01 (7)
Skin removed				
Control	1.7±0.5 (7)	3.0±0.9 (7)	10.8±0.8 (7)	0.32±0.04 (7)
+Phentolamine (0.1mg/100ml)	1.5±0.3 (6)	2.8±0.3 (6)	10.1±0.6 (6)	0.30±0.08 (6)

primarily into glycogen and lactate the value would be much lower. To determine which of these possibilities was correct, [U-^{14}C]glucose was added to the perfusate in six experiments, three control and three with insulin, and $^{14}CO_2$ was collected (Table 4). The results indicate that the latter view is correct. During a 30min perfusion 300–400μmol of O_2 was consumed but only 1.3μmol of glucose was oxidized to CO_2. Hence exogenous glucose accounted for less than 4% of the oxidative fuel of the resting hindquarter.

Lactate production was 2.4 and oxygen consumption 9μmol/min per 30g of muscle in the control preparation (Table 3). If one assumes that 6mol of ATP is generated/mol of O_2 consumed and that 1mol of ATP is generated/mol of lactate produced, 96% of the ATP generated by the hindquarter would have been derived oxidatively.

Comparable results were obtained when the hindquarter was perfused after removal of the skin (Table 3), suggesting that the contribution of skin to oxidative metabolism was negligible. The observation that glycerol release was not lower in the skinned animals also indicates that the exclusion of subcutaneous adipose tissue from the circulation was reasonably complete when the skin was not removed.

In several of the experiments in which the animals were skinned, a vasodilator, the α-adrenergic blocker phentolamine (see Nickerson, 1968), was added to the perfusate. This was done to diminish the vasoconstriction and poor initial flow rates that frequently resulted from removal of the skin. The doses used were not large enough to depress glycerol release. Phentolamine caused vasodilatation, as indicated by higher flow rates at a given arterial pressure. It also caused optimum flow rates to be established more rapidly and to be maintained at a more constant value throughout the perfusion period. Further, phentolamine decreased the number of perfusions in which lactate was produced at an excessive rate (one out of eight, as against three out of ten in a control group).

Effect of insulin (*Table* 3). Insulin produced a greater than tenfold increase in glucose uptake, an effect of the expected order. It did not increase lactate production during the initial 15min of perfusion. However, in the second half of the experimental period, lactate production rose to 3.3±0.58μmol/min per 30g of muscle (mean±S.E.M. for seven observations), a value significantly higher than that obtained during the initial 15min ($P<0.02$, paired comparison). It was also significantly higher than the amount, namely 1.8±0.28μmol of lactate/min per 30g of muscle (mean±S.E.M. for eight observations), produced by the control preparations during the corresponding time-period. In a similar series of experiments, one of us (C.R.S.H.) did not obtain the secondary increase in lactate production. Possibly this was related to the use of phentolamine or to the absence of the skin in these studies. Lipolysis as reflected by glycerol release was decreased to 50% of the control value by insulin. Consumption of O_2 was significantly greater (38%) than that of control preparations. Despite this, glucose utilization was far in excess of that which could have been accounted for by lactate production and O_2 consumption even if glucose were the sole oxidative fuel of the hindquarter. This indicates that most of the glucose taken up in the presence of insulin was utilized for anabolic purposes, e.g. glycogen synthesis.

Tissue morphology. Biopsies were taken for electron microscopy after 40–50min of perfusion in five experiments. The tissue appeared normal except in one experiment in which an adequate flow rate was never established patchy swelling of the T tubules was noted in an occasional section. Even in this sample, however, there was no swelling of the mitochondria or dissolution of I bands such as has been noted in the muscles of mice (Moore, Ruska & Copenhaver, 1956) and dogs (Stenger, Spiro, Scully & Shannon, 1962) after several hours of ischaemia.

Efflux of K^+. In the perfused rat liver K^+ efflux is one of the most sensitive indicators of decreased tissue integrity (Ruderman & Herrera, 1968). K^+

Table 4. *Oxidation of exogenous glucose to carbon dioxide by the perfused rat hindquarter*

Hindquarters of 48h-starved rats were perfused with 125ml of a medium containing 5.5mM-glucose and 5–10μCi of [U-^{14}C]glucose. Preparations were skinned. Results for O_2 consumption were obtained in other experiments (see Table 3). For details of collection of CO_2 see the Materials and Methods section. Results are expressed as μmol/30min per 30g of muscle.

	Glucose uptake	Glucose oxidized to CO_2	O_2 consumption	% of O_2 consumption due to glucose oxidation
Control	12	1.5	285	3.4
	33	1.2		2.6
	45	0.3		0.8
+Insulin (12.5munits/ml)	162	2.1	375	3.4
	111	2.1		3.4
	196	0.6		1.0

efflux has also been demonstrated in mammalian muscle when it is deprived of O_2 (Danowski & Elkinton, 1951; Creese, 1954; Rixon & Stephenson, 1956).

In the perfused hindquarter there was no net release of K^+ during the perfusion period (Table 5). The mean K^+ concentration at the end of the wash-out period was 5.82μmol/ml and it was nearly the same 35min later. In accord with the findings of others (Kamminga, Willebrands, Groen & Blickman, 1950; Willebrands, Groen, Kamminga & Blickman, 1950; Zierler, 1959; Andres, Baltzan, Cader & Zierler, 1962), insulin caused a substantial net uptake of K^+ that was detectable after 5min. Insulin had no effect on plasma K^+ when erythrocyte-containing perfusate was recycled in the absence of tissue.

Maintenance of integrity during a 2h *perfusion.* When the perfusion period was extended to 125min, the hindquarter showed no gross signs of deterioration. The animal's feet remained pink and the flow rate and arterial pressure were relatively constant. The tissue also maintained its rate of O_2 consumption and the ability to take up glucose at an increased rate in the presence of insulin (Table 6). Lactate production was not constant, however. In both the presence and the absence of insulin the increase in lactate in the perfusate was greater during the second hour of the perfusion. The significance of this pattern is not clear, although it may reflect some tissue deterioration.

When the hindquarter was perfused with a medium containing insulin there was a net uptake of K^+ during the first 35–65min. Thereafter perfusate K^+ tended to increase. Possible factors contributing to this haemolysis of erythrocytes, which occurs in all perfusions, and a fall in perfusate pH owing to lactate accumulation. With regard to the latter mechanism, in comparable experiments we have observed an even greater increase in perfusate K^+ when the pH of the medium was adjusted to 7.1–7.2. The contribution of altered tissue integrity to the increase in K^+ concentration in the perfusate is not known. However, it is noteworthy that, even if the entire increment in perfusate K^+ resulted from tissue leakage, less than 5% of the total tissue K^+ would have been lost.

Effect of nerve stimulation on the metabolism of the perfused hindquarter. As shown in Table 7 the hindquarter preparation responds to bilateral sciatic-nerve stimulation with large changes in the metabolism of glucose, lactate and O_2. Stimulation was continuously applied during the period 20–40min after the beginning of the perfusion at a rate of five stimuli/s. During the first 1min of stimulation the muscular contractions were rather violent, but after this initial phase a uniform intensity of contractions was maintained for at least 15min. In some perfusions the contractions weakened during the 35–40min period, probably because of fatigue. Stimulation caused a tenfold increase in the rate of lactate production and a two- to four-fold increase in O_2 consumption. In the presence of excess of insulin (25munits/ml) electrical stimulation caused no extra glucose uptake whereas in the absence of insulin, stimulation caused an increase in glucose uptake from 0.75 to 4.22μmol/min per 30g of muscle, in agreement with similar findings by Szabo *et al.* (1969). The increased glucose uptake could account for no more than 35% of the increased lactate production. Thus endogenous glycogen must have contributed to a large extent to the lactate formed by the exercising muscle (see Havel, Pernow & Jones, 1967). During the last 5min of stimulation (results not shown) there was a marked decrease in lactate production and an increase in glucose uptake in the absence of insulin.

Comparison with rat hindquarter in vivo. Lactate and O_2 were measured in samples of blood taken from the aorta and vena cava of intact rats that had been starved for 48h. To compare these values with those obtained from the isolated hindquarter, the flow rate *in vivo* was calculated from the data of

Table 5. *Effect of insulin on net uptake of* K^+ *by isolated perfused rat hindquarter*

Results are the means±S.E.M. with the numbers of experiments in parentheses. Time 0min represents the beginning of the period during which the perfusate was recycled. Insulin (12.5 units/ml) was present in the initial medium.

	Serum K^+ (μmol/ml)			
Time ...	0min	5min	20min	35min
Control	5.82±0.19 (9)	5.97±0.13 (12)	5.84±0.14 (11)	5.80±0.16 (10)
+Insulin	5.78±0.14 (7)	5.62±0.14 (8)	5.17±0.17* (7)	—

* Significantly different from control: $P<0.02$ (*t* test).

Table 6. *Performance of isolated hindquarter during perfusion for* 2h

The perfusion was carried out under standard conditions in the presence of 10mM-glucose. The erythrocytes used in the experiments had not been fully aged and produced 0.4μmol of lactate/ml in 30min in the presence of glucose. No correction was made for this glycolysis by the erythrocytes. The values are means of two experiments. In the experiments where insulin was present, further glucose (5μmol/ml) was added at 65min.

	Insulin added (12.5munits/ml)				No insulin added	
Time after start of perfusion (min)	Concn. in medium (mM)			O_2 consumption (μmol/min per 30g of muscle)	Concn. in medium (mM)	
	Glucose	Lactate	K^+		Glucose	Lactate
5	9.4	3.4	5.2		11.1	1.9
20				11.4		
35	6.8	4.0	4.9		10.3	2.6
50				7.7		
65	4.9 [9.9]	5.7	5.0		9.3	2.8
80				9.6		
95	7.0	7.4	5.5		8.2	4.9
110				9.8		
125	5.0	9.5	6.2		7.5	6.4

Sapirstein, Sapirstein & Bredemeyer (1960) by assuming that the tissue drained by the vena cava below the entrance of the iliolumbar vessels comprised one-third of the carcass and skin mass. On this basis a blood flow of 7.3ml/min was calculated for the hindquarter of a 181g rat.

Comparative results for lactate production and O_2 consumption are given in Table 8. The hindquarter *in vivo* had a similar rate of lactate production and a slightly higher rate of O_2 consumption than the isolated hindquarter perfused with 5mM-glucose. More than 70% of the oxidative metabolism of the hindquarter *in vivo* was due to ketone-body metabolism, however (see below). When 1.8mM-acetoacetate was added to the medium perfusing the isolated hindquarter, ketone-body metabolism accounted for a similar proportion of O_2 consumption and the absolute rate of O_2 utilization was even higher than that of the hindquarter *in vivo*.

Comparative results for isolated red and white rat adductor muscle fibres incubated *in vitro* (Beatty, Peterson & Bocek, 1963) are also presented in Table 8. The fibres had higher rates of glucose utilization and lactate production than the perfused hindquarter. Their rates of O_2 consumption also may have been higher. In these respects the isolated fibres resemble exercising muscle (see Table 7).

Ketone-body metabolism. It has long been appreciated that ketone bodies may be a major oxidative fuel of muscle during diabetes and starvation (see review by Williamson & Hems, 1970). Measurements of ketone-body utilization and O_2 consumption by hindquarters of 48h-starved rats *in vivo* (Table 9) support this view. If the acetoacetate and 3-hydroxybutyrate taken up by the hindquarter were completely oxidized, ketone-body metabolism would have accounted for 77% of the O_2 consumed. Of this total, acetoacetate accounted for 84% and 3-hydroxybutyrate 16%, even though acetoacetate comprised only 33% of the total ketone bodies in the arterial circulation.

Acetoacetate also appeared to be the preferred fuel of the isolated perfused hindquarter (Table 9).

Table 7. *Effects of bilateral sciatic-nerve stimulation on lactate production, glucose uptake and oxygen consumption in the isolated perfused rat hindquarter*

All preparations had skin removed. After an initial 20min equilibration period of recycling perfusion, the sciatic nerves were stimulated at a rate of 5/s for 20min (see the Materials and Methods section for details). The results are calculated for the period of perfusion between 20 and 40min of the experiment and are expressed as μmol/min per 30g of muscle (means±S.E.M. with the numbers of observations in parentheses). The initial glucose concentration was 5mM. Phentolamine was 3 1μM throughout.

Experimental conditions	Lactate production	Glucose uptake	O_2 consumption		
			At rest	After 9min stimulation	After 19min stimulation
Resting	2.34±0.72 (6)	0.75±0.30 (6)	7.3±0.8 (4)	—	—
Stimulated	22.7 ±1.3 (4)	4.22±0.26 (4)	—	28.6±5.0 (4)	23.6±1.8 (4)
Resting, +insulin (25munits/ml)	1.96±0.21 (6)	6.42±0.33 (6)	9.1±1.2 (5)	—	—
Stimulated, +insulin (25munits/ml)	23.8 ±1.3 (4)	6.94±0.78 (6)	—	22.7±1.8 (5)	19.4±0.7 (3)

Table 8. *Fuel metabolism of perfused rat hindquarter, rat hindquarter* in vivo *and isolated rat muscle fibres*

Results are expressed as μmol/min per 30g of 'muscle' (means±S.E.M. with the numbers of experiments in parentheses). Results for the hindquarter *in vivo* were obtained from animals starved for 48h and anaesthetized with pentobarbital. The results are based on arteriovenous differences. Flow rate was assumed to be 7.3ml/min (see the text). Results for the perfused hindquarter are based on measurements made during the first 15min of the experimental period. Animals had been starved for 48h. Results for rat adductor muscle are taken from Beatty *et al.* (1963).

	Glucose uptake	Lactate production	O_2 consumption
Perfused rat hindquarter			
Glucose (5mM)	0.5±0.3 (6)	2.4±0.3 (8)	9.0±1.2 (8)
Glucose (5mM)+aceto-acetate (1.8mM)	1.0±0.3 (5)	1.8±0.8 (5)	13.1±1.1 (5)
Rat hindquarter *in vivo*		2.1±0.3 (14)	11.8±1.4 (8)
Rat adductor muscle fibres			
Red	3.7 (12)	4.8 (13)	17.0 (6)
White	3.2 (12)	6.1 (13)	17.0 (6)

Tissues perfused with 1.8mM-acetoacetate removed 2.15μmol of this substrate/min per 30g of muscle. Of this 0.45μmol was converted into 3-hydroxybutyrate and 2.06μmol was presumably oxidized. If the latter assumption is correct, about 60% of the O_2 consumption would have been due to acetoacetate oxidation. When present at an equivalent concentration, 3-hydroxybutyrate was taken up at only one-fifth of the rate for acetoacetate. When the contributions of the two ketone bodies were added together, their oxidation theoretically could have accounted for 91% of the O_2 consumption. Once again most of this was due to acetoacetate metabolism.

Acetoacetate utilization was not diminished when 1.3mM-oleate was added to the perfusate. Oleate was also without effect when lower concentrations of acetoacetate were used (results not shown). Insulin failed to decrease acetoacetate removal even though it increased glucose utilization from 1 to nearly 6μmol/min per 30g of muscle.

DISCUSSION

Flow rate. The results establish the efficacy of the isolated perfused rat hindquarter preparation for physiological and biochemical studies. The addition of a vasodilator (phentolamine) may not be essential, but in general a good flow rate (8–12ml/min) was established more rapidly when phentolamine was present, especially when the skin was removed from the preparation. Apart from abolishing vasoconstriction no effects of phentolamine were observed. Thus it does not appear to interfere with metabolic studies, although effects on lipolysis and other metabolic processes have been reported (see Himms-Hagen, 1967; Mayhew, Wright & Ashmore, 1969).

Effect of insulin on oxygen consumption. Although some results suggest (see Krahl, 1961) that increased O_2 consumption accompanies the increased uptake of glucose by muscle caused by insulin, there appears to be no study dealing with the quantitative relations of these effects in a preparation which

Table 9. *Ketone-body metabolism of rat hindquarter* in vivo *and isolated perfused hindquarter*

Results are expressed as μmol/min per 30g of muscle (means±S.E.M. with the numbers of experiments in parentheses). Results from perfusion experiments were obtained during the first 15min of the experimental period. Values for 3-hydroxybutyrate release in the presence of acetoacetate and for 3-hydroxybutyrate disappearance are corrected for 3-hydroxybutyrate release by control hindquarter preparations. Listed 3-hydroxybutyrate concentrations are those of the D-isomer. Values for acetoacetate disappearance are corrected for spontaneous loss in the presence of erythrocytes. Standard perfusing medium contained 4% albumin, 5.5mM-glucose and 0.3–0.5mequiv. of free fatty acid/l. See the text for further details. Animals used in studies *in vivo* had arterial concentrations of 3-hydroxybutyrate and acetoacetate of 1.52 and 0.77mmol/l respectively. The calculations used in the experiments *in vivo* are described in the legend to Table 7 and in the text.

	Acetoacetate uptake	3-Hydroxybutyrate uptake	Net ketone-body utilization	O_2 consumption	% of O_2 consumption due to ketone-body oxidation
Hindquarter *in vivo*					
48h-starved rat	2.12±0.15 (19)	0.36±0.29 (19)	2.48±0.41 (19)	13.0±0.2 (7)	77
Perfused hindquarter					
Control	—	−0.21±0.03 (11)	—	9.0±0.5 (7)	—
Acetoacetate (1.8mM)	2.51±0.15 (6)	−0.45±0.05 (6)	2.06±0.17 (6)	13.0±0.9 (6)	61±5 (6)
3-Hydroxybutyrate (2.1mM)	−0.09±0.02 (5)	0.52±0.26 (5)	0.43±0.19 (5)	—	—
Acetoacetate (2.1mM)+ 3-hydroxybutyrate (1.8mM)	2.54±0.18 (4)	0.30±0.25 (4)	2.84±0.35 (4)	12.8±1.1 (4)	91±7 (4)
Acetoacetate (1.8mM)+ oleate (1.3mM)	2.43±0.26 (4)	−0.37±0.07 (4)	2.06±0.22 (4)	11.9 (2)	71 (2)
Acetoacetate (1.7mM)+ insulin (12.5munits/ml)	2.61±0.20 (10)	−0.46±0.06 (10)	2.16±0.14 (10)	13.1±0.8 (10)	67±5 (10)

simulates conditions *in vivo* as closely as the isolated perfused hindquarter. In all likelihood most of the extra glucose taken up in the presence of insulin is converted into glycogen. Two mol of ATP are required/mol of glucose incorporated into glycogen. On the assumption that this ATP is generated oxidatively, an increase in O_2 consumption of about 2.2μmol/min per 30g of muscle would be expected. In fact the increase was greater (3.5μmol/min per 30g of muscle; Table 3). It is possible that insulin enhances other energy-requiring activities in muscle (see Walaas, Walaas & Wick, 1969).

Origin of glycerol. Whether the glycerol released by the hindquarter (0.22μmol/min per 30g of muscle) originated from the small amount of adipose tissue or from intracellular muscle triglyceride is still an open question.

Glucose as a fuel of resting muscle. The finding that added glucose made no more than a negligible contribution to the fuel of respiration corresponds to similar observations on the human forearm by Andres, Cader & Zierler (1956) and on the dog hindlimb muscles *in situ* by Chapler & Stainsby (1968) and Bass & Hudlicka (1960). Since the glucose 6-phosphate derived from exogenous glucose and that derived from endogenous glycogen are presumably part of a common pool, the results suggest also that glycogen was not an important metabolic fuel.

Free fatty acids and ketone bodies as metabolic fuels. The oxidation by muscle of fatty acids has been well established (see Fritz, 1961; Drummond, 1969). The utilization by muscle of ketone bodies was first recorded by Snapper & Grünbaum (1927) (for review see Williamson & Hems, 1970). It is generally considered that ketone bodies are an important fuel for muscle in diabetic animals and man. Their importance as a muscle fuel during starvation has been less emphasized, although several reports to this effect have appeared (Gammeltoft, 1949–50; Owen & Reichard, 1970). The present study indicates that ketone bodies and in particular acetoacetate are the major oxidative fuels of resting muscle in the 48h-starved rat.

The fact that acetoacetate is utilized more rapidly than 3-hydroxybutyrate, when they are present at comparable concentrations, is probably due to the low activity of 3-hydroxybutyrate dehydrogenase in muscle (Lehninger, Sudduth & Wise, 1960), since 3-hydroxybutyrate must be converted into acetoacetate before it is further metabolized. The observation that the [3-hydroxybutyrate]/[acetoacetate] ratio of rat muscle *in vivo* is significantly higher than that of arterial plasma

(N. B. Ruderman, unpublished work) is consistent with this view.

The finding that acetoacetate utilization was not inhibited by oleate or insulin plus glucose suggests that it is oxidized in preference to these fuels. Acetoacetate is also utilized in preference to free fatty acids by the perfused rat heart (Williamson & Krebs, 1961; Hall, 1961; Olson, 1962). On the other hand insulin has been shown to inhibit ketone-body utilization in this preparation (Williamson & Krebs, 1961).

Crystalline porcine insulin low in glucagon and treated with trypsin (lot PJ 5589) was kindly provided by Dr W. Shaw of Eli Lilly Co., Indianapolis, Ind., U.S.A. Phentolamine was a gift from Ciba Ltd., Duxford, Cambridge, U.K. We are grateful to Professor H. A. Krebs and Dr D. H. Williamson for their advice and criticisms throughout this study and to Dr M. Eseri for performing the electron-microscopic investigations. N.B.R. (on leave of absence from the Joslin Research Laboratory, Department of Medicine, Harvard Medical School, Boston, Mass. 02115, U.S.A.) was the recipient of a research and development award from the American Diabetes Association and a grant from the Burroughs-Wellcome Trust. This work was supported by grants to Professor H. A. Krebs from the Medical Research Council, the U.S. Public Health Service (Grant no. AM11748-03), the British Diabetes Association and the Diabetes Foundation Inc., Boston, Mass., U.S.A.

REFERENCES

Adam, H. (1963). In *Methods of Enzymatic Analysis*, p. 573. Ed. by Bergmeyer, H. U. New York and London: Academic Press.

Andres, R., Baltzan, M. A., Cader, G. & Zierler, K. L. (1962). *J. clin. Invest.* **41**, 108.

Andres, R., Cader, G. & Zierler, K. L. (1956). *J. clin. Invest.* **25**, 671.

Bass, A. & Hudlicka, O. (1960). *Physiologia bohemoslov.* **9**, 401.

Beatty, C. H., Peterson, R. D. & Bocek, R. M. (1963). *Am. J. Physiol.* **204**, 939.

Best, C. H. (1926). *Proc. R. Soc.* B, **99**, 375.

Blixenkrone-Møller, N. (1938). *Hoppe-Seyler's Z. physiol. Chem.* **253**, 261.

Bollman, J. L. & Flock, E. V. (1944). *Am. J. Physiol.* **142**, 290.

Burn, J. H. & Dale, H. H. (1926). *J. Physiol., Lond.*, **61**, 185.

Chapler, C. K. & Stainsby, W. N. (1968). *Am. J. Physiol.* **215**, 995.

Creese, R. (1954). *Proc. R. Soc. Med.* B, **142**, 497.

Danowski, T. S. & Elkinton, J. R. (1951). *Pharmac. Rev.* **3**, 42.

Drummond, G. (1969). *Fortschr. Zool.* **18**, 359.

Forsander, O., Räihä, N. & Soumalainen, H. (1960). *Hoppe-Seyler's Z. physiol. Chem.* **318**, 3.

Fritz, I. B. (1961). *Physiol. Rev.* **41**, 52.

Gammeltoft, A. (1949–50). *Acta physiol. scand.* **19**, 270.

Garland, P. B. & Randle, P. J. (1962). *Nature, Lond.*, **196**, 987.

Greene, E. C. (1959). *Anatomy of the Rat.* New York: Hafner Publishing Co.

Hall, L. M. (1961). *Biochem. biophys. Res. Commun.* **6**, 177.

Havel, R. H., Pernow, B. & Jones, N. L. (1967). *J. appl. Physiol.* **23**, 90.

Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966). *Biochem. J.* **101**, 287.

Himms-Hagen, J. (1967). *Pharmac. Rev.* **19**, 367.

Hohorst, H. J. (1963). In *Methods of Enzymatic Analysis*, p. 266. Ed. by Bergmeyer, H. U. New York and London: Academic Press.

Houghton, C. R. S. & Ruderman, N. B. (1970). *Biochem. J.* **121**, 15 P.

Houghton, C. R. S., Ruderman, N. B. & Hems, R. (1970). *Diabetologia*, **6**, 632.

Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.

Kamminga, C. E., Willebrands, A. F., Groen, J. & Blickman, J. R. (1950). *Science, N.Y.*, **111**, 30.

Krahl, M. E. (1961). *The Action of Insulin on Cells*, p. 15. New York and London: Academic Press.

Kratzing, C. C. (1961). In *Biochemists' Handbook*, p. 795. Ed. by Long, C. London: E. and F. N. Spon Ltd.

Krebs, H. A., Dierks, C. & Gascoyne, T. (1964). *Biochem. J.* **93**, 112.

Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.

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

Lamprecht, W. & Stein, P. (1963). In *Methods of Enzymatic Analysis*, p. 610. Ed. by Bergmeyer, H. U. New York and London: Academic Press.

Lamprecht, W. & Trautschold, I. (1963). In *Methods of Enzymatic Analysis*, p. 453. Ed. by Bergmeyer, H. U. New York and London: Academic Press.

Lehninger, A. L., Sudduth, H. C. & Wise, J. B. (1960). *J. biol. Chem.* **235**, 2450.

Mahler, R. J., Szabo, O. & Penhos, J. C. (1968). *Diabetes*, **17**, 1.

Mayhew, D., Wright, P. & Ashmore, J. (1969). *Pharmac. Rev.* **21**, 183.

Moore, D. H., Ruska, H. & Copenhaver, W. M. (1956). *J. biophys. biochem. Cytol.* **2**, 755.

Mortimore, G. E., Tietze, F. & Stetten, D. (1959). *Diabetes*, **8**, 307.

Nickerson, M. (1968). In *Pharmacological Basis of Therapeutics*, p. 546. Ed. by Goodman, L. S. & Gilman, A. New York: Macmillan Co.

Olson, R. E. (1962). *Nature, Lond.*, **195**, 597.

Owen, O. E. & Reichard, G. A. (1970). *Clin. Res.* **18**, 461.

Rixon, R. H. & Stephenson, J. A. F. (1956). *Can. J. Biochem. Physiol.* **34**, 1069.

Robinson, D. S. & Harris, P. M. (1959). *Q. Jl exp. Physiol.* **44**, 80.

Ruderman, N. B. & Herrera, M. G. (1968). *Am. J. Physiol.* **214**, 1346.

Ruderman, N. B., Houghton, C. R. S. & Hems, R. (1970). *Diabetologia*, **6**, 645.

Ruderman, N. B., Toews, C. J., Lowy, C., Vreeland, J. & Shafrir, E. (1970). *Am. J. Physiol.* **219**, 51.

Sabatini, D. D., Miller, F. & Barnett, R. J. (1964). *J. Histochem. Cytochem.* **12**, 57.
Sapirstein, L. A., Sapirstein, E. H. & Bredemeyer, A. (1960). *Circulation Res.* **8**, 135.
Snapper, I. & Grünbaum, A. (1927). *Biochem. Z.* **185**, 223.
Stenger, R. J., Spiro, D., Scully, R. E. & Shannon, J. M. (1962). *Am. J. Path.* **40**, 1.
Szabo, A. J., Mahler, R. J. & Szabo, O. (1969). *Horm. metab. Res.* **1**, 156.
van Slyke, D. D. & Neill, J. M. (1924). *J. biol. Chem.* **61**, 523.
Walaas, O., Walaas, E. & Wick, A. N. (1969). *Diabetologia*, **5**, 79.
Willebrands, A. F., Groen, J., Kamminga, C. E. & Blickman, J. R. (1950). *Science, N.Y.*, **112**, 277.
Williamson, D. H. & Hems, R. (1970). In *Essays in Cell Metabolism*, p. 257. Ed. by Bartley, W., Kornberg, H. L. & Quayle, J. R. London: Wiley–Interscience Publishers.
Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962). *Biochem. J.* **82**, 90.
Williamson, J. R. & Krebs, H. A. (1961). *Biochem. J.* **80**, 540.
Wollenberger, A., Ristau, O. & Schoffa, G. (1960). *Pflügers Arch. ges. Physiol.* **270**, 399.
Zierler, K. L. (1959). *Am. J. Physiol.* **197**, 524.