Biochem. J. (1980) **186**, 525–534
Printed in Great Britain

Insulin Resistance in Soleus Muscle from Obese Zucker Rats

INVOLVEMENT OF SEVERAL DEFECTIVE SITES

Marco CRETTAZ, Marc PRENTKI, Daniel ZANINETTI and Bernard JEANRENAUD
Laboratoires de Recherches Médicales, Geneva University Medical School, 64 Avenue de la Roseraie, 1205 Geneva, Switzerland

(*Received* 4 *July* 1979)

1. The effect of insulin upon glucose transport and metabolism in soleus muscles of genetically obese (*fa/fa*) and heterozygote lean Zucker rats was investigated at 5–6 weeks and 10–11 weeks of age. Weight-standardized strips of soleus muscles were used rather than the intact muscle in order to circumvent problems of diffusion of substrates. 2. In younger obese rats (5–6 weeks), plasma concentrations of immunoreactive insulin were twice those of controls, whereas their circulating triacylglycerol concentrations were normal. Insulin effects upon 2-deoxyglucose uptake and glucose metabolism by soleus muscles of these rats were characterized by both a decreased sensitivity and a decrease in the maximal response of this tissue to the hormone. 3. In older obese rats (10–11 weeks), circulating concentrations of insulin and triacylglycerols were both abnormally elevated. A decrease of 25–35% in insulin-binding capacity to muscles of obese rats was observed. The soleus muscles from the older obese animals also displayed decreased sensitivity and maximal response to insulin. However, at a low insulin concentration (0.1 m-i.u./ml), 2-deoxyglucose uptake by muscles of older obese rats was stimulated, but such a concentration was ineffective in stimulating glucose incorporation into glycogen, and glucose metabolism by glycolysis. 4. Endogenous lipid utilization by muscle was calculated from the measurements of O_2 consumption, and glucose oxidation to CO_2. The rate of utilization of fatty acids was normal in muscles of younger obese animals, but increased in those of the older obese rats. Increased basal concentrations of citrate, glucose 6-phosphate and glycogen were found in muscles of older obese rats and may reflect intracellular inhibition of glucose metabolism as a result of increased lipid utilization. 5. Thus several abnormalities are responsible for insulin resistance of muscles from obese Zucker rats among which we have observed decreased insulin binding, decreased glucose transport and increased utilization of endogenous fatty acid which could inhibit glucose utilization.

Hyperinsulinaemia and insulin resistance are common features in most types of human and animal obesities (Assimacopoulos-Jeannet & Jeanrenaud, 1976). In obese hyperinsulinaemic animals, the decreased ability of liver (Soll & Kahn, 1975; Le Marchand-Brustel *et al.*, 1978), adipose tissue (Olefsky, 1976; Kobayashi & Olefsky, 1978) and muscle (Forgue & Freychet, 1975; Olefsky *et al.*, 1976; Le Marchand-Brustel *et al.*, 1978) to bind insulin was proposed as the major factor in bringing about insulin resistance. Subsequent results have cast doubts on the paramount importance of this defect and suggest the existence of additional defects distal to the insulin–receptor interaction (Assimacopoulos-Jeannet & Jeanrenaud, 1976). For example, in adipose tissue of obese animals, the observed decreased fatty acid synthesis and pentose phosphate shunt activity found associated with the state of insulin resistance have been attributed to unknown intracellular alterations (Olefsky, 1977; Richardson & Czech, 1978). In muscle, which, *in vivo*, accounts for a much greater proportion of glucose utilization than does adipose tissue (Cahill, 1971), defects other than the decreased number of insulin-receptor sites have also been suggested (Becker *et al.*, 1978; Czech *et al.*, 1978; Le Marchand-Brustel *et al.*, 1978; Kemmer *et al.*, 1979). However, the only substantiated one has been the presence of a decreased glucose transport in soleus muscles of genetically obese mice (Cuendet *et al.*, 1976). No further investigations on possible intracellular abnormalities have been undertaken.

0306-3283/80/020525-10 $1.50/1

The aims of the present study were therefore to study changes in insulin binding, glucose transport and intracellular metabolism in soleus muscle of genetically obese hyperinsulinaemic Zucker (*fa/fa*) rats, and, in particular, to determine whether a modification of lipid metabolism might contribute to the impaired glucose metabolism. An attempt has been made to evaluate qualitatively the relative contribution of the observed defects in the establishment and evolution of muscle insulin resistance. As intact soleus muscles of adult rats present critical problems of substrate diffusion (Chaudry & Gould, 1969), a new soleus preparation has been developed to overcome this difficulty and permit comparison between muscles of young and older animals.

Preliminary reports of this work have been presented (Crettaz *et al.*, 1978; Crettaz & Jeanrenaud, 1978).

Materials and Methods

Animals

Normal female albino rats derived from a Wistar strain and bred in these laboratories were used. They weighed between 65 and 75g when intact soleus muscle was used, and between 150 and 250g when strips (see below) of these muscles were studied. Genetically obese (*fa/fa*) female rats and their lean littermate controls (FA/?) were purchased from the Centre de Sélection et d'Elevage d'Animaux de Laboratoire (CNRS, Orléans, France). All animals had free access to a standard laboratory chow and were maintained in a constant-temperature (23°C) animal quarter with a fixed (12h) artificial light cycle.

Incubations of intact, or strips of, soleus muscles

Rats were killed by cervical dislocation. When intact soleus muscles were incubated, a technique previously described for the mouse muscle was used without major modifications (Cuendet *et al.*, 1976). In other experiments, in order to circumvent the problem of diffusion of substrates, small strips of soleus muscles were incubated. Hindlimbs were fixed and dissected out to expose the soleus muscles. By using a curved needle, a thread was placed around the outer third of the proximal tendon and ligated. The tendon was cut above the knot and, by pulling the thread gently towards the distal tendon, a strip of muscle (25–35mg) was isolated and separated from the overall muscle mass. A second thread was placed around the outer third of the distal tendon that was ligated and cut beyond the knot. Another strip of soleus muscle was prepared from the opposite side of the same muscle, then from the other leg, so that four strips of soleus muscles were obtained from each animal within 7–8min. Strips of the soleus muscle were weighed on a torsion balance. With practice, strips of soleus muscles weighing between 25 and 35mg could regularly be obtained, larger muscles being discarded. Once prepared, strips of soleus muscles were removed from the iso-osmotic 0.9% NaCl solution where they had been stored at room temperature and were blotted and lightly stretched on a stainless-steel holder as described by Cuendet *et al.* (1976). Incubations were carried out in a shaking incubator at 37°C, in 25ml Erlenmeyer flasks containing 4ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932) with 1.5% defatted bovine serum albumin (Chen, 1967). Each flask was gassed with O_2/CO_2 (19:1) for 5min, and then sealed with rubber stoppers. Actual incubations were preceded by one or two 15min preincubations, at the end of which flasks and medium were changed and the preparations gassed as mentioned above. The addition of substrate and insulin is specified in the various Tables or Figures. When the effects of insulin were tested, the hormone was added during both the final preincubation and incubation periods.

Biochemical measurements

Results have been expressed per mg of muscle wet wt. The rate of incorporation of glucose into glycogen was measured by ^3H incorporation from D-[5-^3H]glucose into glycogen, and the rate of glycolysis determined as the production of ^3H_2O from D-[5-^3H]glucose (Cuendet *et al.*, 1976). Glucose transport and phosphorylation were measured with 2-deoxy-D-[1-^{14}C]glucose (0.1–0.2μCi/ml) (Kipnis & Cori, 1960) in the presence of [6,6′ (n)-^3H]sucrose (1–2μCi/ml) as extracellular marker and 1mM-pyruvate as energy source. Oxidation of [U-^{14}C]glucose (1μCi/ml) was measured by collecting $^{14}CO_2$ and counting it for radioactivity as previously described by Cuendet *et al.* (1976). The yield with this method (about 85%) was determined by the recovery of $NaH^{14}CO_3$ (25nCi/ml) added to 4ml of incubation medium.

At the end of the incubations, muscles were frozen, kept in liquid N_2, and homogenized (glass homogenizer) in 6% (w/v) $HClO_4$ and centrifuged (2000*g*; 10min) on the same day of the experiment. Supernatants were neutralized with K_2CO_3 and stored at −20°C for subsequent measurements. Creatine phosphate and ATP were determined fluorimetrically in the same samples by using hexokinase and glucose 6-phosphate dehydrogenase (Lamprecht *et al.*, 1974). ADP and AMP (Jaworek *et al.*, 1974), citrate (Passonneau & Brown, 1974) and glucose 6-phosphate (Lang & Gerhard, 1974) were determined fluorimetrically. Intracellular potassium content was measured as follows. Muscles were incubated in a medium containing [U-^{14}C]sucrose (1μCi/ml) as extracellular marker. At the end of the incubation, muscles were rapidly blotted and homo-

genized (1 ml of double-distilled water). After centrifugation (2500*g* for 15 min), supernatants (0.1 ml) were used for the measurement of potassium by flame photometry, or counted for radioactivity in a liquid-scintillation spectrometer (model no. 3880; Packard Instruments, Downers Grove, IL, U.S.A.). Intracellular potassium was determined by subtracting the extracellular potassium content from total potassium content in the homogenate. Lactate dehydrogenase was measured in the incubation medium and muscle homogenized in 100mM-K_2HPO_4/NaH_2PO_4 buffer (pH 7.5) containing 5mM-EDTA (Bergmeyer & Bernt, 1974*a*).

Muscle proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Muscle glycogen content was assayed as described by Chan & Exton (1976). Muscle triacylglycerol content (10–20mg of tissue) was measured as described by Le Marchand *et al.* (1973).

O_2 consumption of isolated muscles

Measurements of O_2 consumption were made in an 8ml air-tight chamber thermostatically controlled at 37°C. Two strips of soleus muscle were stretched on a glass holder, placed in the chamber and preincubated for 20–30min with recirculating medium (50ml, pumped at 30ml/min). The medium was continuously gassed with O_2/CO_2 (19:1). O_2 disappearance from the medium was recorded at time intervals. In order to carry out this measurement, the circulation of the medium was stopped, and the O_2 present in the medium of the isolated chamber was measured for 4–5min under continuous stirring (magnet stirrer) by using a specific oxygen electrode. The O_2 disappearance from the medium without muscle was recorded before (or after) each experiment and never exceeded 10% of muscle O_2 consumption. The oxygen electrode was previously standardized with gas mixtures containing different proportions of O_2 (30, 60 and 95%).

Binding of insulin to isolated muscles

Porcine monocomponent insulin was iodinated and purified as previously described by Maldonato *et al.* (1976). After a 30min preincubation without hormone at 24°C in a bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 2mM-pyruvate, 1.5% defatted bovine albumin and 1mg of bacitracin/ml, ^{125}I-labelled insulin (180–200mCi/mg) was added to a final concentration of 0.05–0.10nM with various concentrations of unlabelled insulin. Non-specific binding was measured in the presence of 8500nM-unlabelled insulin. At the end of the incubation, muscles were washed three times (15min per wash) with 10ml of 0.9% NaCl (4°C) containing 2.5mg of human serum albumin/ml. Muscles were then counted for their radioactive content in a gamma spectrometer. ^{125}I-labelled-insulin degradation was determined by trichloroacetic acid precipitation and binding to anti-insulin serum. Precipitation with trichloroacetic acid (0.5ml; 12%, w/v) was carried out with 0.1ml of incubated (without muscle) or non-incubated (control) medium. After centrifugation, pellets were washed with trichloroacetic acid (1ml; 6%, w/v) and then counted for radioactivity. Alternatively, samples (0.1ml) of medium containing ^{125}I-labelled insulin were incubated for 24h at 4°C with an excess of antiserum. Free ^{125}I-labelled insulin was then sedimented with dextran-coated charcoal and counted for radioactivity (Herbert *et al.*, 1965).

Plasma measurements

Before isolation of soleus muscles, blood was collected in heparinized tubes from cut carotid arteries. Blood samples were centrifuged (2000*g* for 15min) at 4°C and plasma was used for determination of glucose (Bergmeyer & Bernt, 1974*b*), immunoreactive insulin (Herbert *et al.*, 1965) (with rat insulin standards) and triacylglycerols (Le Marchand *et al.*, 1973).

Chemicals

Porcine monocomponent insulin, proinsulin and glucagon were generous gifts from Dr. J. Schlichtkrull, Novo Research Institute (Copenhagen, Denmark). Radioactive products were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Bacitracin was obtained from Medial SA (Geneva, Switzerland). Bovine serum albumin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Enzymes and cofactors were obtained from Boehringer (Mannheim, W. Germany). Other chemicals (analytical grade) were purchased from E. Merck (Darmstadt, Germany) or Fluka A.G. (Buchs, Switzerland).

Results

Integrity and insulin-sensitivity of strips of soleus muscles

Glucose uptake in whole soleus muscles from rats above 80–100g body wt. has been shown to be limited by diffusion (Chaudry & Gould, 1969). Since we wished to study changes in metabolism of the muscle as obesity developed in the *fa/fa* rats, it was necessary to develop a new preparation using small strips of soleus muscle which did not suffer from diffusion problems. As shown in Fig. 1, total glucose metabolism of large intact soleus muscles was low. In contrast, when strips of soleus muscles were used, glucose metabolism was inversely proportional to muscle weight and became constant within a range of 25–35mg wet wt. Consequently, only preparations ranging from 25 to 35mg were used. The ade-

quacy of the new preparation was further explored by comparing strips with intact soleus muscles of the same weight. First, as shown in Table 1, the concentrations of creatine phosphate, ATP and potassium in incubated strips of soleus muscles were similar to those of either non-incubated or incubated intact soleus muscle. Moreover, in both intact and strips of soleus muscles of the same weight, the release of lactate dehydrogenase into the incubation medium during an incubation period of 60min was negligible (Table 1). Total glucose metabolism (i.e. glucose incorporation into glycogen plus glucose metabolism by glycolysis) by strips of soleus muscles was linear for 120min, and insulin-responsive (results not shown). Actually, as described below, glucose metabolism in soleus strips was sensitive to insulin concentrations as low as 0.1m-i.u./ml (see Table 4). [Lactate]/[pyruvate] ratio measured in the presence of 5mM-glucose and at the end of a 60min incubation was 9.9 for intact and 10.3 for strips of soleus muscles.

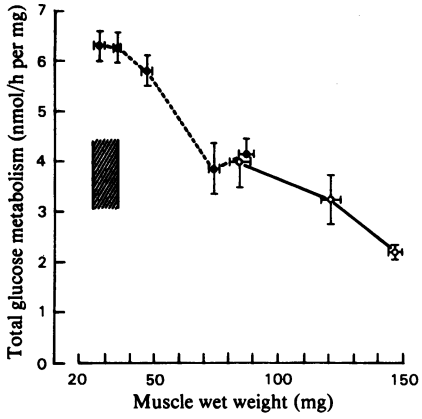

Fig. 1. *Effect of muscle weight on total glucose metabolism by soleus muscle of normal rats*

Intact soleus muscle (○) or strips (●) were incubated at 37°C for 60min in 4ml of bicarbonate-buffered medium containing 5mM-[5-^{3}H]glucose and 1.5% defatted albumin. Total glucose metabolism was calculated from the sum of incorporation of ^{3}H from [5-^{3}H]glucose into glycogen and ^3H_2O. Hatched area indicates the range of muscle weights in which all subsequent studies were carried out. Mean values ± S.E.M. for four to six muscles are shown, expressed per mg wet wt.

Basal characteristics of lean and fa/fa obese rats

During this study, two groups of animals (5–6- and 10–11-week-old) were investigated. As shown in Table 2, 5–6-week-old *fa/fa* rats were hyperinsulinaemic but not hypertriglyceridaemic, whereas at 10–11 weeks of age, they were both hyperinsulinaemic and hypertriglyceridaemic. Despite high insulinaemia, the blood sugar of the obese rats was similar to that of the controls at the two stages of obesity, and not lower, indicating a state of insulin resistance (Table 2).

Basal characteristics of soleus muscles from obese rats

As described in Table 3, wet weight and protein content were similar in muscles from lean and obese rats of both age groups. At 5–6 weeks of age, triacylglycerol content was 2 times higher, whereas at 10–11 weeks of age, it was 3 times higher in muscles of obese rats than in controls.

Insulin-binding studies

In order to keep insulin degradation to a minimum, insulin-binding experiments were performed at low temperature (24°C). In the presence of 0.05–

Table 1. *Creatine phosphate, ATP and potassium contents, and lactate dehydrogenase release in soleus muscles from normal rats*

For non-incubated soleus muscles, rats were anaesthetized with ether and soleus muscles were quickly removed (within 10s) and frozen in liquid N_2. When indicated, muscles were incubated at 37°C in 4ml of bicarbonate-buffered medium containing 5mM-glucose and 1.5% defatted albumin. Lactate dehydrogenase release was measured during pre-incubation and incubation periods. Strips of soleus muscle were prepared as described in the Materials and Methods section. Results are expressed per mg wet wt. and are means ± S.E.M. for six to eight muscles.

Muscle preparation	Time of incubation (min)	Creatine phosphate (nmol/mg)	ATP (nmol/mg)	K content (nequiv./mg)	Lactate dehydrogenase release into medium (% of muscle activity)
Non-incubated soleus	—	13.2 ± 0.6	5.8 ± 0.1	85 ± 2	—
Intact soleus (22–26mg)	60	13.1 ± 1.0	5.0 ± 0.1	75 ± 3	2.6 ± 0.4
Strips of soleus (25–35mg)	15	12.2 ± 1.3	5.0 ± 0.2	—	—
	60	14.1 ± 0.1	5.4 ± 0.1	73 ± 1	4.3 ± 0.2
	120	13.1 ± 1.5	4.9 ± 0.2	69 ± 4	—

Table 2. *Body weight and plasma concentrations of glucose, triacylglycerol and immunoreactive insulin in control and obese fa/fa rats*

Blood was collected from cut carotid arteries, immediately centrifuged and plasma was collected for further determinations. Results are means ± S.E.M. for the numbers of animals indicated in parentheses. Values significantly different from control rats are indicated by *(at $P < 0.02$) and †(at $P < 0.001$).

Animals	Age (weeks)	Body wt. (g)	Plasma glucose (mM)	Plasma triacylglycerol (mM)	Plasma immunoreactive insulin (ng/ml)
Control	5–6	103 ± 1 (29)	5.6 ± 0.2 (7)	0.50 ± 0.07 (5)	1.7 ± 0.2 (4)
Obese (*fa/fa*)		147 ± 3 (28)†	6.2 ± 0.3 (8)	0.74 ± 0.10 (7)	6.4 ± 1.3 (4)*
Control	10–11	175 ± 2 (38)	6.0 ± 0.2 (8)	0.50 ± 0.05 (8)	2.1 ± 0.3 (4)
Obese (*fa/fa*)		308 ± 6 (42)†	6.5 ± 0.2 (8)	1.72 ± 0.27 (7)†	9.4 ± 1.1 (4)†

Table 3. *Wet weight and contents of protein and triacylglycerol in soleus muscles from control and obese (fa/fa) rats*

Soleus muscles were isolated as described in the Materials and Methods section. The results are for non-incubated muscles, and are means ± S.E.M. for the numbers of animals indicated in parentheses. Values significantly different from control muscles are indicated by * (at $P < 0.02$), † (at $P < 0.005$) and ‡ (at $P < 0.001$).

Animals	Age (weeks)	Wet wt. (mg)	Protein content (% of wet wt.)	Triacylglycerol content (nmol/mg wet wt.)
Control	5–6	37.9 ± 0.9 (24)	16.4 ± 0.7 (6)	5.4 ± 0.6 (10)
Obese (*fa/fa*)		36.5 ± 1.1 (24)	16.3 ± 0.4 (6)	11.1 ± 1.2 (16)‡
Control	10–11	70.6 ± 1.7 (25)	17.6 ± 0.3 (8)	7.1 ± 0.6 (12)
Obese (*fa/fa*)		76.3 ± 1.3 (25)*	16.1 ± 0.3 (8)†	21.2 ± 2.0 (14)‡

0.1 nM-^{125}I-labelled insulin, binding of the hormone to strips of normal soleus muscles was time-dependent, a plateau of binding being reached after 4–5 h of incubation. Non-specific binding, measured in the presence of 8500 nM-unlabelled insulin, was 18–24% of total binding. Insulin degradation in the medium, estimated by trichloroacetic acid precipitation and by loss of immunoreactivity with anti-insulin serum, was negligible (1.4 and 3.9% respectively). Unlabelled insulin was more potent than proinsulin in inhibiting ^{125}I-labelled-insulin binding (i.e. a 50% decrease in ^{125}I-labelled-insulin binding being observed after addition of 9 nM-insulin and 110 nM-proinsulin). Glucagon (67 μM) did not prevent the binding of labelled insulin to soleus muscles. As shown in Fig. 2, muscles of 10–11-week-old obese animals bound less insulin than control muscles at each insulin concentration tested. Insulin binding in the presence of 0.06 nM-^{125}I-labelled insulin only (not shown in Fig. 2) was 0.068 ± 0.004 and 0.043 ± 0.002 fmol/mg (means ± S.E.M. for four muscles) to muscles of control and *fa/fa* rats respectively. This decreased binding capacity never exceeded 25–35%. It is worthwhile noting that total specific insulin binding to normal rat soleus muscles was similar to that of normal mice soleus muscles, i.e. about 5 fmol/mg wet wt. (Le Marchand-Brustel *et al.*, 1978).

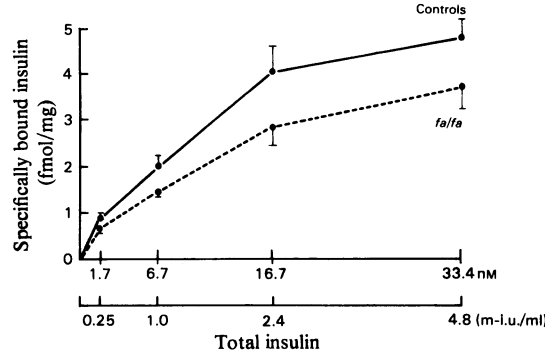

Fig. 2. *Specific insulin binding to strips of soleus muscle from 10–11-week-old lean control and obese (fa/fa) rats*

Muscles were incubated at 24°C for 5 h in 4 ml of bicarbonate-buffered medium containing 0.05 nM-^{125}I-labelled insulin plus various concentrations of unlabelled insulin, 2 mM-unlabelled pyruvate, 1 mg of bacitracin/ml and 1.5% defatted albumin. At the end of incubation, muscles were washed as indicated in the Materials and Methods section. Non-specific binding (18–24% of total binding) has been subtracted from each point. Points are mean values ± S.E.M. for four muscles.

Uptake of 2-deoxyglucose by muscles from obese rats

Basal and insulin-stimulated 2-deoxyglucose uptake by muscles from either normal or 10–11-week-old obese rats was linear for 60 min; uptake by muscle from control rats was similar in the presence of 1 and 10 m-i.u. of insulin/ml (results not shown). As shown in Table 4, basal 2-deoxyglucose uptake was similar in muscles of 5–6-week-old control and obese rats, but insulin-sensitivity and maximal res-

ponse were both impaired in muscles of obese rats. These defects were also observed in muscles of 10–11-week-old obese animals, although they were now accompanied by a marked decrease in the basal uptake of the sugar.

Glucose incorporation into glycogen, and glucose metabolism by glycolysis in muscles from obese rats

As shown in Table 4, newly synthesized glycogen in normal muscles was stimulated by insulin at concentrations as low as 0.1 m-i.u./ml, maximal stimulation being reached at 0.25 m-i.u./ml. In marked contrast and in both age groups, glucose incorporation into glycogen in muscles from obese rats was both less sensitive (a shift to the right of the insulin dose–response curve) and less responsive (a marked decrease in maximal stimulation) to insulin than that in controls. In control rats of both ages, the insulin dose–response curve for glucose utilization in glycolysis had the same characteristics as that for glycogen synthesis (Table 4). In muscles from 5–6-week-old obese rats, only a shift to the right of the insulin dose–response curve was noted, as 0.1 m-i.u. of insulin/ml was ineffective in stimulating glycolysis, but the stimulation obtained at the highest concentration of the hormone used (1 m-i.u./ml) was similar to that of controls. At 10–11 weeks of age, muscles from obese animals were still less insulin-sensitive than in controls. In addition, however, the maximal response to insulin never reached that observed in controls (Table 4), even when a concentration of insulin as high as 10 m-i.u./ml was used [the latter data, not shown in Table 4, were as follows: insulin-stimulated glycolysis was 8.2 ± 0.5 nmol/h per mg (control) and 5.4 ± 0.4 nmol/h per mg (*fa/fa*); insulin-stimulated incorporation of glucose into glycogen was 2.20 ± 0.30 nmol/h per mg (control) and 1.42 ± 0.30 nmol/h per mg (*fa/fa*)].

Intracellular abnormalities of muscles of obese rats

In order to decide whether the 'glucose–fatty acid cycle' (Randle *et al.*, 1963, 1966) by which the oxidation of fatty acids leads to inhibition of glucose utilization exists in muscles of obese rats and could play a role in the previously observed insulin resistance, the intracellular concentrations of citrate, glucose 6-phosphate and glycogen were measured. It was of initial interest to observe (Table 5) that, by mimicking increased fatty acid utilization by incubation of strips of normal soleus muscle with acetate, glucose metabolism via glycolysis was inhibited at a time when intracellular concentrations of both citrate and glucose 6-phosphate were increased. At 5–6 weeks of age, basal concentrations of cit-

Table 4. *Insulin effect on 2-deoxyglucose uptake, glucose incorporation into glycogen, glucose metabolism via glycolysis and total glucose metabolism in strips of soleus muscle from control and obese (fa/fa) rats*

Uptake of 2-deoxyglucose by muscle was measured at 37°C for 30 min in 4 ml of bicarbonate-buffered medium containing 1 mM-2-deoxy-D-[1-^{14}C]glucose, [6,6′(n)-^{3}H]sucrose, 1 mM-unlabelled pyruvate and 1.5% defatted albumin. Glycogen synthesis was measured as the incorporation of 5 mM-[5-^{3}H]glucose into glycogen. Glucose metabolism via glycolysis was measured as the production of $^{3}H_2O$ from 5 mM-[5-^{3}H]glucose. Total glucose metabolism is the sum of glycogen synthesis plus glycolysis. Experimental conditions are given in the legend to Fig. 1. Results are means ± S.E.M. for the numbers of experiments indicated in parentheses. Values significantly different from their respective controls are indicated by *(at $P < 0.05$), †(at $P < 0.02$) and ‡(at $P < 0.005$).

Animals	Age (weeks)	Addition of insulin (m-i.u./ml)	2-Deoxyglucose uptake (nmol/30 min per mg)	Glucose into glycogen (nmol/h per mg)	Glucose via glycolysis (nmol/h per mg)	Total glucose metabolism (nmol/h per mg)
Controls	5–6	0	0.69 ± 0.12 (8)	0.42 ± 0.05 (4)	3.0 ± 0.2 (8)	3.4 ± 0.2 (4)
		0.1	1.53 ± 0.17 (7)	1.89 ± 0.09 (4)	5.0 ± 0.2 (4)	6.9 ± 0.2 (4)
		0.25	1.97 ± 0.06 (7)	2.30 ± 0.37 (4)	6.3 ± 0.3 (4)	8.9 ± 0.5 (4)
		1	2.04 ± 0.15 (8)	2.75 ± 0.16 (4)	6.4 ± 0.4 (8)	9.2 ± 0.3 (4)
Obese (*fa/fa*)	5–6	0	0.61 ± 0.04 (8)	0.34 ± 0.07 (4)	3.3 ± 0.4 (8)	3.7 ± 0.5 (4)
		0.1	1.05 ± 0.10 (7)*	0.50 ± 0.06 (4)‡	3.8 ± 0.4 (4)*	4.3 ± 0.5 (4)‡
		0.25	1.22 ± 0.10 (7)‡	1.03 ± 0.20 (4)*	4.9 ± 0.7 (4)	5.9 ± 0.9 (4)*
		1	1.39 ± 0.17 (8)†	1.60 ± 0.17 (4)‡	5.7 ± 0.2 (8)	7.3 ± 0.3 (4)‡
Controls	10–11	0	1.15 ± 0.08 (11)	0.61 ± 0.13 (10)	4.2 ± 0.4 (12)	4.8 ± 0.3 (10)
		0.1	1.56 ± 0.08 (6)	1.48 ± 0.11 (5)	5.8 ± 0.4 (7)	7.2 ± 0.3 (5)
		0.25	2.16 ± 0.18 (6)	2.15 ± 0.22 (5)	7.7 ± 0.6 (8)	9.8 ± 0.4 (5)
		1	2.37 ± 0.12 (11)	2.31 ± 0.16 (10)	8.0 ± 0.5 (12)	10.3 ± 0.6 (10)
Obese (*fa/fa*)	10–11	0	0.60 ± 0.05 (11)‡	0.49 ± 0.02 (10)	3.5 ± 0.2 (11)	4.0 ± 0.2 (10)*
		0.1	0.98 ± 0.10 (6)‡	0.55 ± 0.06 (5)‡	3.3 ± 0.3 (7)‡	3.9 ± 0.3 (5)‡
		0.25	1.25 ± 0.24 (6)†	0.79 ± 0.12 (5)‡	4.9 ± 0.5 (7)‡	5.6 ± 0.5 (5)‡
		1	1.53 ± 0.08 (11)‡	1.34 ± 0.18 (10)‡	5.2 ± 0.5 (12)‡	6.6 ± 0.3 (10)‡

rate, glucose 6-phosphate and glycogen were identical in muscles of both control and obese rats (Table 6). In marked contrast, at 10–11 weeks of age, basal concentrations of citrate, glucose 6-phosphate and glycogen were clearly higher in muscles from obese animals than in controls (Table 6). The concentration of intracellular citrate was unaffected by the addition of insulin, and remained higher in muscles of 10–11-week-old rats than in their respective controls. As expected, glucose 6-phosphate concentrations in control muscles rose in the presence of the hormone, but this hormone-induced rise was smaller in muscles of obese rats of both age groups (Table 6).

Glucose oxidation and O_2 consumption of muscles from obese rats

In order to know whether higher citrate concentrations measured in muscles of 10–11-week-old obese rats were related to increased lipid utilization, measurement of fatty acid oxidation was necessary. As higher concentrations of triacylglycerol were measured in soleus muscles of obese rats (Table 3), oxidation of ^{14}C-labelled fatty acids could not be used, because of the probability of this substrate being present at different specific radioactivities in obese and control rats. Since carbohydrates and lipids are the major substrates for muscle respiration (Neely & Morgan, 1974), measurement of glucose oxidation and O_2 consumption could provide an indirect estimate of endogenous lipid utilization. No depletion of unlabelled glycogen was measured during a 60min incubation (results not shown), and even net glycogen deposition was observed in muscles incubated with insulin (Table 6), suggesting that muscle glycogen contributed little to glucose oxidation in the presence of extracellular glucose. Moreover, a 90–120min preincubation period of muscles in the presence of [U-^{14}C]glucose was needed to obtain plateau values of $^{14}CO_2$ production (results not shown). This suggested that such a preincubation time was necessary to obtain constant specific radioactivities of all metabolic intermediates contributing to $^{14}CO_2$ formation. With this experimental design, it was observed that [U-^{14}C]glucose oxidation to $^{14}CO_2$ was comparable in muscles of 5–6-week-old obese rats with those of controls. In contrast, muscles from obese rats at 10–

Table 5. *Effect of acetate on glycolysis and concentrations of glucose 6-phosphate and citrate in strips of soleus muscles from normal rats*

Muscles were incubated as indicated in Table 1, with 1m-i.u. of insulin/ml. After 60min of incubation, muscles were frozen in liquid N_2 and then homogenized in 6% (w/v) $HClO_4$. Glucose 6-phosphate and citrate were measured in neutralized supernatants. Results are means ± S.E.M. for five muscles. Values significantly different are indicated by *(at $P < 0.02$), †(at $P < 0.005$) and ‡(at $P < 0.001$).

	Glucose via glycolysis (nmol/h per mg)	Glucose 6-phosphate (nmol/mg)	Citrate (nmol/mg)
No addition	10.5 ± 0.5	0.321 ± 0.009	0.133 ± 0.009
Acetate (1.2mM)	7.0 ± 0.5†	0.411 ± 0.026*	0.282 ± 0.015‡

Table 6. *Concentrations of citrate, glucose 6-phosphate and glycogen in strips of soleus muscles from control and obese (fa/fa) rats*

Muscles were incubated as indicated in Table 1. Glucose 6-phosphate, citrate and glycogen were measured after 60min of incubation, as described in Table 4 and the Materials and Methods section. Results are means ± S.E.M. for the numbers of experiments indicated in parentheses. Values significantly different from muscles of control rats indicated by *(at $P < 0.05$), †(at $P < 0.02$), ‡(at $P < 0.01$) and §(at $P < 0.001$).

Animals	Age (weeks)	Addition of insulin (m-i.u./ml)	Glucose 6-phosphate (nmol/mg)	Citrate (nmol/mg)	Glycogen (nmol of glucose/mg)
Control	5–6	0	0.132 ± 0.006 (4)	0.098 ± 0.008 (4)	20.2 ± 1.7 (8)
		1	0.350 ± 0.021 (4)	0.107 ± 0.005 (4)	28.5 ± 4.6 (8)
Obese (*fa/fa*)	5–6	0	0.129 ± 0.013 (4)	0.106 ± 0.011 (4)	24.2 ± 3.4 (8)
		1	0.267 ± 0.024 (4)*	0.107 ± 0.003 (4)	27.5 ± 3.0 (8)
Control	10–11	0	0.134 ± 0.007 (12)	0.118 ± 0.010 (8)	20.0 ± 1.8 (8)
		1	0.350 ± 0.017 (12)	0.130 ± 0.012 (8)	27.1 ± 2.4 (8)
Obese (*fa/fa*)	10–11	0	0.170 ± 0.010 (12)‡	0.173 ± 0.017 (8)†	34.4 ± 2.1 (8)§
		1	0.256 ± 0.011 (12)‡	0.178 ± 0.016 (8)*	36.5 ± 3.0 (7)*

Table 7. *Glucose oxidation and O_2 consumption in strips of soleus muscles from control and obese (fa/fa) rats*
After a 120min preincubation period in the presence of 5mM-[U-^{14}C]glucose, $^{14}CO_2$ formation from labelled glucose was measured during the following 60min, as described in the Materials and Methods section. The yield (85%) was determined in each experiment (see the Materials and Methods section) and used to calculate actual glucose-oxidation values. Results are means ± S.E.M. for the numbers of experiments indicated in parentheses. Values significantly different from muscle of control rats are indicated by *(at $P < 0.005$).

Animals	Age (weeks)	Addition of insulin (m-i.u./ml)	Glucose oxidation (nmol of glucose/h per mg)	O_2 consumption (nmol/h per mg)	Contribution of glucose to O_2 consumption (%)
Control	5–6	0	0.95 ± 0.08 (5)	44 ± 4 (7)	13
		1	1.84 ± 0.11 (8)	43 ± 3 (7)	25
Obese (*fa/fa*)	5–6	0	0.86 ± 0.10 (6)	42 ± 3 (7)	12
		1	1.52 ± 0.11 (6)	43 ± 3 (7)	22
Control	10–11	0	1.12 ± 0.06 (8)	39 ± 2 (6)	17
		1	1.78 ± 0.09 (7)	40 ± 3 (6)	27
Obese (*fa/fa*)	10–11	0	0.58 ± 0.03 (7)*	40 ± 2 (6)	9
		1	1.27 ± 0.07 (8)*	41 ± 2 (6)	18

11 weeks of age oxidized less glucose than controls, both in the absence and in the presence of insulin (Table 7). O_2 consumption by strips of soleus muscles was constant over 3h of incubation (results not shown). No difference in O_2 utilization was observed between muscles of control and obese rats incubated without or with insulin, and measured at both 37°C (Table 7) and 30°C (results not shown).

Discussion

New technique for soleus-muscle incubation

This study presents a new method for the investigation of soleus muscle metabolism in the rat. As previously reported by Chaudry & Gould (1969) and confirmed in these experiments, investigations of the relatively thick intact soleus muscle from rats are hampered by problems of diffusion of substrates. The proposed technique consists of using strips (25–35mg wet wt.) of soleus muscles that are separated, with the corresponding parts of their tendons, from the whole soleus mass. The advantages of this new technique are as follows: (1) muscles of initially different weights can be compared; (2) the problem of diffusion of substrates is diminished and standardized; (3) the muscle weight of this preparation is 3–4 times greater than that of intact mouse soleus muscle, thus making the measurement of intracellular metabolites easier; (4) the muscle preparation is biochemically well-preserved and responsive to physiological concentrations of insulin. As soleus muscle consists of intermediate fibres (Baldwin *et al.*, 1972), it may be more representative of overall skeletal-muscle mass than highly specialized muscles, such as heart or diaphragm.

Insulin binding

In agreement with other studies in adipose tissue (Kono & Barham, 1971; Gliemann *et al.*, 1975; Olefsky, 1976; Czech *et al.*, 1977; Kobayashi and Olefsky, 1978) and in muscle (Le Marchand-Brustel *et al.*, 1978), our observations suggest the existence of insulin spare receptors in soleus muscle, as 20–30% of total specific insulin-binding sites seem to be sufficient to elicit maximal stimulation of glucose metabolism. On the other hand, in muscles of 10–11-week-old obese rats (the age at which insulin resistance is most marked), only a 25–35% decrease in insulin binding was measured. This decrease was observed over a wide range of insulin concentrations, suggesting that the total binding capacity (i.e. probably the number of receptors) may be diminished. This small decrease in insulin binding, together with the existence of spare receptors, indicates that insulin-receptor abnormality is not the only determinant of insulin resistance in soleus muscles of obese rats and suggests the existence of defect(s) distal to the insulin–receptor interaction.

Uptake of 2-deoxyglucose and glucose metabolism

2-Deoxyglucose uptake by muscles of obese rats from the two age groups was characterized by a decrease in both insulin-sensitivity and insulin-responsiveness. These defects could be due to either abnormal coupling between the insulin–receptor complex and this particular pathway and/or abnormal glucose transport itself, although the differentiation of these two possibilities cannot be made at present. Such an abnormal insulin effect on 2-deoxyglucose uptake occurred early in the development of the obesity syndrome and can conceivably explain the observed impaired glucose metabolism. In marked contrast, at the later phase of the obesity syndrome, glucose metabolism by muscles of obese rats was less sensitive and less responsive to insulin than 2-deoxyglucose uptake, and, in particular, low insulin concentration (0.1m-i.u./ml) stimulated 2-deoxy-

glucose uptake, but was ineffective in stimulating glucose incorporation into glycogen or glycolysis. These observations strongly suggest the existence of additional defect(s) not directly related to glucose transport and/or phosphorylation.

Defects in intracellular metabolism

As previously reported (Bray, 1977; Maleviak *et al.*, 1977; Kemmer *et al.*, 1979) and confirmed by this study, circulating triacylglycerols, and muscle triacylglycerols in obese rats were statistically higher than those in control animals. Moreover circulating non-esterified fatty acids are increased in obese rats (Bray, 1977; Maleviak *et al.*, 1977). These observations lead us to hypothesize that lipid utilization by muscle of obese rats could be increased and thereby alter glucose metabolism by the mechanism of the glucose–fatty acid cycle described by Randle *et al.* (1963) in heart and diaphragm. The possible existence of such a mechanism was indicated during this study by the observation obtained with strips of normal soleus muscles incubated with acetate and insulin. As previously reported with acetoacetate (Cuendet *et al.*, 1975; Maizels *et al.*, 1977), concomitant increases in concentrations of citrate and glucose 6-phosphate and decreased glucose metabolism via glycolysis were observed. Assuming that glucose and endogenously derived fatty acids were the main substrates for respiration with muscle preparations (Neely & Morgan, 1974), the extent of lipid utilization was indirectly estimated from measurements of glucose oxidation and O_2 consumption. In muscles of young obese and control rats, overall glucose oxidation and O_2 consumption were similar, indicating very similar rates of lipid utilization. In contrast, in muscles of 10–11-week-old obese rats, O_2 consumption was unchanged compared with controls, but glucose oxidation was significantly decreased, indicating that the rate of lipid utilization was greater. Such an increase would be small, since soleus muscles were tested under resting conditions, in which lipid utilization accounted for about 80% of the O_2 consumption. In spite of decreased incorporation of glucose into glycogen measured in incubated muscles of older obese rats, their total glycogen content was greater than in the controls. This observation favours the view that there is increased lipid utilization by muscle *in vivo*, perhaps owing to increased plasma non-esterified fatty acid concentrations which may lead to the sparing of glycogen stores (Rennie *et al.*, 1976).

It should be noted that when glucose oxidation was unchanged (5–6 weeks), intracellular concentrations of citrate and glucose 6-phosphate measured in basal conditions were similar in muscles of control and obese rats. In contrast, in muscles of older (10–11-week-old) obese rats, when fatty acid utilization was calculated to be enhanced, basal concentrations of citrate and glucose 6-phosphate in these muscles were higher than those of controls, in keeping with the glucose–fatty acid-cycle hypothesis. However, insulin did not increase glucose 6-phosphate concentrations in muscles of obese rats to higher values than those of controls, as expected if citrate was causing inhibition of phosphofructokinase activity (Randle *et al.*, 1966). On the contrary, in the presence of maximally effective insulin concentrations, glucose 6-phosphate concentrations in muscles from obese rats of both ages were increased to a lesser degree than those of controls. This suggests that, in the presence of the hormone, a major defect(s) before the phosphofructokinase step was present at the early phase of the syndrome and, in spite of increased citrate concentrations, remained predominant at the later phase.

In the absence of insulin, glucose oxidation by muscles of older (10–11 weeks) obese rats was much more impaired than glucose metabolism to glycogen and via glycolysis. This is in accordance with previously reported impairment of lactate oxidation in perfused hindlimb of obese rats (Kemmer *et al.*, 1979) and could suggest an inhibition of pyruvate dehydrogenase as another site of intracellular inhibition of glucose metabolism.

Taken together, these data suggest that: (*a*) the small decrease in insulin binding measured in soleus muscle from genetically obese (*fa/fa*) rats is not the only determinant of insulin resistance; (*b*) defects beyond the insulin–receptor complexes also appear to be of importance in bringing about the resistance of glucose metabolism to insulin. Among these defects, the two most important ones should be noted: firstly, abnormal insulin-stimulated glucose transport occurring from the early phase of the syndrome onwards, although its precise nature remains to be established; secondly, a subsequent increase in fatty acid utilization inhibitory to glucose metabolism, according to the concept of the glucose–fatty acid cycle. Under the present experimental conditions, it is hard to decide to what extent this intracellular defect could contribute to muscle insulin resistance, as it could be underestimated when studying a resting-muscle preparation. It is probable that in working muscles, in which glucose oxidation gives a higher contribution to O_2 consumption, increased triacylglycerol breakdown would be responsible to a larger extent for insulin resistance in muscles of obese rats.

We thank Dr. R. M. Denton for fruitful discussions and criticisms. We are greatly indebted to Mrs. F. Monsch for her excellent technical assistance. We thank Mrs. C. McVeigh for typing this manuscript. This work was supported by grant no. 3.154.0.77 of the Swiss National Science Foundation, Berne, Switzerland and by a grant-in-aid from Nestlé S.A., Vevey, Switzerland, and carried

out within the 'Fondation pour Recherches Médicales', Geneva, Switzerland.

References

Assimacopoulos-Jeannet, F. & Jeanrenaud, B. (1976) *Clin. Endocrinol. Metab.* **5**, 337–365

Baldwin, K. M., Klinkerfuss, G. H., Terjung, R. L., Molé, P. A. & Holloszy, J. O. (1972) *Am. J. Physiol.* **222**, 373–378

Becker, S. G., Smollin, P. F., Richardson, D. K. & Czech, M. P. (1978) *Horm. Metab. Res.* **10**, 204–208

Bergmeyer, H. U. & Bernt, E. (1974*a*) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd English edn., pp. 574–579, Academic Press, New York and London

Bergmeyer, H. U. & Bernt, E. (1974*b*) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd English edn., pp. 1205–1215, Academic Press, New York and London

Bray, G. A. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 148–153

Cahill, G. F., Jr. (1971) in *Muscle Metabolism during Exercise* (Pernow, B. & Saltin, B., eds.), pp. 103–110, Plenum Press, New York and London

Chan, T. M. & Exton, J. H. (1976) *Anal. Biochem.* **71**, 96–105

Chaudry, I. H. & Gould, M. K. (1969) *Biochim. Biophys. Acta* **177**, 527–536

Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181

Crettaz, M. & Jeanrenaud, B. (1978) *Diabetologia* **15**, 225

Crettaz, M., Freychet, P. & Jeanrenaud, B. (1978) *Diabetes* **27**, 453

Cuendet, G. S., Loten, E. G. & Renold, A. E. (1975) *Diabetologia* **11**, 336

Cuendet, G. S., Loten, E. G., Jeanrenaud, B. & Renold, A. E. (1976) *J. Clin. Invest.* **58**, 1078–1088

Czech, M. P., Richardson, D. K. & Smith, C. J. (1977) *Metab. Clin. Exp.* **26**, 1057–1078

Czech, M. P., Richardson, D. K., Becker, S. G. & Walters, C. G. (1978) *Metab. Clin. Exp.* **27**, 1967–1981

Forgue, M.-E. & Freychet, P. (1975) *Diabetes* **24**, 715–723

Gliemann, J., Gammeltoft, S. & Vinten, J. (1975) *J. Biol. Chem.* **250**, 3368–3374

Herbert, V., Lau, K. S., Gottlieb, C. W. & Bleicher, S. J. (1965) *J. Clin. Endocrinol. Metab.* **25**, 1375–1384

Jaworek, D., Gruber, W. & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd English edn., pp. 2127–2131. Academic Press, New York and London

Kemmer, F. W., Berger, M., Herberg, L., Griess, F. A., Wirdeier, A. & Becker, K. (1979) *Biochem. J.* **178**, 733–741

Kipnis, D. M. & Cori, C. F. (1960) *J. Biol. Chem.* **235**, 3070–3075

Kobayashi, M. & Olefsky, J. M. (1978) *Am. J. Physiol.* **235**, E53–E62

Kono, T. & Barham, F. W. (1971) *J. Biol. Chem.* **246**, 6210–6216

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Lamprecht, W., Stein, P., Heinz, F. & Weisser, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd English edn., pp. 1777–1779, Academic Press, New York and London

Lang, G. & Gerhard, M. (1974) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd English edn., pp. 1238–1242, Academic Press, New York and London

Le Marchand, Y., Singh, A., Assimacopoulos-Jeannet, F., Orci, L., Rouiller, C. & Jeanrenaud, B. (1973) *J. Biol. Chem.* **248**, 6862–6870

Le Marchand-Brustel, Y., Jeanrenaud, B. & Freychet, P. (1978) *Am. J. Physiol.* **234**, E348–E358

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Maizels, E. Z., Ruderman, N. B., Goodman, M. N. & Lau, D. (1977) *Biochem. J.* **162**, 557–568

Maldonato, A., Trueheart, P. A., Renold, A. E. & Sharp, G. W. G. (1976) *Diabetologia* **12**, 471–481

Maleviak, M. I., Griglio, S., Mackay, S., Lemonnier, D. & Rosselin, G. (1977) *Diabete Metab.* **3**, 81–89

Neely, J. R. & Morgan, H. E. (1974) *Annu. Rev. Physiol.* **36**, 413–459

Olefsky, J. M. (1976) *J. Clin. Invest.* **57**, 842–851

Olefsky, J. M. (1977) *Endocrinology* **100**, 1169–1177

Olefsky, J., Bacon, V. C. & Baur, S. (1976) *Metab. Clin. Exp.* **25**, 179–192

Passonneau, J. V. & Brown, J. G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd English edn., pp. 1565–1569, Academic Press, New York and London

Randle, P., J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963) *Lancet* **i**, 785–790

Randle, P. J., Garland, P. P., Hales, C. N., Newsholme, E. A., Denton, R. M. & Pogson, C. I. (1966) *Recent Prog. Horm. Res.* **22**, 1–44

Rennie, M. J., Winder, W. W. & Holloszy, J. O. (1976) *Biochem. J.* **156**, 647–655

Richardson, D. K. & Czech, M. P. (1978) *Am. J. Physiol.* **234**, E182–E189

Soll, A. H. & Kahn, C. R. (1975) *J. Biol. Chem.* **250**, 4702–4707