The Effect of Noradrenaline on Glyceride Synthesis and Oxidative Metabolism *in vitro* in the Brown Fat of Newborn Rabbits

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1. The effect of noradrenaline on the synthesis of glyceride from [U-¹⁴C]glucose and on gas exchange in the brown fat of newborn rabbits *in vitro* was investigated. 2. The specific radioactivity of L-glycerol 3-phosphate was lower than that of lactate, presumably because glycerol derived from glyceride was rephosphorylated by glycerokinase. 3. In the basal state more than 25% of the total respiration was due to pyruvate oxidation. Noradrenaline stimulated glyceride synthesis and total respiration without changing the proportion of the total respiration due to pyruvate oxidation. 4. The extra ADP released by noradrenaline stimulation of glyceride synthesis could not have supported more than 2% of the observed increase in substrate oxidation if mitochondria from brown-fat-cells remain fully coupled in the stimulated state, but could have supported about one-third of the observed increase if they become uncoupled in the presence of noradrenaline.

Noradrenaline stimulates O₂ consumption and lipolysis and increases the incorporation of [¹⁴C]-glucose into triglyceride glycerol in the brown fat of newborn rabbits (Knight & Myant, 1970). Since ATP is required for triglyceride synthesis, acceleration of the breakdown and resynthesis of triglyceride (triglyceride cycle) would increase the supply of ADP needed for the respiration of coupled mitochondria. Fisher & Ball (1967) have shown that noradrenaline leads to an acceleration of the triglyceride cycle in white fat and that this would release enough ADP to maintain the whole of the observed increase in O₂ consumption if respiration in the mitochondria of white fat is coupled to phosphorylation. This raises the question of the quantitative importance of the triglyceride cycle in maintaining the high rate of O₂ consumption in the brown fat of newborn rabbits in the presence of noradrenaline. The calculations of Knight & Myant (1970) suggest that the stimulatory effect of noradrenaline on the triglyceride cycle could not account for more than a very small fraction of the increase in O₂ consumption if respiration in the brown fat of newborn rabbits is fully coupled. However, these calculations were based on rates of triglyceride synthesis estimated from the incorporation of [¹⁴C]glucose into triglyceride glycerol. This method of estimating triglyceride synthesis would give erroneously low values if carbon sources other than [¹⁴C]glucose contribute to the triglyceride glycerol of brown fat.

In this paper we describe experiments on brown

fat from newborn rabbits in which we have measured the specific radioactivity of some intermediates between [¹⁴C]glucose and glyceride glycerol, including that of L-glycerol 3-phosphate, the precursor of glyceride glycerol. Estimates of the rate of synthesis of triglyceride based on the specific radioactivity of the L-glycerol 3-phosphate pool and the incorporation of ¹⁴C into glyceride glycerol, in the presence and in the absence of noradrenaline, confirm our previous suggestion that stimulation of the triglyceride cycle can play little part in maintaining the high O₂ consumption of the brown fat of newborn rabbits in the presence of noradrenaline, unless the mitochondria are uncoupled.

METHODS

Two-day-old Dutch rabbits were taken from their mother on the morning of the experiment and kept at 37°C until required. The rabbits were killed by breaking their necks and their interscapular and cervical brown-fat-pads were removed immediately and put into Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1964), at about 10°C. Two sets of experiments were carried out, one in which triglyceride synthesis and O₂ consumption were measured concurrently, and another in which the specific radioactivity of hexose monophosphates in the incubation mixture, as well as that of glucose, lactate and L-glycerol 3-phosphate, was measured.

Combined measurement of triglyceride synthesis and gas exchange. (a) Procedure. Since the amount of brown fat that can be incubated in a manometer flask is too small

to provide enough L-glycerol 3-phosphate for measurement of specific radioactivity, O_2 consumption and triglyceride synthesis were measured separately in different samples from the same pool of tissue. In order to ensure that triglyceride synthesis and O_2 consumption were measured during similar periods, the flasks containing the tissue for measurement of triglyceride synthesis were gassed and equilibrated for the same length of time as the manometer flasks. The amount of [^{14}C]glucose incorporated into triglyceride glycerol during the period of gassing and equilibration was found by including an extra flask in which the reaction was stopped at the end of the period of equilibration.

(b) Incubations. For each experiment brown fat from three rabbits was chopped with a tissue chopper (Mollwain & Buddle, 1953) into pieces weighing 3–5 mg, pooled and divided into three random portions (about 2 g each). Different animals were used in each experiment. The pieces from each portion of tissue were transferred to a weighed 250 ml conical flask containing 35 ml of incubation medium consisting of Krebs–Ringer bicarbonate buffer, pH 7.4, containing half the recommended concentration of $CaCl_2$, together with glucose (10 mM), [$U-^{14}C$]glucose (0.25 $\mu Ci/ml$), insulin (0.1 unit/ml) and 5% bovine serum albumin (Cohn fraction V). The flasks were reweighed. About 150 mg of tissue was taken from each of two of the flasks, weighed and transferred separately to two Dixon–Keilin manometer flasks (Dixon & Keilin, 1933; modified by Flatt & Ball, 1963) containing 2.9 ml of the incubation medium. The three conical flasks and the two manometer flasks were gassed with $O_2 + CO_2$ (95:5) for 20 min at 37°C with shaking. The manometers were then closed and the flasks sealed with rubber caps. After equilibration for 10 min, 0.1 ml of incubation medium containing 6 μg of noradrenaline was tipped from the side arm into the main compartment of one manometer flask and 1.1 ml of the same solution was injected through the rubber seal of the conical flask from which the tissue in the manometer flask had been taken. Then 0.1 ml of incubation medium was added from the side arm to the main compartment of the second manometer flask and 1.1 ml of incubation mixture was injected into the corresponding conical flask. The third conical flask was removed from the water bath and the contents were separated by filtration followed by freezing, as described below. The remaining flasks were incubated at 37°C with shaking for a further 60 min. At the end of the incubation the reactions in the two manometer flasks were stopped by the addition of 0.75 ml of 3 M- H_2SO_4 from the side-arm stopper (Flatt & Ball, 1963) and the contents of the two conical flasks were filtered rapidly with suction through a warmed Buchner funnel. The filter paper in the Buchner funnel, together with the pieces of brown fat, was immediately frozen between the faces of an aluminium clamp cooled in liquid N_2 . Both tissue and filtrate were kept at $-15^\circ C$ until analysed. O_2 consumption and the output of CO_2 and $^{14}CO_2$ in the manometer flasks were measured by the method of Flatt & Ball (1963), with the modifications described by Knight & Myant (1970).

Measurement of the specific radioactivity of metabolites of glucose, including hexose monophosphate. (a) Procedure. Since the concentrations of glucose 6-phosphate and fructose 6-phosphate in brown fat are very low, these

hexose monophosphates were isolated and assayed together. The specific radioactivity of lactate released during an incubation with [^{14}C]glucose will rise until isotopic equilibrium is reached. The brown fat was therefore preincubated in a medium containing [^{14}C]glucose and was then transferred to fresh medium of similar composition for further incubation. The specific radioactivity of the lactate released during the second incubation could thus be compared with that of glucose 6-phosphate+fructose 6-phosphate and that of L-glycerol 3-phosphate estimated at the end of the incubation.

(b) Incubations. For the preincubation of the brown fat, about 10 g of whole tissue was transferred to a 250 ml conical flask containing 40 ml of the incubation medium described above. The flask was gassed for 10 min with $O_2 + CO_2$ (95:5) at 37°C with shaking, sealed with a rubber cap and incubated with shaking for a further 30 min. The tissue was then removed from the medium, blotted and divided into two roughly equal portions. Each portion was chopped into pieces weighing 3–5 mg with the tissue chopper. The pieces from each portion were transferred to weighed 250 ml conical flasks each containing 35 ml of fresh incubation medium and the flasks were reweighed. Noradrenaline was added to one flask to give a final concentration of 2 $\mu g/ml$. Both flasks were gassed with $O_2 + CO_2$ (95:5) for 10 min at 37°C with shaking, sealed and incubated for a further period. Tissue and medium were separated as described above and were kept frozen for further analysis.

Analysis of tissue and incubation mixture. In the experiments in which triglyceride synthesis and O_2 consumption were measured concurrently, the frozen pieces of tissue obtained by filtration were scraped from the filter paper under liquid N_2 and powdered in a percussion mortar cooled in liquid N_2 . Lipids were extracted from a weighed sample (100–200 mg) of the powdered tissue and were analysed for total lipid glycerol and for incorporation of ^{14}C into lipid glycerol by the method of Knight & Myant (1970). Another weighed sample (40–60 mg) of powdered tissue was dissolved in 11 ml of 1 M-KOH by heating at 90°C for 30 min. When cool, the precipitated soap was removed by filtration and the filtrate was assayed for protein. Ice-cold 5% (w/v) $HClO_4$ (1 ml/g of tissue) was added to the remainder of the powdered tissue in an homogenizing tube kept at $-15^\circ C$. The mixture was allowed to thaw slowly at 4°C and was then homogenized with a close-fitting glass plunger. The homogenate was centrifuged at 0°C, and the clear liquid between the sediment and the floating layer of fat was removed with a pipette. The sediment was homogenized with an equal volume of 5% (w/v) $HClO_4$ and the clear liquid obtained after centrifugation was added to the first extract. The pooled extracts were neutralized with ice-cold saturated $KHCO_3$ and the precipitate of $KClO_4$ was then removed by centrifugation at 0°C. The specific radioactivity of the L-glycerol 3-phosphate in the supernatant from this centrifugation was measured by the method of Denton & Randle (1967). The frozen incubation medium, obtained as the filtrate from the Buchner funnel, was thawed and deproteinized by the addition of enough $HClO_4$ to give a final concentration of 5% (w/v). The specific radioactivities of lactate and glycerol in the protein-free filtrate were determined by the method of Denton & Randle (1967). The specific radioactivity of the glucose was

measured in the eluate from the Dowex 1 column used for isolation of lactate from the medium.

In the experiment in which specific radioactivities of glucose, glucose 6-phosphate+fructose 6-phosphate, L-glycerol 3-phosphate and lactate were measured, the specific radioactivities of lactate and glucose in the incubation medium were determined by the procedure described above. The frozen tissue was powdered and extracted as described above, and the specific radioactivities of glucose 6-phosphate+fructose 6-phosphate and L-glycerol 3-phosphate were determined in the same supernatant from the final centrifugation by a modification of the methods of Denton & Randle (1967). After assay of the amounts of glucose 6-phosphate+fructose 6-phosphate and L-glycerol 3-phosphate present in the supernatant, known amounts of glucose 6-phosphate (about 5 μ mol) and L-glycerol 3-phosphate (about 10 μ mol) were added as carriers. The mixture was then submitted to the procedure of Denton & Randle (1967) for the removal of lactate and fructose 1,6-diphosphate, the conversion of glucose 6-phosphate+fructose 6-phosphate into fructose 1,6-diphosphate and the isolation and assay of the specific radioactivity of fructose 1,6-diphosphate (from which the specific radioactivity of glucose 6-phosphate+fructose 6-phosphate in the original extract from the frozen tissue could be calculated). The L-glycerol 3-phosphate recovered from the Dowex 1 column used for the isolation of fructose 1,6-diphosphate was hydrolysed to glycerol, and the glycerol was rephosphorylated after purification by column chromatography (Denton & Randle, 1967). The composition of the solution of rephosphorylated L-glycerol 3-phosphate was such that very little of the L-glycerol 3-phosphate was retained on a column of Dowex 1 (formate form). Interfering anions were therefore removed by passing the solution through a 40 cm $\times$ 3 cm column of Sephadex G-15. The fractions containing L-glycerol 3-phosphate were applied to a 20 cm $\times$ 1 cm column of Dowex 1 (formate form). The L-glycerol 3-phosphate was eluted with 1M-ammonium formate-formic acid buffer, pH 4.0, and its specific radioactivity assayed. The specific radioactivity in the original extract was calculated from the known dilution with carrier.

Assay methods. Glucose, lactate, L-glycerol 3-phosphate, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate were assayed by the enzymic methods described in Bergmeyer (1965). Glycerol was assayed by the method of Garland & Randle (1962). Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951). Radioactivity was assayed in a Packard Tri-Carb liquid-scintillation spectrometer. Up to 1 ml of an aqueous sample was added to 10 ml of the scintillation fluid described by Smith, Easter & Dils (1966). All counting rates were corrected for quenching by means of an internal standard of [14 C]hexadecane.

Calculations. The amounts of 14 C incorporated into glyceride glycerol in the tissue, and into glycerol in the medium, during the final hour of incubation were calculated by subtracting the 30 min from the 90 min values. The amount of L-glycerol 3-phosphate carbon incorporated into glyceride glycerol and the amount released into the medium as glycerol were calculated from the 14 C incorporation during the final hour into glyceride, and into medium glycerol, and the mean of the 30 and 90 min specific radioactivities of L-glycerol 3-

phosphate. The amount of pyruvate oxidized to CO_2 was calculated by dividing the amount of $^{14}\text{CO}_2$ produced during the final hour of incubation in the manometer flask by the specific radioactivity of lactate (assumed to be the same as that of pyruvate) at 90 min in the corresponding conical flask.

The ADP required for respiration was calculated on the assumption that 130 mol and 20 mol of ADP are required for the oxidation of 1 mol respectively of fatty acid and triose phosphate in fully coupled mitochondria, and that 6 mol and 3 mol of ADP are required for the production of ATP at substrate level during the oxidation of 1 mol respectively of fatty acid and triose phosphate in uncoupled mitochondria. The amount of triose phosphate oxidized was assumed to be the same as that of pyruvate oxidized. It was also assumed that the net requirement of ADP for the formation of lactate from glucose is 1 mol/mol of lactate formed and that the net production of ADP during the formation of triose phosphate from glucose is 1 mol/mol of triose phosphate formed. The ADP formed during the synthesis of glyceride was calculated on the assumption that all the glyceride formed was triglyceride and that the synthesis of each mol of triglyceride requires the net hydrolysis of 7 mol of ATP to ADP.

Materials. Nicotinamide nucleotides, ATP, L-noradrenaline bitartrate, bovine serum albumin and DL-glycerol 3-phosphate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and all the enzymes used were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Pig insulin (pro actrapid) free from glucagon was obtained from Novo Industries Medical Sources Department, London E.2, U.K. Dowex 1 ion-exchange resin (AG 1-X4; 200-400 mesh; Cl^- form) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and was converted into the formate form with 3M-sodium formate. Sephadex G-15 was obtained from Pharmacia, Uppsala, Sweden. [14 C]Glucose and [14 C]hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The [14 C]glucose was freed from [14 C]glycerol by the method of Denton & Randle (1967).

RESULTS

Effect of noradrenaline on glyceride synthesis and substrate oxidation. Six experiments were performed to test the effect of noradrenaline on glyceride synthesis and substrate oxidation in brown fat. For each experiment pieces of brown fat from the same pool of tissue were incubated with [14 C]glucose under conditions in which the incorporation of 14 C into glyceride glycerol and the output of CO_2 and $^{14}\text{CO}_2$ could be measured concurrently.

Table 1 shows the effect of noradrenaline on glyceride synthesis. Net synthesis of glyceride during the final hour of incubation was calculated from the amount of L-glycerol 3-phosphate incorporated into glyceride glycerol. The 14 C incorporated into phospholipid glycerol was included in this calculation, but the error due to this

Table 1. *Effect of noradrenaline on the incorporation of L-glycerol 3-phosphate into glyceride glycerol in the brown fat of newborn rabbits*

Six experiments were carried out. For each, pieces of brown fat from a pool of tissue derived from three rabbits were incubated in a medium containing [^{14}C]glucose in conical flasks for measurement of glyceride synthesis and in Dixon-Keilin flasks for measurement of gas exchange during the same period of incubation. Each pool was derived from different animals. Noradrenaline was added after 30 min preincubation to one conical flask and to one Dixon-Keilin flask in each set. In three of the six experiments specific radioactivities of L-glycerol 3-phosphate were determined in pooled extracts from the three flasks incubated under similar conditions. Newly formed glyceride glycerol released into the medium was measured in three experiments. Mean values are given with s.e.m. * $P < 0.001$; ** $P < 0.05$ (paired t test for difference between noradrenaline '+' and noradrenaline '-'). Calculations and other experimental details are given in the Methods section.

Incubation period (min)	Noradrenaline	Incorporation of ^{14}C into glyceride glycerol ($\mu\text{g-atom of C/h per 10 mg of protein}$)	L-Glycerol 3-phosphate/glucose specific-radioactivity ratio	Incorporation of L-glycerol 3-phosphate into glyceride glycerol ($\mu\text{g-atom of C/h per 10 mg of protein}$)	Newly formed glyceride glycerol in medium ($\mu\text{g-atom of C/h per 10 mg of protein}$)
0-30	-	0.22 ± 0.04	0.44 ± 0.05	-	-
30-90	-	0.73 ± 0.11	0.42 ± 0.03	1.76 ± 0.26	0.12 ± 0.09
30-90	+	$1.19 \pm 0.13^*$	$0.61 \pm 0.05^*$	$2.32 \pm 0.31^{**}$	0.37 ± 0.09

approximation must have been small, since incorporation of [^{14}C]glucose into the phospholipids of the brown fat of newborn rabbits is less than 10% of that into glyceride glycerol (Knight & Myant, 1970). In each experiment noradrenaline stimulated incorporation of L-glycerol 3-phosphate into glyceride glycerol.

The amount of ^{14}C incorporated into glyceride glycerol during an incubation of brown fat with [^{14}C]glucose is the net result of glyceride synthesis and loss of glyceride glycerol from the newly formed glyceride due to continuous lipolysis during the incubation. To determine the amount of newly formed glyceride that was hydrolysed during the incubation, the amount of [^{14}C]glycerol released into the medium was measured in three of the six experiments. Significant amounts of [^{14}C]glycerol were released from the tissue in all flasks, and the amount released was increased by noradrenaline (Table 1). In one flask to which noradrenaline was added, nearly one-third as much ^{14}C was released in the form of free glycerol as was retained in the glyceride glycerol of the tissue.

Table 2 shows the effect of noradrenaline on gas exchange, output of $^{14}\text{CO}_2$ and oxidation of substrates in the incubations of brown fat in the experiments shown in Table 1. Noradrenaline increased the uptake of O_2 and the output of CO_2 and $^{14}\text{CO}_2$, but had no consistent effect on the specific radioactivity of $^{14}\text{CO}_2$. The proportion of the total CO_2 that was derived from [^{14}C]glucose was therefore not significantly affected by noradrenaline. Since fatty acid synthesis in brown fat from newborn rabbits is very low (Knight & Myant, 1970), it is reasonable to assume that the glucose 6-phosphate oxidative pathway is negligible and hence that all

the glucose carbon oxidized to CO_2 is derived from pyruvate. If it is also assumed that the specific radioactivity of the pyruvate entering the tri-carboxylic acid cycle is the same as that of lactate in the medium, then the output of CO_2 from pyruvate can be estimated from the output of $^{14}\text{CO}_2$ and the specific radioactivity of lactate. Values for the rates of oxidation of glucose and pyruvate, calculated on the basis of these assumptions, are shown in Table 2. More than one-quarter of the total CO_2 output in the absence of noradrenaline was derived from pyruvate. The addition of noradrenaline caused a two- to three-fold increase in the output of CO_2 from pyruvate, but had no significant effect on the proportion of the total CO_2 derived from pyruvate; in the six pairs of flasks the proportion of the total CO_2 output derived from pyruvate was 28% in the absence of noradrenaline and 29% in its presence.

Specific radioactivities of metabolites of [U- ^{14}C]glucose. In the experiments shown in Tables 1 and 2 the specific radioactivity of lactate during the final hour of incubation was lower than that of the added [^{14}C]glucose, and the specific radioactivity of L-glycerol 3-phosphate was lower than that of lactate. Table 3 shows the results from experiments in which the specific radioactivity of glucose metabolites was investigated in more detail. Brown fat was preincubated with [U- ^{14}C]glucose and was then incubated for different periods, under conditions similar to those used in the experiments described above. There was little difference between the specific radioactivity of glucose 6-phosphate + fructose 6-phosphate and that of glucose after incubation for 35, 60 and 70 min, with or without added noradrenaline. In the absence of nor-

Table 2. Effect of noradrenaline on O₂ uptake, CO₂ output, ¹⁴CO₂ output and substrate oxidation in the brown fat of newborn rabbits

Experimental details are given in Table 1 and in the Methods section. Gas exchange, ¹⁴CO₂ output, lactate release and substrate oxidation refer to values obtained in the final hour of incubation. Glucose oxidized to CO₂ was calculated as ¹⁴CO₂ output/glucose specific radioactivity. In three of the six experiments specific radioactivities of lactate were determined in pooled extracts from the three flasks incubated under similar conditions. Mean values are given with s.e.m. * *P* < 0.01; ** *P* < 0.05 (paired *t* test for difference between noradrenaline '+' and noradrenaline '-').

Noradrenaline	Gas exchange ($\mu\text{mol/h}$ per 10 mg of protein)		$^{14}\text{CO}_2$ output (% of dose of [^{14}C]glucose/h per 10 mg of protein)	Lactate/glucose specific-radioactivity ratio	Lactate released ($\mu\text{mol/h}$ per 10 mg of protein)	Amount of substrate oxidized (μg -atom of C/h per 10 mg of protein)		
	O_2 uptake	CO_2 output				Glucose	Pyruvate	Total minus pyruvate
-	14.3 $\pm$ 2.3	13.4 $\pm$ 2.1	1.50 $\pm$ 0.21	0.69 $\pm$ 0.01	1.16 $\pm$ 0.09	2.7 $\pm$ 0.37	3.8 $\pm$ 0.53	9.6 $\pm$ 1.04
+	37.2 $\pm$ 2.6*	30.6 $\pm$ 2.9*	4.49 $\pm$ 1.02**	0.86 $\pm$ 0.02*	2.23 $\pm$ 0.36	7.8 $\pm$ 1.76**	8.9 $\pm$ 1.9**	21.7 $\pm$ 1.41*

adrenaline the lactate/glucose specific-radioactivity ratio was 0.68 and the L-glycerol 3-phosphate/glucose specific-radioactivity ratio was 0.32. In the presence of noradrenaline these ratios were 0.82 and 0.40 respectively. Noradrenaline had no significant effect on the L-glycerol 3-phosphate/lactate specific-radioactivity ratio. The incubation time had no effect on the specific radioactivity of lactate or L-glycerol 3-phosphate.

DISCUSSION

The results in Table 1 show that noradrenaline in the presence of insulin stimulates O₂ uptake, CO₂ output and glyceride synthesis in the brown fat of newborn rabbits. The results of the present work are therefore in essential agreement with our previous observations, in which glyceride synthesis was estimated from the incorporation of [¹⁴C]-glucose into glyceride glycerol without correction for any possible dilution of the specific radioactivity of L-glycerol 3-phosphate with carbon from endogenous sources (Knight & Myant, 1970).

Since the hydrolysis of brown-fat triglyceride is stimulated by exposure of the whole animal to the cold (Hull & Segall, 1966), and by noradrenaline *in vitro*, it is generally believed that fatty acid, released within the fat-cell, supplies the energy for heat production in brown fat. Our observations suggest that, in the brown fat of newborn rabbits, pyruvate oxidation accounts for at least one-quarter of the CO₂ produced in the unstimulated state and that the proportion of the total energy derived from pyruvate oxidation does not change significantly when respiration is increased by noradrenaline to two–three times the resting rate. Fatty acid oxidation may account for a larger proportion of the total respiration at higher rates of O₂ consumption, such as those observed by Fain, Reed & Saperstein (1967) in isolated brown-fat-cells in the presence of adrenaline. However, Prusiner, Cannon & Lindberg (1968a) have shown that iodoacetate inhibits the respiration of hamster brown fat in the presence of noradrenaline. They suggest that a high rate of fatty acid oxidation in brown fat cannot be maintained unless there is a continuous supply of pyruvate for the formation of the oxaloacetate required to combine with the acetyl-CoA arising from β-oxidation.

If it is assumed that fatty acid oxidation accounts for all the respiration of brown fat other than that due to pyruvate oxidation, then the values in the last column of Table 2 provide an estimate of the rate of fatty acid oxidation in the presence and in the absence of noradrenaline. Ball & Jungas (1961) have pointed out that the synthesis of 1 mol of triglyceride from free fatty acid results in the net hydrolysis of 7 mol of ATP to ADP. It is therefore

Table 3. *Specific radioactivities of metabolites of [U-¹⁴C]glucose formed during incubation of the brown fat of newborn rabbits*

Brown fat was preincubated for 40 min in medium containing [U-¹⁴C]glucose. The tissue was then chopped into small pieces and incubated for 35, 60 or 70 min with or without noradrenaline. The specific radioactivities of L-glycerol 3-phosphate and of hexose monophosphates (glucose 6-phosphate + fructose 6-phosphate) in the tissue, and of glucose and lactate in the medium, were assayed at the end of the incubation. Experimental details of the incubations and methods of assay are given in the Methods section. Since the incubation time did not influence the results significantly, mean values with s.e.m. are given for the three incubation times.

Noradrenaline	Specific-radioactivity ratios			
	Hexose monophosphates/glucose	Lactate/glucose	L-Glycerol 3-phosphate/glucose	L-Glycerol 3-phosphate/lactate
—	0.96 ± 0.06	0.68 ± 0.03	0.32 ± 0.04	0.48 ± 0.08
+	0.92 ± 0.04	0.82 ± 0.05	0.40 ± 0.05	0.49 ± 0.04

possible to calculate the increase in ADP production due to stimulation of glyceride synthesis by noradrenaline if it is assumed that all the glyceride formed during the incubation is triglyceride. It is also possible to calculate the increase in ADP formation that would be required to support the increase in substrate oxidation due to noradrenaline, when the mitochondria are fully coupled and when they are uncoupled (see the Methods section for the assumptions on which these calculations are based). The results of these calculations are shown in Table 4. If, in the conditions of incubation used in the present work, respiration in mitochondria from brown-fat-cells is fully coupled to phosphorylation, both in the resting and the stimulated state, then the extra amount of ADP produced by stimulation of the triglyceride cycle in the presence of noradrenaline could only support about 2% of the observed increase in substrate oxidation. If, on the other hand, the mitochondria are completely uncoupled, enough ADP would be generated by the increase in glyceride synthesis to support nearly one-third of the increase in oxidation. The measurement of glyceride synthesis from the incorporation of [¹⁴C]glycerol 3-phosphate would not reveal a cycle of partial lipolysis and resynthesis of triglyceride via diglyceride. It is difficult to envisage, however, that such a cycle could consume all the ATP produced by coupled mitochondria in brown fat.

In mitochondria isolated from the brown fat of guinea pigs (Hohorst & Stratmann, 1967), rats (Aldridge & Street, 1968) and newborn rabbits (Guillory & Racker, 1968), respiration in the resting state is coupled to phosphorylation provided that the mitochondria are prepared and tested under special conditions. Williamson, Prusiner, Olson & Fukami (1970) have shown that respiration in isolated brown-fat-cells is stimulated by uncouplers. This suggests that, in the resting state, the flow of electrons in the intact brown-fat-cell, as well as in the isolated mitochondrion, is controlled by

the supply of ADP rather than by the supply of substrate for oxidation. However, the question as to how far mitochondria from brown-fat-cells remain coupled when stimulated by noradrenaline cannot yet be answered. Oligomycin, an inhibitor of oxidative phosphorylation, partially inhibits the respiration of isolated brown-fat-cells when their respiration is maximally stimulated by noradrenaline (Prusiner, Eisenhardt, Rylander & Lindberg, 1968b), suggesting that the mitochondria remain at least partially coupled in the stimulated state. On the other hand, the transient stimulatory effect of uncoupling agents on the O₂ consumption of brown-fat-cells diminishes when their respiration is stimulated progressively by increasing doses of noradrenaline (Prusiner *et al.* 1968b). Moreover, Williamson *et al.* (1970) have shown that when noradrenaline is added to a suspension of brown-fat-cells there is an immediate change in their cytochrome *b* from a reduced to a more oxidized state. The most reasonable interpretation of this observation is that the basal respiration of brown-fat-cells is restrained by something other than substrate supply, presumably by the supply of ADP, and that noradrenaline releases respiration from this restraint by loosening the coupling of respiration to phosphorylation. Possibly mitochondria from brown-fat-cells remain coupled in the presence of noradrenaline but with looser coupling than in the resting state, either because of a decrease in the coupling at each of the three phosphorylation sites, or, as suggested by Christiansen, Pedersen & Grav (1969), because of complete loss of coupling at one or two sites. If this is so, the amount of ADP required to support oxidation of substrates in brown fat in the presence of noradrenaline must be between the two values shown in Table 4.

Carbon from endogenous sources could enter the L-glycerol 3-phosphate pool through a number of metabolic pathways. The glycogen present in

Table 4. *ADP produced by the triglyceride cycle compared with that required to support the oxidation of carbohydrate and fatty acid in the brown fat of newborn rabbits*

Values were calculated from the results of the three experiments in which release of newly formed glyceride glycerol into the medium was measured. Total L-glycerol 3-phosphate esterified was estimated as the sum of L-glycerol 3-phosphate incorporated into glyceride and newly formed glyceride glycerol released into the medium. For calculations see the Methods section. Mean values are given with s.e.m.

Noradrenaline	L-Glycerol 3-phosphate esterified ($\mu\text{mol/h}$ per 10 mg of protein)	ADP formed ($\mu\text{mol/h}$ per 10 mg of protein)	ADP required to support O_2 consumption ($\mu\text{mol/h}$ per 10 mg of protein)					
			Coupled mitochondria			Uncoupled mitochondria		
			Fatty acid	Pyruvate	Total	Fatty acid	Pyruvate	Total
-	0.42 $\pm$ 0.09	2.9 $\pm$ 0.65	72.8 $\pm$ 11.0	22.1 $\pm$ 2.3	94.9 $\pm$ 11.6	3.4 $\pm$ 0.5	3.3 $\pm$ 0.3	6.7 $\pm$ 0.5
+	0.79 $\pm$ 0.22	5.5 $\pm$ 1.52	180.9 $\pm$ 18.6	46.2 $\pm$ 11.1	227.1 $\pm$ 29.1	8.4 $\pm$ 0.9	6.9 $\pm$ 1.7	15.3 $\pm$ 2.0
Increase due to noradrenaline	0.37	2.6			132.2			8.6

the brown fat of newborn rabbits (Dawkins & Hull, 1964) could give rise to glucose 1-phosphate, which would then enter the pathway to pyruvate via glucose 6-phosphate. Oxaloacetate arising from gluconeogenesis could be converted into L-glycerol 3-phosphate via glyceraldehyde phosphate. The enzymes required for this pathway are likely to be present in the brown fat of newborn rabbits, since the brown fat of newborn rats contains phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (Hahn & Greenberg, 1968) and is capable of converting pyruvate into triglyceride glycerol (Hahn, Greenberg, Dobiášová & Drahotka, 1968). Finally, glycerol released by the hydrolysis of triglyceride could be phosphorylated, since glycerokinase activity is present in the brown fat of newborn rabbits (Knight & Myant, 1970). The results presented in Table 3 show that little or no endogenous carbon enters the glycolytic pathway before fructose 6-phosphate, either in the absence or in the presence of noradrenaline, since the specific radioactivity of glucose 6-phosphate + fructose 6-phosphate was almost as high as that of [^{14}C]glucose. Endogenous carbon must, however, enter the glycolytic pathway between fructose 6-phosphate and pyruvate, since the specific radioactivity of lactate was significantly lower than that of glucose. In this respect the brown fat of newborn rabbits differs from that of the white fat of adult rats, in which the specific radioactivity of lactate is the same as that of glucose after incubation in a medium containing [U- ^{14}C]glucose (Denton & Randle, 1967). The contribution of oxaloacetate to the pyruvate pool cannot be estimated because the specific radioactivity of oxaloacetate was not measured. However, it is possible to estimate the flow of carbon through pyruvate from sources other than glucose, if it is assumed that the contribution of the glucose 6-phosphate oxidative pathway is negligible in the brown fat of newborn rabbits. The results of such calculations are shown in Table 5. In the absence of noradrenaline about one-third of the carbon flow through pyruvate was derived from non-glucose sources. Noradrenaline induced a twofold increase in total flow through pyruvate with no change in the flow from non-glucose sources, suggesting that the increase was due entirely to increased glycolysis.

The low specific radioactivity of L-glycerol 3-phosphate compared with that of lactate presumably reflects the activity of glycerokinase. The presence of significant glycerokinase activity in brown fat means that [^{14}C]glycerol released from newly synthesized glycerides can be reconverted into glycerides via L-glycerol 3-phosphate. Hence the present calculations, which are based on the net accumulation of ^{14}C in glyceride glycerol and free glycerol, could underestimate the true rate of esterification. The specific radioactivity of L-glycerol

Table 5. *Flow of carbon through pyruvate from glucose and from endogenous sources other than glucose*

Values are taken from Tables 1 and 2. Flow through pyruvate from endogenous sources other than glucose is estimated as: (total flow through pyruvate) $\times$ (1 - lactate/glucose specific-radioactivity ratio).

Noradrenaline	Total flow through pyruvate ($\mu\text{g-atom of C/h}$ per 10 mg of protein)	Lactate/glucose specific-radioactivity ratio	Flow through pyruvate from non-glucose carbon ($\mu\text{g-atom of C/h}$ per 10 mg of protein)
—	6.8	0.67	2.24
+	13.6	0.82	2.45

3-phosphate was over ten times that of the glycerol released and did not change during the incubations. [^{14}C]Glycerol from the medium cannot therefore provide more than a negligible proportion of the total glycerol 3-phosphate esterified. However, since newly synthesized glycerides can be hydrolysed before they mix with the bulk of the intracellular triglyceride (Knight, 1971), the possibility that some glycerol of high specific radioactivity is phosphorylated before it can equilibrate with glycerol released into the medium cannot be excluded. Nevertheless at least 50% of the non-radioactive L-glycerol 3-phosphate that is esterified is derived from glycerol (Table 3, last column). This would require a minimum glycerokinase activity in brown fat, in the presence and in the absence of noradrenaline, of 0.16 and 0.12 $\mu\text{mol/h}$ per 10 mg of protein respectively. The maximum glycerokinase activity in brown fat from newborn rabbits is 0.6 $\mu\text{mol/h}$ per 10 mg of protein, and the true value is probably much lower than this (Knight & Myant, 1970). It would appear therefore that, even if some glycerol of high specific radioactivity is rephosphorylated, the amount that can be recycled is too small to invalidate the conclusions drawn above. In the brown fat of rats, on the other hand, glycerokinase activity is considerable (Treble & Ball, 1963; Knight & Myant, 1970), and would therefore be expected to lead to a much greater dilution of the radioactivity in the L-glycerol 3-phosphate pool and recycling of [^{14}C]glycerol by rephosphorylation of the glycerol resulting from lipolysis. This may explain why a stimulatory effect of noradrenaline on [^{14}C]glucose incorporation into triglyceride glycerol is not detectable in the brown fat of rats (Steiner & Cahill, 1966).

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