A Comparison between the Effects of Cold Exposure *in vivo* and of Noradrenaline *in vitro* on the Metabolism of the Brown Fat of New-born Rabbits

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1. Exposure of new-born rabbits to the cold leads to an increase in the incorporation of [^{14}C]glucose into the glycerol of brown-fat triglyceride, but has no effect on [^{14}C]glucose incorporation into triglyceride of white fat or liver. The effect of cold exposure on brown-fat triglyceride is abolished by cutting the cervical sympathetic nerve. 2. Brown fat incorporates very little [^{14}C]glucose into triglyceride fatty acids, either *in vivo* or *in vitro*. 3. Noradrenaline added to incubations of brown fat from new-born rabbits stimulates O_2 consumption, CO_2 output and incorporation of glucose into triglyceride glycerol. The effects of noradrenaline *in vitro* are therefore consistent with the hypothesis that noradrenaline mediates the response of the brown fat of new-born rabbits to cold exposure. 4. Glycerokinase is present in the brown fat of new-born rabbits, but its activity is much less than that of the glycerokinase in the brown fat of adult rats. 5. Insulin has no effect on O_2 consumption, CO_2 output or glucose uptake in brown fat of new-born rabbits. 6. It is concluded that the thermogenic response of new-born rabbits to cold exposure is accompanied by a selective acceleration of the triglyceride cycle in brown fat. However, resynthesis of triglyceride would not account for more than 1% of the O_2 consumed *in vitro* by new-born rabbit brown fat in the presence of noradrenaline if respiration remains coupled to phosphorylation.

When new-born rabbits are exposed to a cool environment they increase their heat production without shivering. This response to cold is due largely to stimulation of the oxygen consumption of interscapular and cervical brown fat (Heim & Hull, 1966). In adult cold-adapted rats the increase in the respiration of brown fat during cold exposure is accompanied by an increase in the rate of resynthesis of triglyceride (Steiner & Cahill, 1964; Himms-Hagen, 1965; Steiner, Schönbaum, Johnson & Sellers, 1968). In the present paper we show that a similar process occurs in the brown fat of new-born rabbits and that acceleration of glyceride synthesis depends upon the presence of an intact sympathetic nerve supply.

It is generally thought that the response of brown fat *in vivo* to cold exposure is mediated by local secretion of noradrenaline at sympathetic nerve endings near the surfaces of the fat-cells. In support of this view we show in the present work that the metabolic effects of noradrenaline *in vitro* on the metabolism of brown fat from new-born rabbits are similar to the effects of cold-exposure on brown fat in the intact animal. A brief account of part of this work has already been published (Knight, 1967).

METHODS

For the experiments on incorporation of [^{14}C]glucose *in vivo*, new-born rabbits of the Dutch strain were removed from their mother on the morning of the experiment and each animal was paired with another of a similar weight from the same litter. One rabbit from each pair was put in an incubator at 36°C and the other was kept at 22°C . After 4 h, each animal was given an intraperitoneal injection of [$\text{U-}^{14}\text{C}$]glucose ($5\mu\text{Ci}$ with $1.7\mu\text{mol}$ of glucose) in 0.5 ml of 0.9% NaCl. The rabbit was then replaced in its incubator until it was killed. The rabbits were killed by breaking their necks, and their interscapular and cervical brown fat pads, perirenal fat pads and livers were removed as quickly as possible and weighed in beakers containing chloroform-methanol (2:1, v/v). Blood was taken by heart puncture immediately before the animals were killed.

The cervical sympathetic nerves were exposed on both sides while the rabbit was anaesthetized with ether. The nerve on one side was cut at the level of the upper border of the sternum; that on the other side was left intact. Muscle and skin were sutured in separate layers. After the operation, the animals were left overnight at 36°C and returned to the mother for a single feed on the following morning. They were then put in an incubator at 22°C and given intraperitoneal [$\text{U-}^{14}\text{C}$]glucose as described

above. At various times after the injection the animals were killed by breaking their necks and the anterior lobe of the cervical brown fat (Hull & Segall, 1965) was removed from both sides of the neck.

For the preparation of tissue samples for incubation, tissues were removed from rabbits aged 2–5 days immediately after breaking their necks. The tissue was chopped with a tissue chopper (McIlwain & Buddle, 1953) into pieces weighing 3–5 mg. A portion of the chopped tissue was transferred to a lens paper supported on a wire sieve, weighed on a torsion balance and allowed to float off the lens paper into the incubation medium. A weighed portion of the remaining chopped tissue was transferred into 10 ml of chloroform–methanol (2:1, v/v) and set aside for subsequent analysis. For assay of glycerokinase, the tissue was homogenized in a glass homogenizer with 4 vol. of ice-cold sucrose (0.25 M) containing 5 mM-EDTA. The supernatant obtained after centrifugation of the homogenate at 11 000 g for 20 min at 4°C was used for the assay.

Incubations. The incubation medium was Krebs–Ringer bicarbonate buffer (pH 7.4) with half the recommended concentration of CaCl_2 , bovine serum albumin (5%) and glucose (10 mM). All incubations were carried out at 37°C in the manometer flasks described by Dixon & Keilin (1933), as modified by Flatt & Ball (1963). The main compartment of the flask contained 2.9 ml of incubation medium with 50–80 mg of tissue. In all experiments except those with [^{14}C]glycerol, 0.05 ml of [^{14}C]glucose (20 mM in 0.9% NaCl with $1 \mu\text{Ci}$) with either 0.05 ml of 0.9% NaCl or 0.05 ml of a solution of insulin (6.3 units/ml in 3 M-HCl in 0.9% NaCl) were added from the side-arm after gas equilibration.

O_2 consumption and CO_2 output were measured concurrently in the same incubation by the method of Flatt & Ball (1963). Hyamine hydroxide instead of KOH was used as CO_2 absorber in the stopcock attached to the centre well of the Dixon–Keilin manometer flask, since this was found to be more satisfactory for radioassay of $^{14}\text{CO}_2$. The flasks were gassed with $\text{O}_2 + \text{CO}_2$ (95:5) for 20 min at 37°C before measurement of gas exchange. The subsequent steps for measuring O_2 consumption and CO_2 output were those described by Flatt & Ball (1963), except that the flasks were left overnight at room temperature before the last manometric reading was taken, to ensure complete absorption of CO_2 by the hyamine hydroxide.

Separation of tissue from medium at the end of the incubation could not be achieved by filtration because the final acidification necessary for liberation of all the CO_2 led to precipitation of the albumin in the medium. After the final manometric reading as much of the tissue as possible, together with denatured albumin, was transferred with a spatula to 5 ml of water in a stoppered tube. Enough 5 M-NaOH was added to the remaining contents of the flask to redissolve the albumin, and the resulting solution was added to the contents of the stoppered tube. The pH was adjusted to 5 with 5 M-NaOH, when most of the albumin dissolved, and the floating pieces of albumin-free tissue were removed with a spatula. The amount of glyceride glycerol in the tissue recovered from the incubation medium was determined by assaying the glycerol in a sample of the lipid hydrolysate after removal of the fatty acids (see below). The amount of glyceride glycerol in the weighed portion of tissue set aside in chloroform–methanol (2:1, v/v) was determined by the same procedure.

Incorporation of [^{14}C]glucose into glyceride glycerol and glyceride fatty acid could then be expressed in terms of fresh weight of tissue.

Analysis of tissues obtained after cold or warm exposure of rabbits. The weighed tissues were homogenized in 10 vol. of chloroform–methanol (2:1, v/v) in a glass tube. The homogenate was filtered and the residue homogenized with a further 10 vol. of chloroform–methanol. The combined extracts were washed once with 0.2 vol. of 0.9% NaCl and three times with the 'upper phase' solvent of Folch, Lees & Sloane-Stanley (1957). There was no detectable radioactivity in the aqueous phase from the final wash. The washed extract was evaporated to dryness under reduced pressure and the residue was dissolved in chloroform. Radioactivity was assayed in a portion of the chloroform solution and the remainder was submitted to t.l.c. by the two-stage method of Skipski, Smolowe, Sullivan & Barclay (1965), with Kieselgel H slurried in 0.0004% Rhodamine in water. The bands of lipid were located under u.v. light, scraped from the plate and eluted with chloroform. The eluate from the band at the origin, containing the phospholipids, was assayed directly for radioactivity. The eluate containing the triglycerides was evaporated to dryness and the residue was saponified by dissolving it in 2 ml of ethanol containing 0.25 ml of 2.5% (w/v) KOH and heating for 1 h at 60°C. After the addition of 0.5 ml of 6% (v/v) acetic acid, the hydrolysate was evaporated to dryness. After the addition of 1.0 ml of 0.67 M- H_2SO_4 to the residue, the fatty acids were extracted with hexane and assayed for radioactivity. Samples were taken from the aqueous phase for assay of glycerol by the method of Carlson & Wadström (1959), and for radioassay of [^{14}C]glycerol.

Analysis of tissue and medium after incubation. The tissue pieces were washed twice with 10 ml of water and extracted twice with 10 ml of chloroform–methanol (2:1, v/v). They were then ground with a glass rod in a further 10 ml of chloroform–methanol. The suspension was filtered and the residue washed with 5 ml of chloroform–methanol. Water was added to the combined extracts to keep the water/chloroform–methanol ratio at 1:20. The lipid extract was washed and the lipids saponified and assayed for glycerol, [^{14}C]glycerol and ^{14}C -labelled fatty acids as described above. After removal of tissue from the diluted incubation medium, the pH of the medium was adjusted to 7 and the volume made up to 20 ml with water. Samples were taken for determination of free fatty acids, glucose, lactate and glycerol.

Assay methods. For measurement of the specific radioactivity of the plasma glucose, the serum proteins were precipitated with an equal volume of 10% (w/v) trichloroacetic acid and removed by filtration. The filtrate was submitted to descending paper chromatography with ethyl acetate–pyridine–water (8:2:1, by vol.) as solvent. Glucose markers were run on adjacent lanes. The chromatograms were stained with 1.66% phthalic acid and 0.91% aniline in butan-1-ol–ether–water (12:12:1, by vol.). The spot corresponding to the glucose marker was cut out and eluted with 4 ml of 10% (v/v) HCl in ethanol. A sample was taken from each eluate for assay of glucose by comparing the E_{390} of the sample with that of standard amounts of glucose eluted from the same chromatogram. Another sample was taken from the eluate for assay of radioactivity. Free fatty acids were assayed by the

method of Mosinger (1965). Glucose and lactate were assayed by the enzymic methods described by Bergmeyer (1965). Glycerol in the incubation medium was assayed by the method of Garland & Randle (1962).

Glycerokinase was assayed by a modification of the method of Garland & Randle (1962) for the measurement of glycerol. In this method, the phosphorylation of glycerol by ATP and glycerokinase is coupled with the oxidation of NADH by pyruvate formed from phosphoenolpyruvate. Under appropriate conditions, the change in the rate of oxidation of NADH is proportional to glycerokinase activity when the reaction sequence is initiated by addition of glycerol. To increase the sensitivity of the method, NADH was measured with a Locarte fluorimeter, with an LH6 primary filter and an LH2 secondary filter. The fluorimeter was connected to a recorder that gave a linear response to the fluorescence of NADH over the range used. The assay was carried out at 24°C. The assay mixture contained triethanolamine hydrochloride (0.1M, pH 7.7), ATP (2.15mM), phosphoenolpyruvate (0.74mM), NADH (0.15mM), pyruvate kinase (50 µg/ml) and lactic dehydrogenase (25 µg/ml). The assay mixture (0.9ml) was added to the cuvette, followed by 0.1 ml of tissue extract. When a constant rate of NADH oxidation was achieved, 0.02ml of 0.1M-glycerol was added. This resulted in an increase in the rate of oxidation of NADH. Glycerokinase activity was calculated from the change in the rate of oxidation of NADH on addition of glycerol. The assay gave a linear response with 0–2 µg of purified glycerokinase.

Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. For radioassay of free fatty acids and glucose the radioactive material was dissolved in 2 ml of ethanol and transferred to a counting vial with 10 ml of scintillation fluid (0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene.) For radioassay of $^{14}\text{CO}_2$ trapped in the Hyamine hydroxide, grease was removed from the stopcock and the stopcock was soaked for 1 h in a mixture of 2 ml of ethanol and 5 ml of scintillation fluid and then for a further 1 h in 5 ml of fresh scintillation fluid, with a final rinse in 3 ml of scintillation fluid. The pooled washings were transferred to a counting vial. This procedure removed all the radioactivity from the stopcock. For radioassay of glyceride glycerol, 1 ml of the aqueous solution of glycerol was mixed with 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.

The dry samples of Kieselgel powder containing the phospholipids were transferred to counting vials and suspended in 15 ml of a scintillation gel prepared by mixing 40 g of Cab-O-Sil in 1 litre of a solution containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene.

Materials. All enzymes required for assay of glucose, lactate and glycerol were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Pyridine nucleotides, ATP, L-arterenol bitartrate and bovine serum albumin (Fraction V powder) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Pig insulin (pro actrapid, Lot A62), free from glucagon, was obtained from Novo Industries, Medical Sources Department, London E.2, U.K. Hyamine hydroxide [benzyltrimethyl-

(*p*-1,1,3,3-tetramethylbutylcresoxyethoxyethyl)ammonium hydroxide] was obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh 11, U.K. Triton X-100 was obtained from Lennig Chemicals Ltd., London W.C.1, U.K. [^{14}C]Glucose, [^{14}C]glycerol and [^{14}C]hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

The diethyl ether and di-isopropyl ether used for chromatography were purified by passage through a column of activated alumina. Kieselgel H was obtained from E. Merck Laboratory Chemicals, Darmstadt, Germany. 2,5-Diphenyloxazole and Cab-O-Sil were obtained from Packard Instrument Co. Ltd., Wembley, Middx., U.K. 1,4-Bis-(5-phenyloxazol-2-yl)benzene was obtained from Nuclear Enterprises (G.B.) Ltd.

RESULTS

Effect of cold exposure on brown fat in vivo

[^{14}C]Glucose incorporation. The incorporation of [^{14}C]glucose into triglyceride glycerol and fatty acids of brown fat, white fat and liver was measured in 2-day-old rabbits kept at 36°C and 22°C after removal from their mother.

In the cold-exposed and warm-exposed animals the specific radioactivity of the plasma glucose increased to a similar maximum at $\frac{1}{2}$ h after the injection; at 2 h the specific radioactivity of the plasma glucose was lower in the cold-exposed than in the warm-exposed animals (Fig. 1).

In the interscapular brown fat of the cold-exposed animals, the specific radioactivity of the triglyceride glycerol reached a maximum at $1\frac{1}{2}$ h after the injection of [^{14}C]glucose (Table 1). At each of the three time-intervals the difference between the values for incorporation into triglyceride glycerol in the warm-exposed and cold-exposed animals was

Fig. 1. Specific radioactivity of plasma glucose after injection of [^{14}C]glucose into new-born rabbits kept at 22°C (●) or 36°C (○). Each value shows mean $\pm$ S.E.M.

Table 1. *Effect of exposing new-born rabbits to the cold on the incorporation of [^{14}C]glucose into triglyceride glycerol and fatty acids of interscapular brown fat, perirenal white fat and liver*

Results are expressed as % of dose incorporated/mmol of triglyceride (mean $\pm$ s.e.m.). The number of rabbits used for each observation is shown in parentheses. See the Methods section for experimental details.

Time after injection (h)		Brown fat		White fat		Liver	
		Warm	Cold	Warm	Cold	Warm	Cold
$\frac{1}{2}$	Glycerol	0.038 $\pm$ 0.01 (5)	0.160 $\pm$ 0.04 (5)*	0.668 $\pm$ 0.38 (3)	0.170 $\pm$ 0.06 (3)	0.123 $\pm$ 0.03 (5)	0.213 $\pm$ 0.08 (5)
1 $\frac{1}{2}$		0.096 $\pm$ 0.03 (6)	0.619 $\pm$ 0.17 (6)*	0.648 $\pm$ 0.47 (2)	1.941 $\pm$ 0.79 (2)	0.687 $\pm$ 0.09 (7)	0.562 $\pm$ 0.09 (7)
2		0.177 $\pm$ 0.06 (5)	0.557 $\pm$ 0.13 (5)†	1.503 $\pm$ 1.01 (2)	—	1.521 $\pm$ 0.36 (5)	1.192 $\pm$ 0.08 (5)
$\frac{1}{2}$	Fatty acids	0.019 $\pm$ 0.01	0.096 $\pm$ 0.05	0.064 $\pm$ 0.02	0.075 $\pm$ 0.03	0.117 $\pm$ 0.08	0.187 $\pm$ 0.08
1 $\frac{1}{2}$		0.030 $\pm$ 0.01	0.035 $\pm$ 0.01	0.089 $\pm$ 0.04	0.102 $\pm$ 0.05	0.072 $\pm$ 0.04	0.041 $\pm$ 0.03
2		0.023 $\pm$ 0.01	0.033 $\pm$ 0.02	0.237 $\pm$ 0.19	0.153 $\pm$ 0.07	0.040 $\pm$ 0.01	0.052 $\pm$ 0.02

* Value for cold-exposed significantly higher than value for warm-exposed ($P < 0.05$).

† Value for cold-exposed significantly higher than value for warm-exposed ($P < 0.01$).

statistically significant. Incorporation of [^{14}C]glucose into the triglyceride fatty acids of interscapular brown fat was less than that into triglyceride glycerol and was not consistently affected by exposure to the cold. Analysis of the cervical brown fat gave essentially similar results.

The incorporation of [^{14}C]glucose into triglyceride glycerol and fatty acids of white fat and liver was not significantly affected by exposure of the rabbit to the cold (Table 1). Incorporation of [^{14}C]glucose into the phospholipids of brown fat and liver was also measured in the rabbits used for the experiments shown in Table 1. Exposure of the rabbit to the cold had no effect on incorporation into phospholipids in either of these tissues (Table 2).

Triglyceride concentration. The concentration of triglyceride, expressed in terms of fresh weight of tissue, was slightly higher in the warm-exposed than in the cold-exposed animals at all intervals between 3 h and 6 h, but the difference was statistically significant only at 5 $\frac{1}{2}$ h (Table 3). In cold-exposed animals the triglyceride content of the brown fat at 6 h did not differ significantly from that at 3 h. There was, however, a significant increase in the triglyceride content of the brown fat of the warm-exposed animals between 3 $\frac{1}{2}$ and 6 h after exposure to 36°C.

Sympathetic denervation. The effect of sympathetic denervation on the response of brown fat to cold exposure of the rabbit was investigated in seven two-day-old rabbits from the same litter. The cervical sympathetic nerve was cut on one side in all seven animals. In four of the five animals that were subsequently exposed to cold, incorporation of [^{14}C]glucose into triglyceride glycerol was higher in the brown fat on the intact side than on the cut side (Table 4). In the two animals kept at 36°C, incorporation of [^{14}C]glucose into triglyceride glycerol was the same on the two sides.

Effect of noradrenaline and insulin on brown fat in vitro

Fig. 2 shows the effect of increasing concentrations of noradrenaline in the presence of insulin on the metabolism *in vitro* of brown fat from new-born rabbits. O_2 consumption, CO_2 output, and the output of lactate, free fatty acid and glycerol into the medium, were all significantly stimulated. Glucose uptake from the medium tended to be greater in the presence of noradrenaline than in its absence, but the difference was not statistically significant ($P > 0.05$). Noradrenaline also stimulated the incorporation of ^{14}C from glucose into glyceride glycerol. These effects reached a maximum when the noradrenaline concentration was 1 $\mu\text{g}/\text{ml}$. Incorporation of ^{14}C from glucose into glyceride fatty acid was negligible.

Table 2. *Incorporation of [U-¹⁴C]glucose into the phospholipids of interscapular brown fat and liver in warm-exposed and cold-exposed new-born rabbits*Results are expressed as % of dose incorporated/g fresh weight (mean $\pm$ S.E.M.).

Time after injection (h)	Brown fat		Liver	
	Warm	Cold	Warm	Cold
$\frac{1}{2}$	0.005 $\pm$ 0.002	0.011 $\pm$ 0.003	0.013 $\pm$ 0.002	0.010 $\pm$ 0.003
1 $\frac{1}{2}$	0.010 $\pm$ 0.003	0.010 $\pm$ 0.003	0.056 $\pm$ 0.010	0.038 $\pm$ 0.006
2	0.011 $\pm$ 0.003	0.011 $\pm$ 0.002	0.050 $\pm$ 0.010	0.055 $\pm$ 0.007

Table 3. *Triglyceride content of interscapular brown fat in warm-exposed and cold-exposed new-born rabbits*Results are expressed as means $\pm$ S.E.M. Experimental details are given in the Methods section.

Time of exposure (h)	Number of rabbits	Triglyceride content (μ mol/g fresh wt.)	
		Warm	Cold
3	1	352	307
3 $\frac{1}{2}$	3	330 $\pm$ 32*	255 $\pm$ 12
4 $\frac{1}{2}$	5	390 $\pm$ 42	376 $\pm$ 73
5 $\frac{1}{2}$	6	455 $\pm$ 36	320 $\pm$ 45†
6	5	491 $\pm$ 42*	366 $\pm$ 48

* Value for warm-exposed significantly higher at 6 h than at 3 $\frac{1}{2}$ h ($P < 0.05$).† Value for cold-exposed significantly lower than value for warm-exposed ($P < 0.05$).Table 4. *Effect of cervical sympathectomy on the incorporation of [U-¹⁴C]glucose into glycerol of the brown-fat triglyceride of new-born rabbits exposed to a cold environment*Results are expressed as % of the injected dose of [¹⁴C]glucose/mmol of triglyceride. See the Methods section for experimental details.

Rabbit	Temperature	Side cut	Specific radioactivity of glycerol		
			Left	Right	Intact/cut
1	22°C	Left	0.22	1.02	4.6
2		Right	2.36	0.41	5.8
3		Left	0.11	0.47	4.3
4		Left	0.51	1.30	2.5
5	36°C	Left	0.66	0.69	1.0
6		Left	0.53	0.54	1.0
7		Left	0.24	0.20	0.8

Insulin had no effect on O₂ consumption, CO₂ output, glucose uptake, lactate output, free fatty acid output or incorporation of ¹⁴C from glucose into glyceride fatty acid of brown fat *in vitro* (Table 5). Incorporation of ¹⁴C into glyceride glycerol was stimulated by insulin in the presence of noradrenaline ($P < 0.05$), and glucose oxidation was stimulated in the absence of noradrenaline ($P < 0.05$). Insulin depressed glycerol output into the medium in the absence of noradrenaline ($P < 0.05$). The insulin used for the present work did, however, have effects on the metabolism of brown fat from adult rats similar to the effects described by other workers (Shackney & Joel,

1966). Thus, in brown fat from adult rats it stimulated [¹⁴C]glucose uptake and incorporation into glyceride glycerol and fatty acid.

Glycerokinase activity

Calculations based on the incorporation of [¹⁴C]glucose into triglyceride glycerol would underestimate the true rate of glyceride synthesis if brown fat of new-born rabbits contains a glycerokinase, since this would permit the re-esterification of free glycerol derived from the hydrolysis of triglyceride. Treble & Ball (1963) found glycerokinase activity in brown fat of adult rats but

Fig. 2. The influence of noradrenaline in the presence of insulin on the metabolism *in vitro* of brown fat from new-born rabbits, showing effect on: (a) O_2 uptake ($\circ$), CO_2 output ($\bullet$) and R.Q. ($\times$); (b) glucose uptake (Δ) and lactate release ($\blacktriangle$); (c) free fatty acid release ($\square$) and glycerol release ($\blacksquare$); (d) $[^{14}C]$ glucose incorporation into triglyceride glycerol (∇) and fatty acid ($\blacktriangledown$). Values for free fatty acid release are expressed in $\mu\text{equiv./2h}$ per 100mg; all other values are expressed in $\mu\text{mol/2h}$ per 100mg. For experimental details see the Methods section.

Dawkins & Hull (1964) were unable to detect any in new-born rabbit brown fat. In the present work, glycerokinase activity in brown fat and liver of new-born rabbits and adult rats was compared by two methods of assay.

Glycerokinase activity could be demonstrated by enzymic assay in rat and rabbit liver and in rat brown fat, but was not detectable in the brown fat of new-born rabbits (Table 6). However, when pieces of brown fat from new-born rabbits were incubated with $[^{14}C]$ glycerol, phosphorylation of glycerol took place, as is shown by the presence of ^{14}C in CO_2 and glyceride glycerol (Table 7). In the

Table 5. *Effect of insulin on the metabolism in vitro of brown fat from new-born rabbits*

Brown fat was incubated under the conditions described in the Methods section, with and without noradrenaline ($1\mu\text{g/ml}$) in the presence and in the absence of insulin (0.1 units/ml). Other experimental details are given in the Methods section. Values are expressed as $\mu\text{mol/h}$ per 100 mg of fresh tissue (mean $\pm$ s.e.m.), with numbers of incubations in parentheses. Net output of free fatty acids into the medium was calculated from the difference between the amount of free fatty acid in the medium at the beginning and end of the incubation. The amount of $[^{14}C]$ glucose oxidized to CO_2 was calculated from the yield of $^{14}CO_2$ and the specific radioactivity of the $[^{14}C]$ glucose in the medium at the beginning of the incubation.

	Without noradrenaline		With noradrenaline	
	Without insulin	With insulin	Without insulin	With insulin
O_2 consumption	4.85 ± 0.55 (7)	4.90 ± 0.35 (8)	27.8 ± 2.05 (6)	24.9 ± 1.65 (10)
CO_2 output	4.90 ± 0.45 (6)	3.45 ± 0.2 (7)	20.6 ± 1.35 (6)	19.8 ± 1.3 (10)
$[^{14}C]$ glucose oxidized to CO_2	0.080 ± 0.003 (3)	0.106 ± 0.007 (4)	0.430	0.440 ± 0.058 (5)
Glucose uptake	0.80 ± 0.30 (5)	1.00 ± 0.35 (7)	1.95 ± 0.45 (7)	2.15 ± 0.45 (5)
Lactate output	0.48 ± 0.11 (6)	0.56 ± 0.10 (5)	1.02 ± 0.09 (5)	0.86 ± 0.13 (6)
Free fatty acid output	0.47 ± 0.15 (7)	0.46 ± 0.11 (7)	1.70 ± 0.11 (8)	1.40 ± 0.10 (8)
Glycerol output	0.38 ± 0.12 (7)	0.05 ± 0.05 (8)	0.88 ± 0.06 (8)	0.87 ± 0.05 (9)
^{14}C in glyceride glycerol	0.021 ± 0.004 (8)	0.028 ± 0.002 (8)	0.051 ± 0.004 (8)	0.076 ± 0.010 (9)
^{14}C in glyceride fatty acid	0.002 ± 0.0005 (8)	0.003 ± 0.001 (7)	0.002 ± 0.0005 (8)	0.002 ± 0.0005 (9)

absence of noradrenaline the amount of glycerol carbon incorporated into glyceride glycerol was about one-fifth the amount oxidized to CO_2 , and the oxidation of glycerol accounted for about 12% of the total CO_2 output. In the absence of noradrenaline equal amounts of carbon were incorporated from $[^{14}\text{C}]$ glycerol and $[^{14}\text{C}]$ glucose into glyceride glycerol. In the presence of noradrenaline, however, the amount of carbon in glyceride glycerol derived from $[^{14}\text{C}]$ glycerol was only about one-fifth that derived from $[^{14}\text{C}]$ glucose, and the oxidation of $[^{14}\text{C}]$ glycerol accounted for only 1% of the total CO_2 output.

DISCUSSION

In most animal tissues, mitochondrial respiration is thought to be controlled by the supply of ADP for phosphorylation. In adult homothermic animals that have not been cold-adapted, the supply of ADP needed for increased O_2 consumption during exposure to cold is generated from ATP in skeletal muscle by shivering. However, the thermogenic response of the brown fat of new-born animals exposed to the cold takes place without any increase in mechanical work or net synthesis likely to increase the utilization of ATP. Ball & Jungas

(1961) have pointed out that an increased rate of breakdown and resynthesis of triglyceride in adipose tissue would lead to increased production of ADP, since ATP is required for conversion of fatty acids into their CoA esters. An acceleration of the triglyceride cycle would thus permit O_2 consumption to increase in conditions in which mitochondrial respiration is coupled to phosphorylation.

In the present work, cold exposure of new-born rabbits led to a sixfold increase in the maximal incorporation of $[^{14}\text{C}]$ glucose into the triglyceride glycerol of brown fat, but not of white fat or liver. The increased incorporation in brown fat was not due to an effect of cold on the specific radioactivity of the plasma $[^{14}\text{C}]$ glucose, since the plasma glucose specific radioactivity in the cold-exposed and control animals was similar at the time of maximum incorporation of ^{14}C . Nor is it likely to have been due to an increase in the specific radioactivity of the α -glycerophosphate pool in the brown fat. Had such an increase occurred, incorporation of ^{14}C into the brown-fat phospholipids should also have been stimulated by cold exposure. It is likely, therefore, that the increased incorporation of $[^{14}\text{C}]$ glucose into triglyceride was due to stimulation of triglyceride synthesis. If so, the triglyceride cycle in brown fat must have been accelerated, since cold exposure stimulates triglyceride breakdown in the brown fat of new-born rabbits (Hull, 1966; Hardman, Hey & Hull, 1969).

Glucose carbon was not incorporated to more than a slight extent into triglyceride fatty acids of brown fat either *in vivo* or *in vitro*. This suggests that brown fat of new-born rabbits cannot synthesize fatty acids from glucose, and that the fatty acid utilized in the resynthesis of triglyceride is derived from hydrolysis of intracellular triglyceride or from fatty acid (free or esterified) in the plasma. Brown fat of adult rats, on the other hand, incorporates glucose carbon into triglyceride fatty acid *in vivo* (Himms-Hagen, 1965) and *in vitro* (Steiner & Cahill, 1966).

Table 6. *Glycerokinase activity in brown fat and liver of adult rats and new-born rabbits*

Glycerokinase was assayed enzymically by the method described in the Methods section. Values are expressed as μmol of glycerol phosphorylated/h per 100mg of fresh tissue (mean $\pm$ s.e.m.). Numbers of assays are shown in parentheses.

Tissue	Glycerokinase activity
Rat liver	8.6 ± 1.1 (12)
Rat brown fat	4.4 ± 1.2 (4)
Rabbit liver	3.4 ± 0.5 (5)
Rabbit brown fat	<0.4 (8)

Table 7. *The metabolism in vitro of $[^{14}\text{C}]$ glycerol and $[^{14}\text{C}]$ glucose in brown fat from new-born rabbits*

Brown fat was incubated in the medium described in the Methods section, modified by the addition of 5mm-glycerol to all flasks. All flasks contained insulin (0.1 units/ml) with either $[\text{U-}^{14}\text{C}]$ glycerol (0.5 μCi) or $[\text{U-}^{14}\text{C}]$ glucose (2 μCi). The noradrenaline concentration was 1 $\mu\text{g}/\text{ml}$. Other experimental details are given in the Methods section. Values are expressed as μg -atoms of ^{14}C incorporated/h per 100mg of fresh tissue. Values show results from single incubations without noradrenaline and means ($\pm$ s.e.m.) from four incubations with noradrenaline.

Labelled substrate	Noradrenaline	$^{14}\text{CO}_2$ produced	$^{14}\text{CO}_2$ as % of total CO_2	^{14}C in glyceride glycerol
$[\text{U-}^{14}\text{C}]$ Glycerol	—	0.46	12	0.09
	+	0.20 ± 0.01	1	0.10 ± 0.01
$[\text{U-}^{14}\text{C}]$ Glucose	—	0.32	5	0.09
	+	3.10 ± 0.33	12	0.47 ± 0.03

The increase in triglyceride content of the brown fat of animals kept at 36°C suggests that in a thermally neutral environment, when heat production is minimal, synthesis of triglyceride in brown fat exceeds breakdown. This may reflect the fact that when the mother is away from the nest the brown-fat triglyceride of the new-born young is broken down to provide energy for heat production, and that when the mother returns to the nest the triglyceride store is recharged.

The thermogenic response to cold exposure in new-born rabbits is mediated by the sympathetic nerves supplying brown fat (Hull & Segall, 1965). Therefore any biochemical change in brown fat that is essential for the thermogenic response should be abolished by cutting the sympathetic nerves. In the present work, increased incorporation of [¹⁴C]glucose into brown-fat triglyceride during cold exposure did not occur after severance of the sympathetic nerve supplying a circumscribed region of the interscapular brown fat. Our results are therefore consistent with the possibility that an acceleration of the triglyceride cycle is necessary for the thermogenic response to cold in the new-born rabbit.

There is evidence to suggest that local secretion of noradrenaline is responsible for the increase in O₂ consumption of the brown fat of animals exposed to the cold (Hull, 1966). However, Steiner & Cahill (1966) have shown that noradrenaline has no effect *in vitro* on [¹⁴C]glucose incorporation into triglyceride glycerol of brown fat from adult rats, although exposure of the whole animal to the cold stimulates incorporation of [¹⁴C]glucose into both glycerol and fatty acids of brown-fat triglyceride (Steiner & Cahill, 1964; Himms-Hagen, 1965). The results of the present work show no such discrepancy, and are fully consistent with the view that in new-born rabbits the metabolic response of brown fat to cold-exposure is mediated by noradrenaline. When noradrenaline is added at optimum concentration to incubations of new-born rabbit brown fat, O₂ consumption and [¹⁴C]glucose incorporation into triglyceride glycerol are stimulated and lipolysis is accelerated.

Steiner & Cahill (1966) suggested that the failure of noradrenaline to stimulate [¹⁴C]glucose incorporation into triglyceride glycerol in adult rat brown fat is due to the presence of glycerokinase. By permitting the re-esterification of free glycerol released by lipolysis, the glycerokinase in brown fat of adult rats (Treble & Ball, 1963; Fain, Reed & Saperstein, 1967; Table 6 of the present work) would be expected to diminish incorporation of glucose carbon into triglyceride glycerol. This effect would be less marked in new-born rabbit brown fat, in which the activity of glycerokinase is relatively low. The failure of Dawkins & Hull (1964)

to find glycerokinase in new-born rabbits is readily explained by its low activity in this tissue. Glycerokinase activity in new-born rabbit brown fat is below the limits of detection by enzymic methods, such as the one used by Dawkins & Hull (1964), but is high enough to be detected by measurement of the metabolism of [¹⁴C]glycerol.

The absence of a significant effect of insulin on glucose metabolism in new-born rabbit brown fat is surprising in view of the very marked effects of insulin on glucose metabolism in the brown fat of adult rats (Shackney & Joel, 1966; Fain *et al.* 1967).

The stimulatory effect of noradrenaline on the O₂ consumption of new-born rabbit brown fat raises the question of the source of the ADP that would have to be supplied to the mitochondria if respiration remains coupled to phosphorylation in the presence of noradrenaline. Calculations based on the results given in Table 5 indicate that the incorporation of [¹⁴C]glucose from the medium into glyceride glycerol could supply only 1% of the ADP required to support the observed oxygen consumption if the P/O ratio were 3.0. Owing to the presence of glycerokinase activity in the brown fat of new-born rabbits, some of the glycerol of newly synthesized triglyceride must be derived from free glycerol rather than from glucose. Hence the rate of resynthesis of triglyceride must be greater than that calculated from the incorporation of [¹⁴C]glucose from the medium. However, the results shown in Table 7 show that even in the presence of noradrenaline, when the rate of release of free glycerol from triglyceride is greatest, glucose in the medium is more important than glycerol as a source of carbon for triglyceride glycerol. It seems unlikely, therefore, that an acceleration of the triglyceride cycle can account for more than a small fraction of the O₂ consumption of new-born rabbit brown fat in the presence of noradrenaline.

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