Biochem. J. (1986) 238, 395–403 (Printed in Great Britain)

Effect of adenosine deaminase, N^6-phenylisopropyladenosine and hypothyroidism on the responsiveness of rat brown adipocytes to noradrenaline

Julie A. WOODWARD and E. David SAGGERSON
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

1. Adenosine deaminase (1 unit/ml) potentiated the lipolytic action of noradrenaline in adipocytes isolated from brown adipose tissue of 1- and 6-week-old rats by decreasing the EC_{50} (concn. giving 50% of maximal effect) for noradrenaline by 3–4-fold. With cells from neonatal rabbit tissue, adenosine deaminase only had a small, non-significant, effect on the EC_{50} for noradrenaline. 2. Lipolysis in rat brown adipocytes was inhibited by low concentrations of N^6-phenylisopropyladenosine (PIA). Rabbit cells were far less sensitive to PIA. 3. PIA, prostaglandin E_1 and nicotinate all inhibited noradrenaline-stimulated respiration in rat brown adipocytes. 4. Hypothyroidism diminished the maximum response of respiration and lipolysis to noradrenaline in rat cells and increased the EC_{50} for noradrenaline. Responsiveness of lipolysis to noradrenaline was particularly decreased in hypothyroidism and was partially restored by addition of adenosine deaminase. 5. Lipolysis in cells from hypothyroid rats was more sensitive to the anti-lipolytic action of PIA. 6. *Bordetella pertussis* toxin increased lipolysis in the presence of PIA, suggesting an involvement of the N_i guanine-nucleotide-binding protein in the control of brown-adipocyte metabolism.

INTRODUCTION

Heat generation by brown adipose tissue is important during early postnatal life, during cold exposure, on arousal from hibernation, and may also be induced in some animals by overfeeding. As reviewed at length (Nedergaard & Lindberg, 1982; Nicholls & Locke, 1983, 1984; Cannon & Nedergaard, 1985), heat is derived from uncoupled respiration, involving a unique proton-conductance pathway dissociated from oxidative phosphorylation. The major stimulus for thermogenesis appears to be noradrenaline released from the dense sympathetic nerve supply to the tissue. Noradrenaline binding to β-adrenoceptors leads to activation of adenylate cyclase, the cellular concentration of cyclic AMP is elevated, activating protein kinase and hence phosphorylating hormone-sensitive lipase, resulting in lipolysis. It has been suggested (Nicholls & Locke, 1984) that fatty acids activate the proton-conductance pathway in mitochondria. Receptor-mediated control of lipolysis may therefore be central to regulation of thermogenesis. Accompanying these events, there is a substantial increase in blood flow through the tissue (Heim & Hull, 1966; Foster & Frydman, 1978; Yahata *et al.*, 1983).

In white adipose tissue, lipolysis, besides being increased by stimulatory agonists such as the catecholamine hormones, is also decreased by inhibitory agonists such as adenosine, E-series prostaglandins and nicotinic acid. Receptors for these inhibitory agonists appear to be coupled to adenylate cyclase by a guanine-nucleotide-binding protein (N_i) distinct from that (N_s) which couples receptors for stimulatory agonists to the enzyme (Rodbell, 1980; Murayama & Ui, 1983; Olansky *et al.*, 1983; Moreno *et al.*, 1983; Bokoch *et al.*, 1984; Codina *et al.*, 1984). This inhibitory action of adenosine is mediated by the A_1 (Van Calker *et al.*, 1979) or R_i (Londos *et al.*, 1980) type of adenosine receptor.

In vivo (Fredholm *et al.*, 1977; Fredholm & Sollevi, 1981) and *in vitro* (Schwabe *et al.*, 1973; Fain & Wieser, 1975; Hjemdahl *et al.*, 1978), white adipose tissue releases adenosine. *In vivo* this substance probably acts as a 'local hormone' or paracrine agent. Since it is both vasodilatory in white adipose tissue (Sollevi & Fredholm, 1981) and anti-lipolytic, adenosine may play a role in matching rates of blood flow and lipolysis.

In general, the effects of inhibitory agonists are less well understood in brown adipose tissue, and it was the original intention of this study to investigate the role that adenosine and other inhibitory agents might play in control of lipolysis and respiration in brown adipocytes. Szillat & Bukowiecki (1983) showed that 0.1–10 μM-adenosine opposes isoprenaline stimulation of lipolysis and respiration in hamster brown adipocytes and that addition of adenosine deaminase increased sensitivity to isoprenaline. Schimmel & McCarthy (1984) confirmed and extended these studies with hamster cells and showed that cells prelabelled with [^{3}H]adenine released [^{3}H]adenosine on subsequent incubation. At present there is little information concerning the control of adenosine release and metabolism in brown adipose tissue. However, noradrenaline causes a 20–30% decrease in brown-adipocyte ATP content and a 40–100% increase in AMP content (Prusiner *et al.*, 1968; La Noue *et al.*, 1982), and it has been calculated (La Noue *et al.*, 1982) that this represents a 4–5-fold increase in cytosolic AMP, the likely precursor of adenosine via 5′-

Abbreviations used: EC_{50}, the concentration of an agonist that causes 50% of its maximum effect; PGE_1, prostaglandin E_1; PIA, N^6-L-phenylisopropyladenosine.

nucleotidase. Since these changes occur in isolated adipocytes, they are independent of effects on vasculature, and presumably are a consequence of the uncoupling of oxidative phosphorylation and/or increased fatty acid activation that occurs when the thermogenic response is initiated. Heim & Hull (1966) proposed that the increased blood flow through brown adipose tissue on infusion of noradrenaline was secondary to the effect of the hormone on the metabolism of the tissue, i.e. that vasodilation on sympathetic nerve stimulation is regulated by some substance(s), the production of which is a function of the oxygen requirements of the adipocyte (Foster & Depocas, 1980). It is therefore suggested that, by being both vasodilatory and anti-lipolytic, adenosine could play an important role in brown adipose tissue by co-ordinating lipolysis with blood flow, which in turn delivers oxygen for the increased thermogenesis and removes heat.

The first objective of the present study was to extend earlier work by examining the effects of adenosine deaminase and the non-metabolized analogue N^6-phenylisopropyladenosine (PIA) on noradrenaline-stimulated lipolysis and respiration in brown adipocytes from the rat and the neonatal rabbit. These species have not previously been investigated in this regard, and it was thought useful, since the rat interscapular tissue is widely used and since the newborn rabbit, though studied infrequently, possesses considerable quantities of brown adipose tissue (Dawkins & Hull, 1964) and could potentially be a useful animal model of brown adipose tissue in the newborn.

The second objective was to investigate the effect of hypothyroidism on the responsiveness of rat brown adipocytes to noradrenaline. In hypothyroidism the thermogenic response of brown adipose tissue to electrical nerve stimulation or noradrenaline is severely impaired (Mory *et al.*, 1981; Seydoux *et al.*, 1982). Although this might, in part, be attributable to decreased numbers of β-adrenoceptors in the adipocytes, additional changes are implicated in this lesion (Seydoux *et al.*, 1982). In white adipocytes decreased responsiveness to noradrenaline in hypothyroidism is attributed both to a diminution in the ability of β-adenergic receptors to interact productively with N_s (thereby decreasing cyclic AMP production) (Malbon *et al.*, 1984) and to an increased sensitivity to the anti-lipolytic effects of adenosine (Ohisalo & Stouffer, 1979; Malbon & Graziano, 1983; Chohan *et al.*, 1984; Malbon *et al.*, 1985) and other inhibitory agonists (Saggerson, 1986). Accordingly we have investigated the possibility that stimulation of lipolysis and respiration by noradrenaline may be defective in rat brown adipocytes in hypothyroidism and the possibility that altered sensitivity to adenosine may play a part in this lesion. During the course of this study, Sundin *et al.* (1984) demonstrated that noradrenaline stimulation of cyclic AMP elevation, lipolysis and respiration is defective in hypothyroidism. We have confirmed and extended these previous findings.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (150–180 g) bred at University College London were used as normal rats. Male and female Sprague–Dawley rat pups at 7 days old were used when studying brown adipose tissue in the young rat. GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.) was supplied to these animals. For study of hypothyroidism, male Sprague–Dawley rats were selected at age 4 weeks (95–105 g) and then maintained for 4 weeks on a low-iodine version of Rat & Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.) and drinking water containing 0.01% (w/v) 6-n-propyl-2-thiouracil. Ethanol was used initially to dissolve the propylthiouracil and hence was also present in the drinking water at 0.3% (v/v). Euthyroid controls were also selected at 95–105 g and then maintained on the normal version of the No. 3 Breeding Diet with 0.3% ethanol added to the drinking water. All rats were maintained at 20–21 °C on a 13 h-light/11 h-dark cycle with light from 06:00 to 19:00 h.

Pregnant New Zealand White rabbits were obtained from Buxted Rabbits (Buxted, Sussex, U.K.), maintained at 18–19 °C on the same light/dark cycle as the rats, and fed on Rabbit Maintenance Diet (Special Diet Services). After birth, male or female young rabbits were used aged 1–10 days.

Chemicals

Chemicals were obtained and treated as described by Fernandez & Saggerson (1978), Honnor & Saggerson (1980) and Saggerson (1980). In addition, prostaglandin E_1 (PGE_1), nicotinic acid and soya-bean trypsin inhibitor were from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Purified *Bordetella pertussis* toxin was purchased from the Centre for Applied Microbiology & Research, Porton Down, Wilts, U.K. Before use in lipolysis and O_2-uptake studies, adenosine deaminase (EC 3.5.4.4) was centrifuged in an Eppendorf 5412 centrifuge at 6500 $g_{av.}$ for 3 min to remove $(NH_4)_2SO_4$. The pellet was dissolved in 0.15 M-NaCl and the enzyme standardized spectrophotometrically at 265 nm and 25 °C by the method of Kalckar (1947). A unit of adenosine deaminase is the amount of enzyme required to deaminate 1 μmol of adenosine/min at 25 °C in this assay.

Isolation of brown adipocytes

The procedure originally described by Fain *et al.* (1967) and elaborated by Nedergaard & Lindberg (1982) was used throughout. Three or four adult rats were killed by cervical dislocation; ten young rats or single young rabbits were decapitated. Brown adipose tissue was taken from interscapular and cervical depots, trimmed of connective tissue, cut into small pieces, transferred to 50 ml silicone-treated flasks and disaggregated with collagenase (2 mg/ml) in a final volume of 3 ml of Krebs–Ringer bicarbonate (Krebs & Henseleit, 1932) containing fatty acid-poor albumin (40 mg/ml), soya-bean trypsin inhibitor (0.3 mg/ml), 5 mM-glucose and 5 mM-fructose. The disaggregation was carried out in a slowly shaking water bath (approx. 80 cycles/min) at 37 °C for 1 h. The flask contents were continuously gassed with O_2/CO_2 (19:1) and every 10 min were shaken vigorously by hand for 15 s. The digest was then filtered through nylon mesh and the filtrate centrifuged at 500 $g_{av.}$ for 90 s. The resulting layer of floating cells was washed once with Krebs–Ringer bicarbonate containing fatty acid-poor albumin (10 mg/ml) and then suspended as a stock in the same medium, which was again filtered through nylon mesh.

On average, approx. 30–40% of the tissue mass was recovered in adipocyte layer. The cells were judged to be viable, since all preparations sustained linear rates of lipolysis in the presence and absence of noradrenaline for at least 1 h. With rat cells, maximum rates of lipolysis relative to cell DNA were comparable with those in white adipocytes prepared from the same population of animals. The cell preparation from rats was adjudged to be mainly brown adipocytes by the following criteria. First, respiration was substantially stimulated by noradrenaline (see the Results and discussion section). Second, the brown-adipocyte preparation exhibited clear differences in some key enzyme activities from those found in white-adipocyte preparations from the same population of rats. The specific activity of 5′-nucleotidase in the brown-cell preparation was one-quarter of that in white cells, and the activity ratio 5′-nucleotidase/adenosine deaminase in the brown cells was one-tenth of that in white cells (Z. Jamal & E. D. Saggerson, unpublished work). In addition, the brown-cell preparations exhibited only one-sixth of the activity of microsomal glycerolphosphate acyltransferase and one-third of the activity of Mg^{2+}-dependent phosphatidase phosphohydrolase compared with white-cell preparations (H. Baht & E. D. Saggerson, unpublished work). In the unlikely event that brown adipocytes contained no glycerolphosphate acyltransferase, the maximum contamination of the brown-adipocyte preparation by white cells would be calculated as 17%. This is obviously an overestimate. The preparation of rabbit cells was not characterized with biochemical markers; however, Dawkins & Hull (1964) reported that white adipocytes represent a small proportion of the cell population in the first perinatal week in rabbits. In confirmation of this, the adipocyte pellet from the neonatal-rabbit tissue was always quite obviously brown.

Measurement of lipolysis

Immediately after isolation, 0.25 ml samples of the stock cell suspension were added to 5 ml plastic vials and incubated with shaking (105 cycles/min) at 37 °C in a final volume of 1 ml of Krebs–Ringer bicarbonate medium containing fatty acid-poor albumin (40 mg/ml) and 5 mM-glucose. The contents were continuously gassed with O_2/CO_2 (19:1). Incubation times and other additions to vials are indicated in individual Figure legends. Incubations were terminated by adding $HClO_4$ to a final concentration of 6% (w/v) and were then neutralized and treated as described by Fernandez & Saggerson (1978). Glycerol was assayed enzymically by the method of Garland & Randle (1962).

Measurement of oxygen consumption

O_2 uptake by brown-adipocyte suspensions was measured with stirring at 37 °C in a water-jacketed Perspex chamber with an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) connected to a chart recorder. The instrument was calibrated at the start of each experiment by using spectrophotometrically standardized NADH and phenazine methosulphate (Harper & Saggerson, 1975). The stock cell suspension was kept in ice. By using two oxygen electrodes in parallel, all measurements were completed within 2–3 h without any apparent deterioration of the cells. Samples (0.75 ml) of the stock cell suspension were added to the electrode chamber in a final volume of 3 ml of Krebs–Ringer bicarbonate medium containing fatty acid-poor albumin (40 mg/ml) and 5 mM-glucose. After temperature equilibration, the basal rate of O_2 uptake was measured, after which various additions were made with a micro-syringe and measurements were made after a steady rate of respiration was obtained. The final contents of the chamber are indicated in individual Figures.

Measurement of adipocyte DNA

The DNA content of 50–100 μl samples of the stock cell suspension was determined fluorimetrically by the method of Switzer & Summer (1971).

Statistical methods and presentation of data

Throughout values are shown as means ± S.E.M. Where S.E.M. bars are not shown in Figures, these lie within the area of the symbol. Values of n in legends refer to the numbers of separate cell preparations. Statistical significance was determined by Student's t test for paired or unpaired samples as appropriate.

RESULTS AND DISCUSSION

Effect of adenosine deaminase on lipolysis

Preliminary experiments established that both basal and noradrenaline-stimulated lipolysis was essentially linear for 1 h with our brown-adipocyte preparations. Addition of adenosine deaminase to incubations of rat white adipocytes increases sensitivity of lipolysis to β-adrenergic agonists without changing the maximal response (Schwabe & Ebert, 1974; Fain & Wieser, 1975; Fernandez & Saggerson, 1978) and often, but not invariably, increases the basal rate of lipolysis (Schwabe & Ebert, 1974; Fain & Wieser, 1975; Fernandez & Saggerson, 1978; Aitchison *et al.*, 1982; Saggerson, 1986). In the two studies so far reported using hamster brown adipocytes, adenosine deaminase had no effect on basal rates of lipolysis or respiration (Szillat & Bukowiecki, 1983; Schimmel & McCarthy, 1984) and potentiated effects of isoprenaline in only one study (Schimmel & McCarthy, 1984). From these studies it was not apparent what effect adenosine deaminase had on the EC_{50} and maximal response to β-agonists. We chose to work with the relatively high concentration of 1 unit of adenosine deaminase/ml (approx. 5 μg/ml) because, in preliminary experiments, this concentration stimulated rat brown-adipocyte lipolysis in the presence of a submaximally effective concentration of noradrenaline (0.1 μM). Higher concentrations of adenosine deaminase (5 or 10 units/ml) were no more effective than 1 unit/ml. In all studies shown here with rat or rabbit cells, adenosine deaminase had negligible or only very small effects on basal lipolysis, and it was therefore not feasible to investigate the concentration-dependence of stimulation of basal lipolysis by adenosine deaminase. Fig. 1 shows that adenosine deaminase significantly decreased the EC_{50} for noradrenaline by 3–4-fold with cells from 1- or 6-week-old rats. Although not apparent from the normalized data, the maximal values for noradrenaline-stimulated lipolysis were the same in the presence and the absence of adenosine deaminase. The same 3-fold effect of adenosine deaminase on the EC_{50} for noradrenaline is also seen with 9-week-old rats (see Fig. 5*a*). These experiments may be compared with other work from our

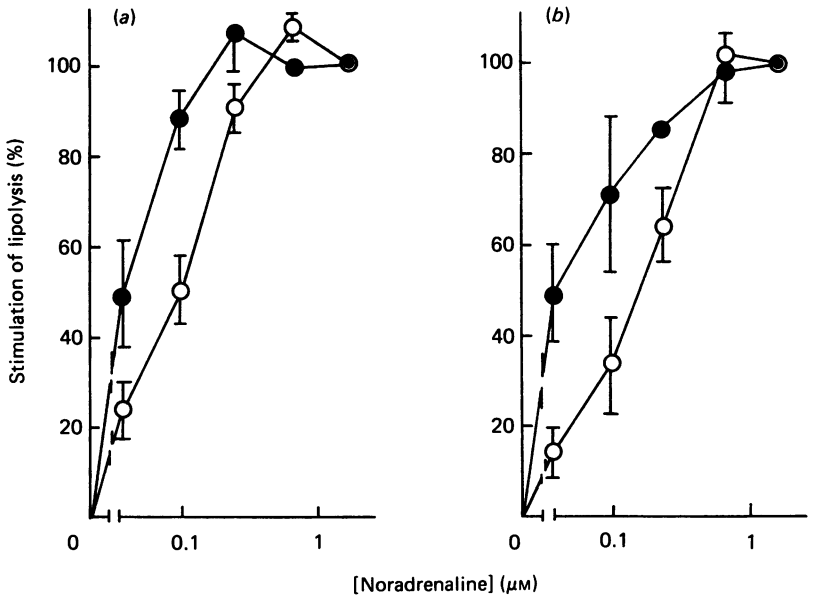

Fig. 1. Effect of adenosine deaminase on sensitivity of brown-adipocyte lipolysis to noradrenaline

Cells were incubated for 1 h with (●) or without (○) adenosine deaminase (1 unit/ml). The values represent increases over the basal rate and are normalized relative to the rate obtained with 3 μM-noradrenaline, which generally gave a rate of lipolysis of approx. 10 times the basal. (*a*) 6-week-old rats ($n = 5$). Adenosine deaminase decreased the EC_{50} for noradrenaline from 114±27 to 41±11 nM ($P < 0.05$). The mean adipocyte DNA per 1 ml incubation was 19.8±2.9 μg. (*b*) 7-day-old rats ($n = 4$). Adenosine deaminase decreased the EC_{50} for noradrenaline from 225±48 to 60±24 nM ($P < 0.05$). The mean adipocyte DNA per 1 ml incubation was 2.7±1.3 μg.

laboratory using white adipocytes from similar animals and incubated under similar conditions. With white cells the shift in the noradrenaline dose–response curve on adding adenosine deaminase is approx. 30-fold (Fernandez & Saggerson, 1978). The relatively small effect of adenosine deaminase with the brown cells might suggest that less adenosine is accumulated, and in this regard it is noteworthy that, although the brown and white tissues contain similar endogenous activities of adenosine deaminase, the specific activity of 5′-nucleotidase in rat whole white adipose tissue is 3.5 times that in brown tissue (Newsholme *et al.*, 1985). We also investigated the effect of adenosine deaminase (1 unit/ml) on noradrenaline-stimulated lipolysis in brown adipocytes from newborn rabbits. There was no effect on basal lipolysis and very little potentiation of the effect of noradrenaline. In five separate experiments (results not shown), adenosine deaminase decreased the EC_{50} for noradrenaline from 255±52 nM to 168±41 nM. These values are not significantly different.

Effect of PIA on lipolysis

It is not appropriate to investigate the effect of adenosine itself, since this substance is likely to be continuously produced and metabolized during the course of cell incubations. The derivative PIA, which is selective for A_1-type adenosine receptors (Londos *et al.*, 1980), is not a substrate for adenosine deaminase (Westermann *et al.*, 1969) and therefore may be tested in conjunction with adenosine deaminase added to remove endogenous adenosine. The effect of this agonist does not appear to have been investigated previously in brown adipose tissue. Fig. 2 shows that, with adenosine deaminase present together with 30 nM-noradrenaline

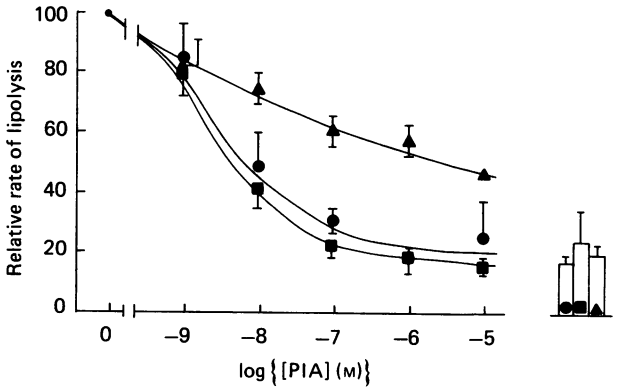

Fig. 2. Anti-lipolytic effect of PIA in rat and rabbit brown adipocytes

Cells were incubated for 1 h with adenosine deaminase (1 unit/ml), the indicated concentration of PIA, and noradrenaline (30 nM for rat cells and 100 nM for rabbit cells). The values are normalized relative to the rates with adenosine deaminase and noradrenaline alone. The histograms indicate basal rates with adenosine deaminase alone. ●, 6-week-old rats ($n = 3$); 19±8 nM-PIA gave 50% inhibition of lipolysis. Adipocyte DNA was 6.54±0.7 μg/1 ml incubation. ■, 7-day-old rats ($n = 4$); 7±2 nM-PIA gave 50% inhibition of lipolysis. Adipocyte DNA was 5.3±1.7 μg/1 ml incubation. ▲, 1–10-day-old rabbits ($n = 5$); 2.6±1.2 μM-PIA gave 50% inhibition of lipolysis. Adipocyte DNA was 12.8±8.1 μg/1 ml incubation.

(this half-maximally stimulates lipolysis; Figs. 1*a* and 1*b*), PIA in the concentration range 10–20 nM inhibited total lipolysis by 50% in cells from 1- or 6-week-old rats. Higher concentrations of PIA decreased lipolysis to rates similar to the basal, but never completely suppressed lipolysis. It can be calculated from these incubation curves that, under these experimental conditions, the EC_{50} values for PIA were 4–5 nM in both sets of rat cells. In preliminary experiments approx. 100 nM-noradrenaline was found to stimulate lipolysis half-maximally in newborn rabbit cells when adenosine deaminase was present. Fig. 2 shows that, although PIA significantly decreased lipolysis elevated by this concentration of noradrenaline in rabbit cells, this species was less sensitive to PIA by a factor of at least 100 compared with the rat. Presumably this explains the absence of any appreciable effect of adenosine deaminase in the rabbit cells (see above). Although it is possible that *in vivo* rabbit cells might release adenosine, which in turn might play a regulatory role, no further studies were performed with this species.

Measurement of O_2 uptake: effects of PIA, PGE_1 and nicotinate

With cells from 6-week-old rats, preliminary experiments established that maximally effective concentrations of noradrenaline generally increased respiration by approx. 10-fold and that adenosine deaminase slightly increased basal respiration and potentiated the effect of noradrenaline. These effects were also found in cells from the 9-week-old euthyroid rats and are elaborated in the next section. Fig. 3 shows typical polarographic traces. As also found by Nedergaard & Lindberg (1982), Szillat & Bukowiecki (1983) and Schimmel & McCarthy (1984) with hamster cells, steady increased rates of respiration were obtained within 2–3 min after addition of the stimulatory agonist. After subsequent addition of an inhibitory agonist (PIA in this case), a new steady rate was obtained within the same time interval. The rate of respiration obtained with a combination of noradrenaline+PIA was the same irrespective of the order of addition of these agonists (compare traces A and C in Fig. 3). Nedergaard & Lindberg (1982) have indicated that inclusion of pyruvate together with monosaccharide substrates enhances stimulation of respiration by noradrenaline. In the system used here, addition of pyruvate only enhanced the effect of noradrenaline by 10–15%, and so was omitted in order to keep the incubation conditions as comparable as possible with preceding lipolysis experiments.

Fig. 4 shows that PIA, PGE_1 and nicotinate all caused substantial inhibition of respiration. In the presence of 10 nM-noradrenaline (which stimulates respiration to approx. 70% of the maximal extent), the following concentrations of PIA, PGE_1 and nicotinate respectively caused 50% inhibition of the noradrenaline effect: 15 ± 6 nM, 1.72 ± 1.15 μM and 61 ± 20 μM. These three inhibitory agonists therefore have a similar potency order in brown and white adipocytes (Butcher, 1970; Londos *et al.*, 1981; Saggerson, 1986). Bertin & Portet (1976) reported that prostaglandin E_2 prevented an

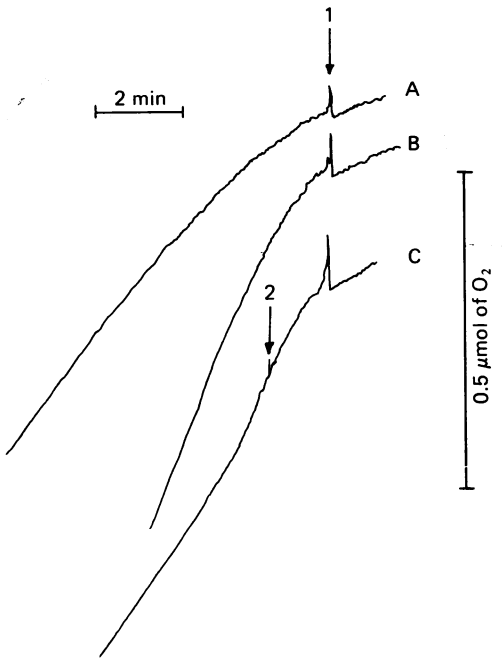

Fig. 3. Oxygen-electrode traces demonstrating the effects of noradrenaline and PIA on rat brown-adipocyte respiration

Cells from 6-week-old rats (18.5 μg of DNA) were preincubated in the electrode chamber in 3 ml of Krebs–Ringer bicarbonate containing 5 mM-glucose, fatty acid-poor albumin (40 mg/ml) and adenosine deaminase (1 unit/ml). In Expt. A the chamber also initially contained 10 μM-PIA. In all three experiments 10 nM-noradrenaline was added at point 1. In Expt. C 10 μM-PIA was added at point 2.

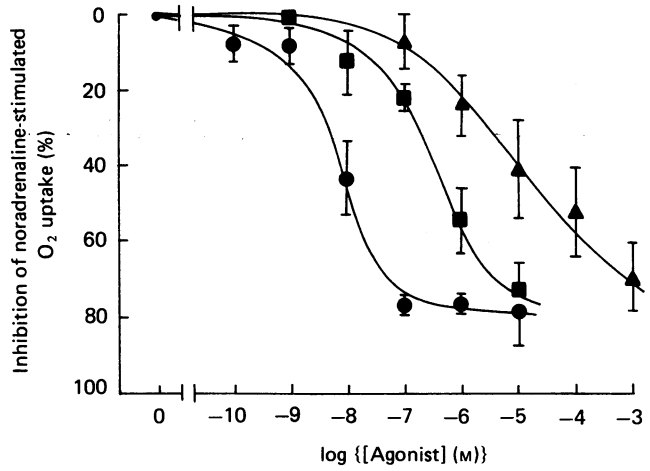

Fig. 4. Inhibition of respiration in rat brown adipocytes by PIA, PGE_1 and nicotinate

Cells from 6-week-old rats (1.7 ± 0.3 μg of DNA/ml) were preincubated in the electrode chamber with 5 mM-glucose, fatty acid-poor albumin (40 mg/ml), adenosine deaminase (1 unit/ml) and the indicated concentrations of the inhibitory agonists; 10 nM-noradrenaline was then added. In the absence of inhibitory agonists, noradrenaline increased respiration over the basal rate by 2.0 ± 0.1 μmol of O_2/min per 100 μg of DNA. The values indicate the percentage inhibition of this noradrenaline-dependent increase. ●, With PIA; ■, with PGE_1; ▲, with nicotinate. ($n = 4$ in each case).

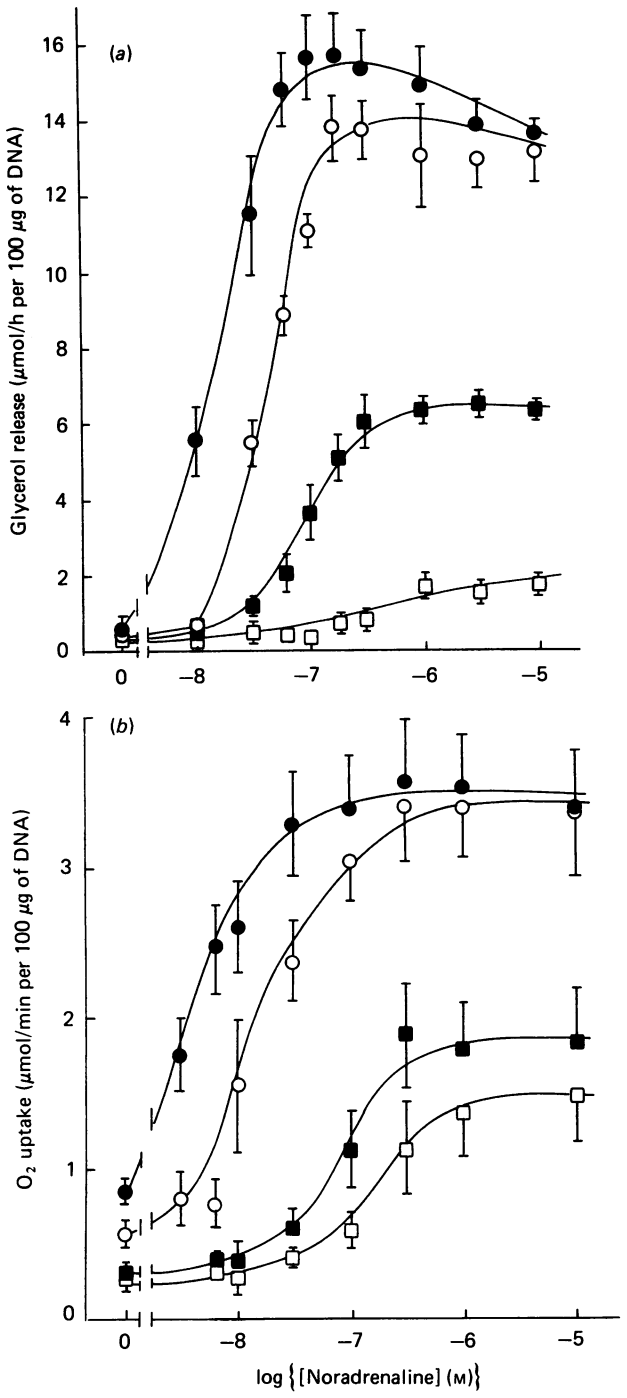

Fig. 5. Effect of adenosine deaminase on stimulation by noradrenaline of lipolysis and respiration in brown adipocytes from euthyroid and hypothyroid rats

Cells were obtained from 8–9-week-old euthyroid or hypothyroid rats. (*a*) Lipolysis measurements. Cells were incubated for 1 h with (●, ■) or without (○, □) adenosine deaminase (1 unit/ml). ○, ● Euthyroid ($n = 4$); adipocyte DNA was 1.8 ± 0.2 μg/1 ml incubation. □, ■, Hypothyroid ($n = 4$); adipocyte DNA was 2.5 ± 0.3 μg/1 ml incubation. (*b*) O_2-uptake measurements. Measurements were made in the absence and presence of adenosine deaminase (1 unit/ml) as indicated in the Materials and methods section and in Fig. 3. Symbols are as for (*a*). For the euthyroid state cells were incubated at 2.3 ± 0.6 μg of DNA/ml ($n = 5$), and for the hypothyroid state at 2.1 ± 0.3 μg of DNA/ml ($n = 4$).

increase in brown-adipocyte cyclic AMP content in response to noradrenaline, but that no effect of nicotinate was obtained. We are unaware of any other studies with nicotinate in brown adipocytes or of any with PGE_1. If, as is proposed for white adipocytes (Kather *et al.*, 1983), the receptors for these three agonists have a common coupling pathway through N_i, removal of endogenous adenosine with adenosine deaminase may facilitate detection of the effects of PGE_1 and nicotinate as well as that of PIA.

Effect of hypothyroidism on noradrenaline stimulation of lipolysis and respiration

Rats maintained on the low-iodine diet with propylthiouracil cease to grow after approx. 3 weeks, and this procedure causes severe depression of the plasma concentrations of thyroid hormones (Chohan *et al.*, 1984).

In accord with previous work with rat brown adipocytes (Sundin *et al.*, 1984) or white adipocytes (Ohisalo & Stouffer, 1979), hypothyroidism resulted in severe resistance of lipolysis to noradrenaline when adenosine deaminase was absent from incubation media (Fig. 5*a*). As found by Ohisalo & Stouffer (1979) with white cells, adenosine deaminase brought about a considerable restoration of responsiveness to noradrenaline. However, this was not a total restoration, since the EC_{50} for noradrenaline in the presence of adenosine deaminase was 107 ± 25 nM in the hypothyroid state, compared with 17 ± 2 nM in the euthyroid animals ($P < 0.02$). This remaining 6-fold difference in EC_{50} may be contrasted with the 37-fold difference in EC_{50} found in white adipocytes from the same populations of euthyroid and hypothyroid rats (Saggerson, 1986). Furthermore, even in the presence of adenosine deaminase, the maximum lipolytic rate with noradrenaline in hypothyroidism was only 50% of that in the euthyroid state, a situation very similar to that seen in white adipocytes from these hypothyroid animals (Saggerson, 1986). In the euthyroid state, adenosine deaminase decreased the EC_{50} for noradrenaline stimulation of lipolysis 3-fold, from 44 ± 6 nM to 17 ± 2 nM ($P < 0.01$). The size of the corresponding effect of adenosine deaminase in the hypothyroid state cannot be determined exactly, but must be well in excess of 100-fold. This suggests that incubations of cells from hypothyroid animals must accumulate more adenosine and/or be more sensitive to the anti-lipolytic effect of adenosine. This latter possibility is borne out by Fig. 6 (see below).

Fig. 5(*b*) shows that adenosine deaminase both increased basal respiration and potentiated stimulation of the process by noradrenaline. In the euthyroid state, adenosine deaminase increased basal respiration from 0.57 ± 0.09 to 0.87 ± 0.07 μmol of O_2 min per 100 μg of DNA ($P < 0.025$ by a paired test). In the hypothyroid state, the corresponding change from 0.24 ± 0.06 to 0.28 ± 0.07 μmol/min per 100 μg of DNA was smaller but still significant ($P < 0.025$ by a paired test). In the euthyroid state, adenosine deaminase decreased the EC_{50} for noradrenaline stimulation of respiration from 15.0 ± 3.8 nM to 4.6 ± 0.4 nM ($P < 0.05$), whereas in hypothyroidism the effect of adenosine deaminase was not significant (a decrease in EC_{50} for noradrenaline from 217 ± 63 nM to 73 ± 20 nM). Although hypothyroidism diminished both the maximum response of respiration to noradrenaline and the sensitivity of the process to this

agonist, adenosine deaminase had a far less pronounced effect on respiration than on lipolysis in hypothyroidism. The reason for this is that, in the absence of adenosine deaminase, respiration still retains some sensitivity to noradrenaline, whereas lipolysis is very insensitive to this agonist in hypothyroidism. There would seem to be two feasible explanations for this. First, the small amount of lipolysis that does occur in the absence of adenosine deaminase might be sufficient to activate the proton-conductance pathway in the hypothyroid cell or, second, most of the adrenergic stimulation of respiration in hypothyroidism might be through an α-adrenergic pathway. This would not be affected by adenosine deaminase and would presumably be independent of the β-adrenergic stimulation of lipolysis. It is noteworthy that micro-electrode measurements in rat brown adipose tissue show that nerve stimulation causes a rapid α-adrenoceptor-mediated depolarization (Girardier & Schneider-Picard, 1983). More directly, hamster brown adipocytes contain α_1-adrenoceptors (Mohell *et al.*, 1983) through which O_2 uptake can be stimulated. This α_1-adrenergic effect in normal animals elicits approx. 25% of the stimulation of respiration that is acheived by occupation of β-adrenoceptors (Schimmel *et al.*, 1983; Mohell *et al.*, 1983). It is also noteworthy that Sundin *et al.* (1984) observed a more pronounced decrease in responsiveness of respiration in hypothyroidism, using the selective β-agonist isoprenaline.

Effect of hypothyroidism on the anti-lipolytic effect of PIA

The dose–response curves for stimulation of lipolysis shown in Fig. 5(*a*) indicated that 30 nM- and 175 nM-noradrenaline would stimulate lipolysis to 75% of maximum in the euthyroid and hypothyroid states respectively. Accordingly, lipolysis in both sets of cells was stimulated to 75% of maximum and the effects of PIA were compared. In white adipocytes the effectiveness of the anti-lipolytic action of adenosine (or PIA) is dependent on the noradrenaline concentration (Stock & Prilop, 1974; Fredholm, 1978) and, as discussed by Chohan *et al.* (1984) and Saggerson (1986), it is therefore important to chose submaximal concentrations of the stimulatory agonist. Furthermore, when comparing different physiological states, these should be at equivalent points on each of the noradrenaline dose–response curves. On this basis, cells from hypothyroid animals were more sensitive to PIA (Fig. 6). It was calculated from the individual inhibition curves that the mean EC_{50} for PIA in the euthyroid state was 9.8 ± 1.4 nM, and 0.6 ± 0.2 nM in hypothyroidism ($P < 0.01$). From Fig. 5(*a*) it may be calculated that in the euthyroid state omission of adenosine deaminase at 30 nM-noradrenaline diminishes lipolysis by approx. 65%. In the hypothyroid state omission of adenosine deaminase at 175 nM-noradrenaline causes an approx. 85% decrease in lipolysis. Fig. 6 shows that these percentage inhibitions would be achieved with PIA concentrations of approx. 60 and 150 nM in the euthyroid and hypothyroid states respectively. It is therefore concluded that the differences between the effect of adenosine deaminase on lipolysis in the two states (Fig. 5*a*) must mainly be attributable to the large alteration in sensitivity to adenosine, rather than to large differences in adenosine accumulation.

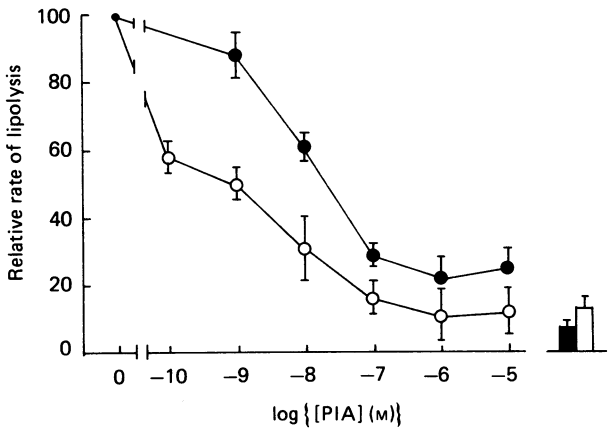

Fig. 6. Anti-lipolytic effect of PIA in brown adipocytes from euthyroid and hypothyroid rats

Cells from 8–9-week-old euthyroid or hypothyroid rats were incubated for 1 h with adenosine deaminase (1 unit/ml), the indicated concentrations of PIA and sufficient noradrenaline to elicit 75% of the maximum rate of lipolysis: 30 nM for euthyroid rats and 175 nM for hypothyroid rats. Values are expressed relative to the rates obtained with noradrenaline alone, which were: euthyroid, 6.8 ± 1.5, and hypothyroid, 3.6 ± 1.0 μmol/h per 100 μg of DNA. The histograms indicate basal rates with adenosine deaminase alone. ●, Euthyroid ($n = 4$); adipocyte DNA was 6.1 ± 0.4 μg/1 ml incubation. ○, Hypothyroid ($n = 3$); adipocyte DNA wa 3.8 ± 0.9 μg/1 ml incubation.

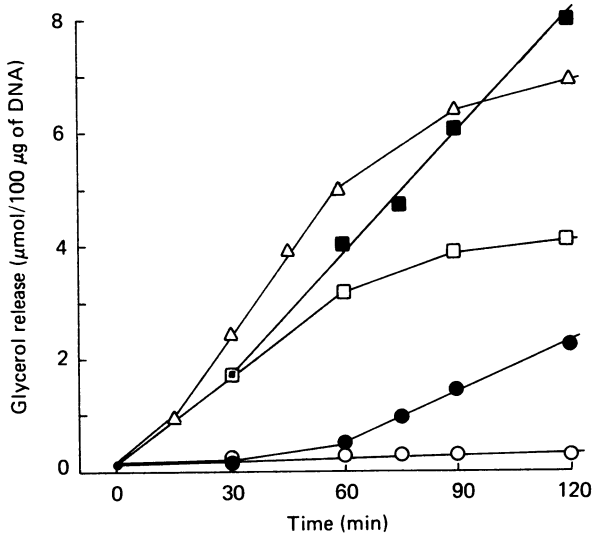

Fig. 7. Effect of pertussis toxin on lipolysis in rat brown adipocytes

Cells from 6-week-old rats were incubated for the indicated times with adenosine deaminase (1 unit/ml) and, where appropriate, noradrenaline (0.1 μM), PIA (1 μM) or pertussis toxin (1 μg/ml). The values are from two similar experiments. The adipocyte DNA was 7.2 ± 2.6 μg/1 ml incubation. ○, With PIA; ●, with PIA + pertussis toxin; △, with noradrenaline; □, with noradrenaline + PIA; ■, with noradrenaline + PIA + pertussis toxin.

Effect of pertussis toxin

The *Bordetella pertussis* exotoxin is reported to catalyse the ADP-ribosylation of the M_r-41000 α-subunit of N_i (Bokoch *et al.*, 1984; Codina *et al.*, 1984) and thereby to attenuate the effect of inhibitory agonists that are normally coupled to adenylate cyclase through this protein (Moreno *et al.*, 1983; Olansky *et al.*, 1983). We are unaware of any investigations using pertussis toxin in brown adipose tissue. Fig. 7 demonstrates that, after a lag period, the toxin increased lipolysis in the presence of a high concentration of PIA (1 μM) in both the presence and the absence of noradrenaline. These data are interpreted as suggesting an involvement of N_i in the regulation of brown-adipocyte lipolysis. It is unclear why the time courses with noradrenaline alone or with noradrenaline + PIA became non-linear after 60 min, whereas that with toxin + noradrenaline + PIA remained linear. However, this phenonemon was seen in both the experiments performed.

Conclusion

This study demonstrates that rat brown-adipocyte incubations contain sufficient adenosine to modify noradrenaline stimulation of lipolysis and respiration. This effect of adenosine is particularly apparent with lipolysis in the hypothyroid state, owing to a substantial increase in responsiveness to this inhibitory agonist. The effect of pertussis toxin suggested an involvement of N_i, and it is also demonstrated that the brown adipocyte responds to other inhibitory agonists (PGE_1 and nicotinate) whose receptors are believed to couple through N_i in white adipose tissue. Further work should investigate whether enhanced responsiveness to PIA in hypothyroidism is due to a change in the abundance of N_i, as suggested for white adipose tissue (Malbon *et al.*, 1985). Also further work should consider whether thyroid status alters the balance between α_1- and β-adrenergic stimulation of respiration and should endeavour to elucidate the nature of the decrease in responsiveness to noradrenaline that is seen in hypothyroidism even in the presence of adenosine deaminase.

This work was supported by the Medical Research Council by a studentship for J.A.W. and a project grant to E.D.S.

REFERENCES

Aitchison, R. E. D., Clegg, R. A. & Vernon, R. G. (1982) Biochem. J. **202**, 243–247

Bertin, R. & Portet, R. (1976) Eur. J. Biochem. **69**, 177–183

Bokoch, G. M., Katada, T., Northup, J. K., Ui, M. & Gilman, A. G. (1984) J. Biol. Chem. **259**, 3560–3567

Butcher, R. W. (1970) in Adipose Tissue, Regulation and Metabolic Functions (Jeanrenaud, B. & Hepp, D., eds.), pp. 5–19, Academic Press, London

Cannon, B. & Nedergaard, J. (1985) Essays Biochem. **20**, 110–164

Chohan, P., Carpenter, C. & Saggerson, E. D. (1984) Biochem. J. **223**, 53–59

Codina, J., Hildebrandt, J. D., Sekura, R. D., Birnbaumer, M., Bryan, J., Manclark, C. R., Iyengar, R. & Birnbaumer, L. (1984) J. Biol. Chem. **259**, 5871–5886

Dawkins, M. R. J. & Hull, D. (1964) J. Physiol. (London) **172**, 216–238

Fain, J. N. & Wieser, P. B. (1975) J. Biol. Chem. **250**, 1027–1034

Fain, J. N., Reed, N. & Saperstein, R. (1967) J. Biol. Chem. **242**, 1887–1894

Fernandez, B. M. & Saggerson, E. D. (1978) Biochem. J. **174**, 111–118

Foster, D. O. & Depocas, F. (1980) Can. J. Physiol. Pharmacol. **58**, 1418–1425

Foster, D. O. & Frydman, M. L. (1978) Can. J. Physiol. Pharmacol. **56**, 110–122

Fredholm, B. B. (1978) Acta Physiol. Scand. **102**, 191–198

Fredholm, B. B. & Sollevi, A. (1981) J. Physiol. (London) **313**, 351–367

Fredholm, B. B., Belfrage, E. & Blaschke, E. (1977) Acta Physiol. Scand. **99**, 313–322

Garland, P. B. & Randle, P. J. (1962) Nature (London) **196**, 987–988

Girardier, L. & Schneider-Picard, G. (1983) J. Physiol. (London) **335**, 629–641

Harper, R. D. & Saggerson, E. D. (1975) Biochem. J. **152**, 485–494

Heim, T. & Hull, D. (1966) J. Physiol. (London) **187**, 271–283

Hjemdahl, P., Fredholm, B. B. & Sollevi, A. (1978) Acta Physiol. Scand. **102**, 81A–81A

Honnor, R. C. & Saggerson, E. D. (1980) Biochem. J. **188**, 757–761

Kalckar, H. M. (1947) J. Biol. Chem. **167**, 445–459

Kather, H., Aktories, K., Schulz, G. & Jacobs, K. H. (1983) FEBS Lett. **161**, 149–152

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

La Noue, K. F., Koch, C. D. & Meditz, R. B. (1982) J. Biol. Chem. **257**, 13740–13748

Londos, C., Cooper, D. M. F. & Wolff, J. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2551–2554

Londos, C., Cooper, D. M. F. & Rodbell, M. (1981) Adv. Cyclic Nucleotide Res. **14**, 163–171

Malbon, C. C. & Graziano, M. P. (1983) FEBS Lett. **155**, 35–38

Malbon, C. C., Graziano, M. P. & Johnson, G. L. (1984) J. Biol. Chem. **259**, 3254–3260

Malbon, C. C., Rapiejko, P. J. & Mangano, T. J. (1985) J. Biol. Chem. **260**, 2558–2564

Mohell, N., Nedergaard, J. & Cannon, B. (1983) Eur. J. Pharmacol. **93**, 183–193

Moreno, F. J., Mills, I., Garcia-Sainz, J. A. & Fain, J. N. (1983) J. Biol. Chem, **258**, 10938–10943

Mory, G., Ricquier, D., Pesquies, P. & Hemon, P. (1981) J. Endocrinol. **91**, 515–524

Murayama, T. & Ui, M. (1983) J. Biol. Chem. **253**, 3319–3326

Nedergaard, J. & Lindberg, O. (1982) Int. Rev. Cytol. **74**, 187–286

Newsholme, E. A., Blomstrand, E., Newell, J. & Pitcher, J. (1985) FEBS Lett. **81**, 189–192

Nicholls, D. G. & Locke, R. H. (1983) in Mammalian Thermogenesis (Girardier, L. & Stock, M. J., eds.), pp. 8–49, Chapman and Hall, London

Nicholls, D. G. & Locke, R. M. (1984) Physiol. Rev. **64**, 1–64

Ohisalo, J. J. & Stouffer, J. E. (1979) Biochem. J. **178**, 249–251

Olansky, L., Myers, G. A., Pohl, S. L. & Hewlett, E. L. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6547–6551

Prusiner, S. B., Cannon, B., Ching, T. M. & Lindberg, O. (1968) Eur. J. Biochem. **7**, 51–57

Rodbell, M. (1980) Nature (London) **284**, 17–22

Saggerson, E. D. (1980) FEBS Lett. **115**, 127–128

Saggerson, E. D. (1986) Biochem. J. **238**, 387–394

Schimmel, R. J. & McCarthy, L. (1984) Am. J. Physiol. **246**, C301–C307

Schimmel, R. J., McCarthy, L. & McMahon, K. K. (1983) Am. J. Physiol. **244**, C362–C368

Schwabe, U. & Ebert, R. (1974) Naunyn-Schmiedeberg's Arch. Pharmacol. **282**, 33–44

Schwabe, U., Ebert, R. & Erbler, H. C. (1973) Naunyn-Schmiedeberg's Arch. Pharmacol. **276**, 133–148
Seydoux, J., Giacobino, J. P. & Girardier, L. (1982) Mol. Cell. Endocrinol. **25**, 213–226
Sollevi, A. & Fredholm, B. B. (1981) Acta Physiol. Scand. **112**, 293–298
Stock, K. & Prilop, M. (1974) Naumyn-Schmiedeberg's Arch. Pharmacol. **282**, 15–31
Sundin, U., Mills, I. & Fain, J. N. (1984) Metab. Clin. Exp. **33**, 1028–1033
Switzer, B. R. & Summer, G. K. (1971) Clin. Chim. Acta **32**, 203–206
Szillat, D. & Bukowiecki, L. J. (1983) Am. J. Physiol. **245**, E555–E559
Van Calker, D., Muller, M. & Hamprecht, B. (1979) J. Neurochem. **33**, 999–1005
Westermann, E., Stock, R. & Bieck, P. (1969) Fettstoffwechsel **5**, 68–73
Yahata, T., Habara, Y. & Kuroshima, A. (1983) Jpn. J. Physiol. **33**, 367–376

Received 16 December 1985/15 April 1986; accepted 1 May 1986