Biochemical Pharmacology, Vol. 31, No. 24, pp. 3961–3971, 1982.
Printed in Great Britain.

0006-2952/82/243961-11 $03.00/0

EFFECT OF N^6-METHYLADENOSINE ON FAT-CELL GLUCOSE METABOLISM

EVIDENCE FOR TWO MODES OF ACTION

JOHN E. SOUNESS,* JOHN E. STOUFFER* and VICTORIA CHAGOYA DE SANCHEZ†‡

* Marrs McLean Department of Biochemistry, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030, U.S.A.

and

† Departamento de Bioenergetica, Centro de Investigaciones en Fisiologia Celular, Universidad Nacional Autonoma de Mexico, Mexico 20, D.F. Mexico

(*Received* 19 *June* 1981; *accepted* 21 *April* 1982)

Abstract—N^6-Methyladenosine strongly stimulates [1-^{14}C]glucose oxidation in rat adipocytes [J. E. Souness and V. Chagoya de Sanchez, *Fedn Eur. Biochem. Soc. Lett.* **125**, 249 (1981)]. The effect of the adenosine analogue is largely independent of its action as an R-site agonist. Removal of endogenous adenosine was a prerequisite for the manifestation of the effect of N^6-methyladenosine. Nucleoside uptake inhibitors, dipyridamole and nitrobenzylthioinosine, did not block the action of N^6-methyladenosine on [1-^{14}C]glucose oxidation. The effect of the adenosine analogue was not greatly influenced by N^6-phenylisopropyladenosine, nicotinic acid or theophylline. Although N^6-methyladenosine stimulated 3-*O*-methylglucose uptake into fat cells, it is uncertain whether this was its only effect on glucose metabolism, in view of the comparable enhancement of hexose transport elicited by N^6-phenylisopropyladenosine, a much weaker stimulator of glucose oxidation. That hexose transport is not the sole site of action of N^6-methyladenosine was supported by the finding that, under conditions which have been proposed to make glucose transport rate limiting, the adenosine analogue only weakly enhanced [1-^{14}C]glucose oxidation. The conversion of glucose carbon 1 to $^{14}CO_2$ was enhanced by N^6-methyladenosine to a greater degree than that of carbon 6, suggesting an increase in pentose phosphate shunt activity. Mechanisms by which this could arise are discussed. Although similarities exist between the effects of insulin and N^6-methyladenosine on adipocyte glucose metabolism, the mechanisms by which they stimulate glucose oxidation appear to be distinct, in view of the additivity of their actions on [1-^{14}C]glucose conversion to $^{14}CO_2$. The results indicate that N^6-methyladenosine affects fat-cell glucose metabolism via two different mechanisms: (1) a weak R-site-dependent mode of action related to stimulation of glucose transport and inhibition of lipolysis, and (2) a strong R-site-independent effect of unknown mechanism.

Adenosine stimulates basal and insulin-stimulated glucose oxidation in rat epididymal adipocytes [1–4]. Although originally postulated that the mechanism of adenosine action is distinct from that by which it inhibits lipolysis [2], the finding that other strong inhibitors of hormone-stimulated cyclic AMP§ production, prostaglandin E (PGE_1), nicotinic acid and N^6-phenylisopropyladenosine (PIA), similarly enhance glucose conversion to $^{14}CO_2$ implicated depression of intracellular cyclic AMP as a probable mechanism [1, 3–6]. Under certain conditions, an inverse correlation has been demonstrated between intracellular cyclic AMP levels and [1-^{14}C]glucose oxidation [1, 5].

Recent work from this laboratory showed that N^6-methyladenosine, an R-site ligand which inhibits adenylate cyclase in isolated fat-cell membrane preparations via a GTP-dependent mechanism [7], strongly stimulates [1-^{14}C]glucose oxidation in isolated rat adipocytes [8]. The effect of N^6-methyladenosine is much stronger than that of PIA, the most potent R-site effector, and stimulation of $^{14}CO_2$ production is observed at lower concentrations of the adenosine analogue than are required to inhibit noradrenaline-stimulated cyclic AMP accumulation. In view of these results, it was proposed that an R-site-independent mechanism mediates N^6-methyladenosine enhancement of [1-^{14}C]glucose oxidation. The aim of the present paper is to corroborate these findings and further characterize the mechanism of action of N^6-methyladenosine.

EXPERIMENTAL

Materials

Crude *Clostridium histolyticum* collagenase (EC 3.4.24.3) (Lot 40C190) was purchased from the Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Bovine serum albumin (Cohn Fraction V),

‡ Address reprint requests to: Dr. Chagoya de Sanchez, Centro de Investigaciones en Fisiologia Celular, Universidad Nacional Autonoma de Mexico, Apartado Postal 70-600, Mexico 20, D.F. Mexico.

§ Abbreviations: cyclic AMP, cyclic 3′:5′ adenosine monophosphate; PIA, N^6-phenylisopropyladenosine; PGE_1, prostaglandin E_1; PMS, phenazine methosulfate; KRB buffer, Krebs–Ringer bicarbonate buffer; NBMPR, nitrobenzylthioinosine; 3-*O*-MG, 3-*O*-methylglucose; and PPP, pentose phosphate pathway.

noradrenaline, insulin (bovine), nicotinic acid, theophylline, cerulenin, phenazine methosulfate (PMS) and adenosine deaminase (EC 3.5.4.4) were supplied by the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All radionuclides were obtained from the Radiochemical Centre (Amersham, Bucks, U.K.). P-L Biochemicals (Milwaukee, WI, U.S.A.) supplied the N^6-methyladenosine, and the N^6-phenylisopropyladenosine (PIA) (Boehringer & Soehne GmbH, Mannheim, Germany) was a gift from D. J. Cadenos of Farmaceuticos Lakeside, Mexico. The dipyridamole used was a product of C. H. Boehringer & Sohn (Ingelheim am Rhein, Germany), the nitrobenzylthioinosine was purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), and the dinonylphthalate was supplied by Eastman Kodak (Rochester, NY, U.S.A.). All other reagents were of the highest grade available.

Methods

Male Wistar rats (200–230 g) were allowed free access to laboratory chow until they were killed. All preparations and incubations were carried out at 37° in bicarbonate-buffered medium [9] containing albumin (3%) and were gassed with O_2/CO_2 (19:1). Isolated fat cells were prepared according to Rodbell [10], modified as described by Taylor and Halperin [1], such that the glucose concentration was 1.67 mM. The cells were washed twice in glucose-free medium. Fat-cell DNA was measured following the method of Sooranna and Saggerson [11].

For glycerol determinations, incubations (2 ml) were terminated by removal of 0.4 ml medium from under the floating cells and rapid addition to 0.6 ml $HClO_4$ (0.5 M). After neutralization of samples, glycerol was determined enzymatically according to Wieland [12]. Fat-cell cyclic AMP content was measured essentially as described by Taylor and Halperin [1]. After a 10-min incubation (1 ml) of fat cells with lipolytic agents and separation of the cells from medium, using the oil-flotation method of Gliemann *et al.* [13], little (<3%) extracellular cyclic AMP was observed and, for this reason, the cyclic nucleotide was routinely assayed in cells plus medium.

In experiments where [^{14}C]glucose conversion to $^{14}CO_2$ was measured, fat cells were incubated in a bicarbonate-buffered medium that contained [^{14}C]glucose and the indicated additions. Incubations were terminated by the addition of 0.5 ml of 0.5 M $HClO_4$. The $^{14}CO_2$ was trapped by hyamine hydroxide (1 M solution in methanol) contained in suspended centre wells. The radioactivity was counted in Aquasol (New England Nuclear Corp., Boston, MA).

The incorporation of [^{14}C]glucose into glyceride-glycerol and glyceride-fatty acids was determined as follows. After incubation and collection of $^{14}CO_2$ was complete, the cell suspension was shaken with 10 ml of a mixture of heptane and isopropanol (1:4) to extract the lipid fraction. The contents of the vial were allowed to stand for 15 min, after which time 4 ml of heptane and 5 ml of water were added. The mixture was shaken vigorously, the phases were allowed to separate, and the upper organic phase was removed to a clean test tube. The heptane layer was then washed two times with 5 ml of water. A 4-ml sample of the extract was then removed to a clean test tube and the heptane was evaporated off. A 2-ml sample of freshly prepared ethanolic potassium hydroxide [prepared by adding 5 parts of aqueous 80% (w/v) KOH to 95 parts of ethanol] was added, the tubes were stoppered, and the lipid was saponified by placing the tubes in a water bath at 60° for 12 hr. After cooling the tubes in ice, 0.75 ml of water was added and the sample was extracted twice with 5 ml of light petroleum (b.p. range 40–60°). These light petroleum fractions were discarded. The remaining ethanolic KOH layer was acidified by adding 0.33 ml of 10 N H_2SO_4 and extracted three times with 5 ml of light petroleum. The combined light petroleum fractions were evaporated to dryness in a scintillation vial. Aquasol was added, and the radioactivity in the glyceride-fatty acid fraction was counted. An aliquot of the remaining acid aqueous fraction, after light-petroleum extraction of fatty acids, was added directly to Aquasol and counted for determination of radioactivity in glyceride-glycerol.

For the quantification of total glucose utilization, detritiation of [5-^{3}H]glucose was measured following the procedure of Brown and Garratt [14]. Briefly, after terminating the cell incubation with 0.5 ml $HClO_4$, an aliquot of the supernatant fraction was removed to a 15-ml Thunberg tube which was heated in a water bath at 60°, evacuated, and sealed. A trough of liquid air was placed under the bulb of the stopper and, after a few minutes, about 0.5 ml of water derived from the incubation medium condensed and froze in the bulb. The ^3H_2O content of an aliquot of the distillate was determined by counting the radioactivity in Aquasol.

Glucose transport was determined according to Siegel and Olefsky [15] using D-3-*O*-methyl[^{14}C]-glucose as substrate. A concentrated fat cell suspension (50 μl) was preincubated with additions for 15 min in Eppendorf microcentrifuge tubes. The reaction was started by rapid addition of 20 μl of the radiolabel (3 nmoles, 0.2 μCi) to the fat-cell suspension. Transport was terminated at the desired time by addition of buffer (0.4 ml) containing 0.3 mM phloretin. To correct for extracellular trapping of water, uptake was measured in parallel incubations in the presence of 50 μM cytochalasin B and subtracted from the other values obtained. Cells were then separated from the buffer by the oil flotation technique [13] and removed with a piece of absorptive material to a scintillation vial. Radioactivity was measured in Aquasol.

^{14}C and ^{3}H radioactivities were measured in a Packard model 2425 Tri-Carb liquid scintillation spectrometer system. Quenching corrections were applied using the channel's ratio method. Statistical comparisons were made using Student's paired *t*-test.

RESULTS

The effects of N^6-methyladenosine on [1-^{14}C]-glucose oxidation, cyclic AMP accumulation and glycerol release, in the presence and absence of noradrenaline, are shown in Table 1. In the absence of noradrenaline, an almost 3-fold stimulation of

Table 1. Effects of noradrenaline and N^6-methyladenosine on conversion of [1-^{14}C]glucose into $^{14}CO_2$, intracellular concentrations of cyclic AMP, and glycerol release*

	Conversion of [1-^{14}C]glucose to $^{14}CO_2$ (μmoles/100 μg DNA)		Intracellular cyclic AMP concentration (nmoles/100 μg DNA)		Glycerol release (μmoles/100 μg DNA)	
Addition	0	N^6-Methyladenosine	0	N^6-Methyladenosine	0	N^6-Methyladenosine
None	0.16 ± 0.06	0.42 ± 0.09†	1.20 ± 0.23	1.20 ± 0.30	21.00 ± 4.04	13.90 ± 3.39†
Noradrenaline	0.13 ± 0.06	0.42 ± 0.10†	23.64 ± 4.76‡	19.78 ± 4.47‡,§	34.07 ± 7.33‖	31.02 ± 6.09‡

* For details of incubation conditions see the Experimental section. Fat cells were incubated for 45 min, in the case of the glucose oxidation and lipolysis studies, and for 10 min in experiments where cyclic AMP was measured. Where indicated, noradrenaline (1 μM) and N^6-methyladenosine (50 μM) were added, together with adenosine deaminase (2 μg/ml). In experiments where glucose oxidation was measured, [1-^{14}C]glucose was present at a concentration of 0.5 mM (0.2 μCi). Results are means ± S.E.M. of three paired experiments.

† Effect of N^6-methyladenosine, $P < 0.02$.

‡ Effect of noradrenaline, $P < 0.02$.

§ Effect of N^6-methyladenosine, $P < 0.05$.

‖ Effect of noradrenaline, $P < 0.05$.

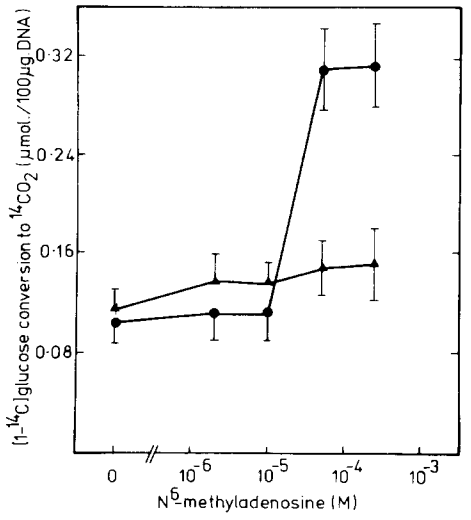

Fig. 1. Effect of N^6-methyladenosine on fat-cell [1-^{14}C]glucose oxidation in the presence and absence of adenosine deaminase. Fat cells were incubated for 45 min in bicarbonate-buffered medium that contained [1-^{14}C]glucose (0.5 mM; 0.2 μCi) and the indicated concentrations of N^6-methyladenosine, in the absence (▲) and presence (●) of adenosine deaminase (2 μg/ml). For further details, see the Experimental section. Results are means ± S.E.M. of three paired experiments.

CO_2 production was elicited by N^6-methyladenosine, an effect which did not concur with a measurable change in intracellular cyclic AMP levels, although a significant fall in glycerol output was observed. The addition of noradrenaline strongly enhanced cyclic AMP production and lipolysis while slightly decreasing [1-^{14}C]glucose oxidation. The strong stimulatory effect of N^6-methyladenosine on [1-^{14}C]glucose conversion to $^{14}CO_2$ was unaltered by noradrenaline in spite of the fact that cyclic AMP levels were high. N^6-Methyladenosine, at the concentration employed (50 μM), only slightly reduced noradrenaline-stimulated glycerol release.

In Fig. 1, the effect of a range of N^6-methyladenosine concentrations on [1-^{14}C]glucose oxidation, in the presence and absence of adenosine deaminase, is presented. In the absence of the enzyme, only a very slight increase in $^{14}CO_2$ production was observed, while the removal of endogenous adenosine facilitated the action of N^6-methyladenosine, with a sharp (3-fold) increase in the rate of glucose oxidation elicited in the concentration range 10–100 μM.

Prostaglandins, nicotinic acid, adenosine and R-site analogues of adenosine, including PIA and N^6-methyladenosine, depress cyclic AMP accumulation by inhibiting adenylate cyclase via a mechanism dependent upon an inhibitory guanine nucleotide regulatory protein (N_i), although prostaglandins, nicotinic acid and nucleosides bind to distinct receptors [16]. If the stimulation of glucose oxidation by N^6-methyladenosine was dependent upon N_i, one would expect a similar-sized response to optimal concentrations of all the aforementioned inhibitors of cyclic AMP accumulation. As can be seen in Table 2, the increased conversion of [1-^{14}C]glucose to $^{14}CO_2$ elicited by N^6-methyladenosine was much greater than that due to nicotinic acid or PIA. The effect of N^6-methyladenosine was not blocked by these N_i-site ligands, suggesting that the R-receptor played, at the most, only a minor role in its mechanism of action.

Theophylline, an R-site antagonist of adenosine action, inhibited [1-^{14}C]glucose oxidation, both in the presence and absence of adenosine deaminase (Fig. 2). In agreement with the suggestion that N^6-methyladenosine stimulation of $^{14}CO_2$ formation from [1-^{14}C]glucose is, to a large part, independent of its R-site action, theophylline had little effect on the N^6-methyladenosine dose–response curve, although a depression of [1-^{14}C]glucose oxidation by the methylxanthine, observable in the absence of the adenosine analogue, was amplified at high (200 μM) concentrations of N^6-methyladenosine.

In Table 3, the effects of the nucleoside transport inhibitors, dipyridamole and nitrobenzylthioinosine, on N^6-methyladenosine-stimulated glucose oxidation are presented. Since endogenous adenosine blocks N^6-methyladenosine action, and the preceding results seemingly relegate R-site involvement to a minor role, it was decided to investigate the possibility that internalization of the adenosine analogue is a requirement for N^6-methyladenosine action. Both dipyridamole and nitrobenzylthioinosine (NBMPR), potent inhibitors of nucleoside transport in mammalian cells [2, 17], failed to block the potentiation elicited by N^6-methyladenosine on $^{14}CO_2$ production from [1-^{14}C]glucose. Dipyridamole had a

Table 2. Effect of N_i-site ligands on basal and N^6-methyladenosine-stimulated [1-^{14}C]glucose oxidation*

	Conversion of [1-^{14}C]glucose to $^{14}CO_2$ (μmoles/100 μg DNA)	
Addition	0	N^6-Methyladenosine
None	0.086 ± 0.012	0.245 ± 0.025
PIA	0.103 ± 0.014	0.248 ± 0.026
Nicotinic acid	0.105 ± 0.013	0.248 ± 0.025

* Fat cells were incubated for 1 hr in bicarbonate-buffered medium containing bovine serum albumin (3%), adenosine deaminase (2 μg/ml) and [1-^{14}C]glucose (0.5 mM; 0.2 μCi). PIA (0.1 μM), nicotinic acid (200 μM) and N^6-methyladenosine (100 μM) were added where indicated. The results are means ± S.E.M. of three paired experiments.

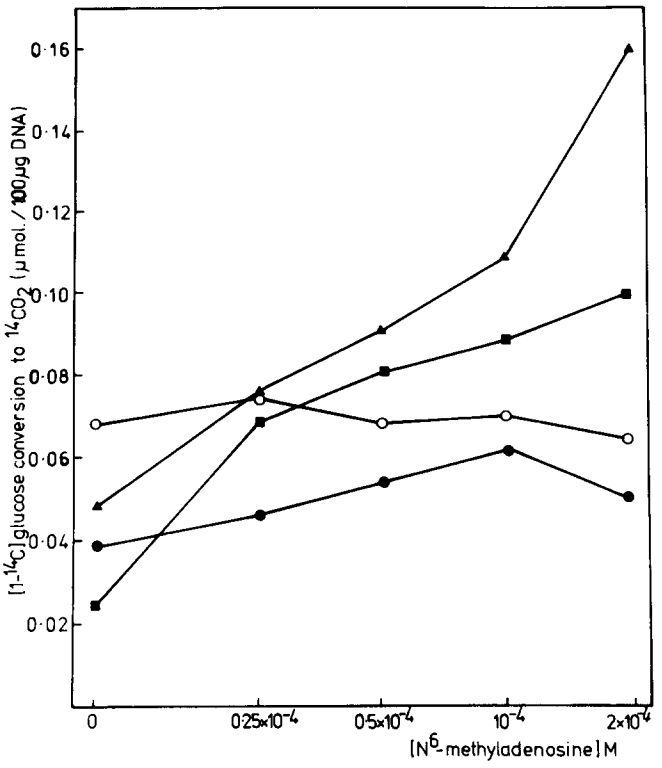

Fig. 2. Effect of theophylline on basal and N^6-methyladenosine-stimulated [1-^{14}C]glucose oxidation in the presence and absence of adenosine deaminase. The conditions employed in the experiments were the same as those described in the legend of Fig. 1. [1-^{14}C]Glucose (0.5 mM; 0.2 μCi) and the indicated concentrations of N^6-methyladenosine were included with no other addition (○), plus adenosine deaminase (2 μg/ml) (▲), plus theophylline (0.5 mM) (●), or with adenosine deaminase and theophylline (■). The results represent the means of three paired experiments.

strong inhibitory effect on glucose oxidation, apparently independently of its effect on nucleoside transport, as evidenced by the depression of $^{14}CO_2$ production, in the absence of endogenous adenosine. The N^6-methyladenosine response in the presence of the drug was similarly decreased but remained almost three times greater than in incubations with dipyridamole alone and 40% higher than the basal value. These results are inconsistent with the hypothesis that internalization is important in the enhancement of [1-^{14}C]glucose oxidation by N^6-methyladenosine.

A 15-min exposure of adipocytes to N^6-methyladenosine, in the presence of adenosine deaminase, followed by an hour-long incubation of adipocytes with [1-^{14}C]glucose in the presence of 2-deoxycoformycin, caused an increase in $^{14}CO_2$ production comparable to the increase in glucose oxidation elicited by the adenosine analogue in the absence of the adenosine deaminase inhibitor (Table 4). The possibility that exposure of N^6-methyladenosine to adenosine deaminase or adenosine deaminase plus albumin might convert the adenosine analogue to a metabolite with properties that include an ability

Table 3. Effects of nucleoside transport inhibitors on basal and N^6-methyladenosine-stimulated [1-^{14}C]glucose oxidation*

Addition	Conversion of [1-^{14}C]glucose to $^{14}CO_2$ (μmoles/100 μg DNA)	
	0	N^6-Methyladenosine
None	0.077 ± 0.021	0.189 ± 0.025
Dipyridamole	0.041 ± 0.010	0.110 ± 0.026
NBMPR	0.068 ± 0.020	0.172 ± 0.029

* Fat cells were incubated for 1 hr in bicarbonate-buffered medium containing bovine serum albumin (3%), adenosine deaminase (2 μg/ml) and [1-^{14}C]glucose (0.5 mM; 0.1 μCi). Dipyridamole (10 μM), nitrobenzylthioinosine (5.7 μM) and N^6-methyladenosine (100 mM) were added where indicated. The results are means ± S.E.M. of three paired experiments.

Table 4. Effects of preincubation with N^6-methyladenosine and subsequent incubation in the presence of 2-deoxycoformycin on [1-^{14}C]glucose conversion to $^{14}CO_2$*

Addition		[1-^{14}C]Glucose conversion to $^{14}CO_2$ (μmoles/100 μg DNA)
Preincubation	Incubation	
None	None	0.115 ± 0.029
N^6-Methyladenosine	N^6-Methyladenosine	0.286 ± 0.057
2-Deoxycoformycin	2-Deoxycoformycin	0.163 ± 0.040
N^6-Methyladenosine + 2-deoxycoformycin	N^6-Methyladenosine + 2-deoxycoformycin	0.166 ± 0.045
N^6-Methyladenosine	N^6-Methyladenosine + 2-deoxycoformycin	0.251 ± 0.063

* Cells were preincubated with the indicated additions for 15 min. [1-^{14}C]Glucose and any agent not included in the preincubation were then added, and $^{14}CO_2$ production over 1 hr was measured as described in the Experimental section. The concentrations of the inclusions were as follows: N^6-methyladenosine (100 μM); 2-deoxycoformycin (50 μM); adenosine deaminase (2 μg/ml) (included throughout); and [1-^{14}C]glucose (0.25 mM; 0.1 μCi). The results are means ± S.E.M. of three paired experiments.

to enhance glucose oxidation was tested by preincubating N^6-methyladenosine (2 mM), in bicarbonate-buffered medium containing albumin (3%) and adenosine deaminase (80 ng/ml) for 15 min in the absence of cells, and, then, adding an aliquot (50 μl) of the mixture to a 2-ml fat-cell incubation [containing 2-deoxycoformycin (50 nM) to which [1-^{14}C]glucose (0.5 mM; 0.1 μCi final concentration) was subsequently added] and incubating for 1 hr. No stimulation of $^{14}CO_2$ production to values above those obtained in the presence of 2-deoxycoformycin, without addition of the preincubated mixture, was observed, seemingly ruling out the possibility that cell-independent conversion of N^6-methyladenosine might produce a molecular species capable of stimulating glucose oxidation (unpublished results).

As seen in Table 5, N^6-methyladenosine stimulated both [1-^{14}C]glucose and [6-^{14}]glucose conversion to $^{14}CO_2$, glyceride-glycerol and glyceride-fatty acids, when the labeled sugars were included at a concentration at which hexose transport was not the rate-limiting step in their further metabolism. In addition, total glucose utilization, as quantified by detritiation of [5-^{3}H]glucose was enhanced (Table 5). The production of $^{14}CO_2$ from [1-^{14}C]glucose as compared to that from [6-^{14}C]glucose, in response to the adenosine analogue, was increased (C_1/C_6 ratio 2.1 and 3.0 respectively). Since $^{14}CO_2$ is specifically removed from [1-^{14}C]glucose by the action of the PPP enzyme, 6-phosphogluconate dehydrogenase, and also by passage through the Krebs cycle, whereas [6-^{14}C]glucose oxidation, in contrast, occurs predominatly in the Krebs cycle, the elevated C_1/C_6 ratio indicates an enhancement of the flux of glucose through the PPP. Employing equation 1 of Katz *et*

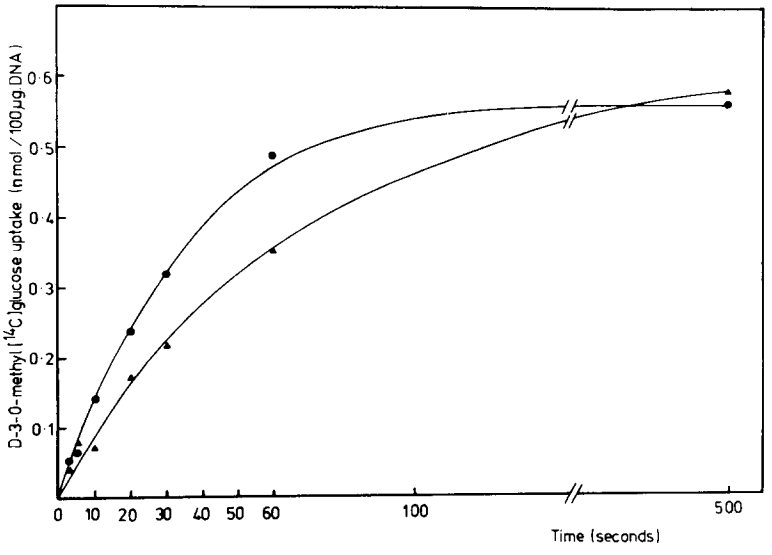

Fig. 3. N^6-Methyladenosine stimulation of 3-*O*-methylglucose uptake by adipocytes. Isolated adipocytes were incubated in the absence (▲) or presence (●) of N^6-methyladenosine for 15 min at 37° in KRB buffer containing 3% albumin and adenosine deaminase (2 μg/ml). Uptake was initiated by the addition of 20 μl of buffer, containing 3 nmoles of 3-*O*-methyl[^{14}C]glucose, to 50 μl of cells. Uptake was terminated as described in the Experimental section. Values are the means of three duplicated experiments.

Table 5. Effect of N^6-methyladenosine on [1-^{14}C]glucose and [6-^{14}C]glucose conversion to $^{14}CO_2$, glyceride-glycerol and glyceride-fatty acid and [5-^{3}H]glucose conversion to ^3H_2O*

	Conversion of labeled glucose (μmoles/100 μg DNA)						
	[^{14}C]Glucose → $^{14}CO_2$		[^{14}C]Glucose → glyceride-glycerol		[^{14}C]Glucose → glyceride-fatty acid		
Addition	Carbon 1	Carbon 6	Carbon 1	Carbon 6	Carbon 1	Carbon 6	[5-^{3}H]Glucose → ^3H_2O
None	0.900 ± 0.129	0.426 ± 0.059 (C_1/C_6 – 2.113)	1.487 ± 0.289	1.020 ± 0.150	0.312 ± 0.095	0.287 ± 0.050	6.218 ± 1.324
N^6-Methyladenosine	1.822 ± 0.456†	0.604 ± 0.047† (C_1/C_6 – 3.017)	2.591 ± 0.634†	1.921 ± 0.369†	0.993 ± 0.370†	1.357 ± 0.610†	13.781 ± 3.673†

* Parallel fat-cell incubations were performed with [1-^{14}C]glucose or [6-^{14}C]glucose (5 mM; 0.2 μCi), or [5-^{3}H]glucose (5 mM; 1 μCi), with or without N^6-methyladenosine for 1 hr at 37°. Adenosine deaminase (2 μg/ml) was included throughout. For details of experimental procedures and subsequent analyses, see the Experimental section. The results are means ± S.E.M. of three paired experiments.

† Effect of N^6-methyladenosine was significant in all cases ($P < 0.01$).

al. [18], the percentage of utilized glucose passing through the PPP was stimulated from 2.9 to 10.5% by N^6-methyladenosine (total glucose utilization, required in the equation, was that obtained from detritiation of [5-^{3}H]glucose).

That N^6-methyladenosine stimulates hexose transport is demonstrated by the increased rate of 3-*O*-methyl-D-[1-^{14}C]glucose (3-*O*-MG) uptake into adipocytes (Fig. 3). The ability of N^6-methyladenosine to enhance hexose transport was shared by PIA, with a significant ($P < 0.05$) increase in 3-*O*-MG uptake being elicited by the R-site ligand (Fig. 4).

In Fig. 5, the effect of phenazine methosulfate (PMS) on N^6-methyladenosine-stimulated glucose oxidation in the presence of NaF is presented. PMS is an electron acceptor that de-inhibits the pentose phosphate pathway by oxidizing NADPH, which inhibits glucose-6-phosphate dehydrogenase, the probable rate-limiting step of the pentose phosphate shunt [19]. NaF blocks glycolysis by inhibiting enolase. Under these conditions, it has been argued [1, 5], glucose transport is made the rate-limiting step in the oxidation of [1-^{14}C]glucose. PMS greatly increased $^{14}CO_2$ production from [1-^{14}C]glucose (0.048 μmole/100 μg DNA to 0.388 μmole/100 μg DNA). In the presence of the electron acceptor, the percentage stimulation of [1-^{14}C]glucose oxidation elicited by N^6-methyladenosine was much lower than in the absence of the drug (50 and 380%, respectively, at 100 μM N^6-methyladenosine). The size of the response to insulin was also reduced by PMS, but to a lesser extent than that to the adenosine analogue.

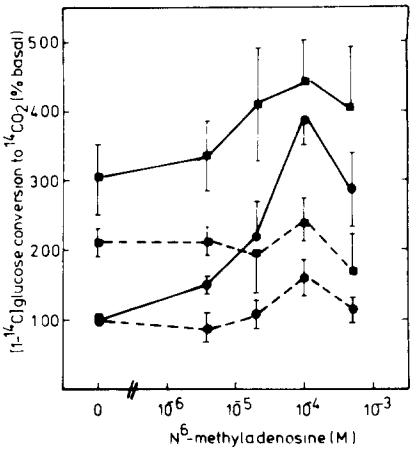

Fig. 5. Effect of N^6-methyladenosine on basal and insulin-stimulated [1-^{14}C]glucose oxidation in the presence and absence of phenazine methosulfate. Fat cells were incubated for 45 min in bicarbonate-buffered medium, containing [1-^{14}C]glucose (0.5 mM; 0.2 μCi) and the stated concentrations of N^6-methyladenosine with (discontinuous line) and without (continuous line) PMS (30 μM) in the presence (■) and absence (●) of insulin (0.2 μg/ml). NaF (20 mM) and adenosine deaminase (2 μg/ml) were included throughout. The results are expressed as a percentage of the basal values obtained in the absence and presence of PMS, which are 0.048 μmole $^{14}CO_2$/100 μg DNA and 0.388 μmole $^{14}CO_2$/100 μg DNA respectively. The results are the means ± S.E.M. of three paired experiments.

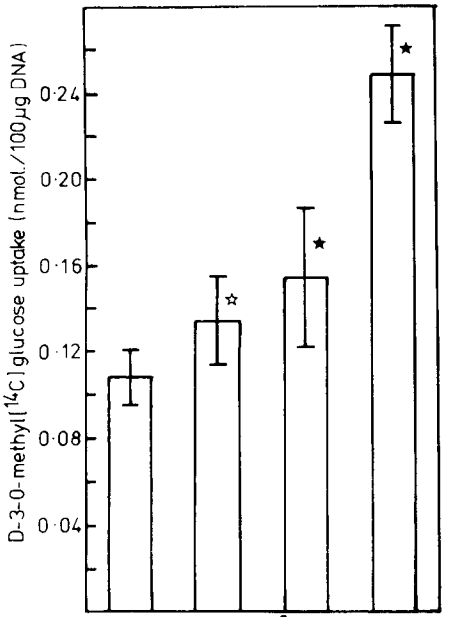

Fig. 4. Comparison of the actions of N^6-methyladenosine, PIA and insulin on 3-*O*-methylglucose uptake by adipocytes. Isolated adipocytes were incubated where indicated with N^6-methyladenosine (100 μM), PIA (0.1 μM) and insulin (0.2 μg/ml) for 15 min at 37° in KRB buffer, and 3-*O*-methylglucose uptake was measured as described in Fig. 3. Adenosine deaminase (2 μg/ml) was included throughout. Values are the means ± S.E.M. of three paired experiments. Effects of addition when compared with the control value: (☆) $P < 0.05$, and (★) $P < 0.01$.

The actions of N^6-methyladenosine and insulin, on [1-^{14}C]glucose oxidation, appear to be additive (Fig. 6). The addition of insulin increased conversion of [1-^{14}C]glucose to $^{14}CO_2$ approximately 5-fold; however, the response to N^6-methyladenosine was preserved, with the form of the dose–response curve being similar in the presence or absence of the hormone (Fig. 6a). Equally, N^6-methyladenosine pushed the insulin dose–response curve up along the response axis to a comparable extent over a wide concentration range (Fig. 6b).

DISCUSSION

It has been claimed that cyclic AMP levels and glucose oxidation are inversely related and that adenosine stimulates basal and insulin-stimulated glucose oxidation by reducing intracellular concentrations of the cyclic nucleotide [1, 3–5]. Two pieces of evidence strongly support this hypothesis. First, other inhibitors of hormone-stimulated cyclic AMP accumulation, namely PGE_1, nicotinic acid and PIA, also enhance glucose oxidation in fat cells [3–5]. Second, adenosine is a more effective stimulator of CO_2 production from [1-^{14}C]glucose in dilute fat-cell incubations [2, 5]. Under the latter conditions, it is argued, less adenosine accumulates in the extracellular medium, causing the intracellular accumulation of cyclic AMP, which, in turn, depresses glucose oxidation, an occurrence which can be reversed by

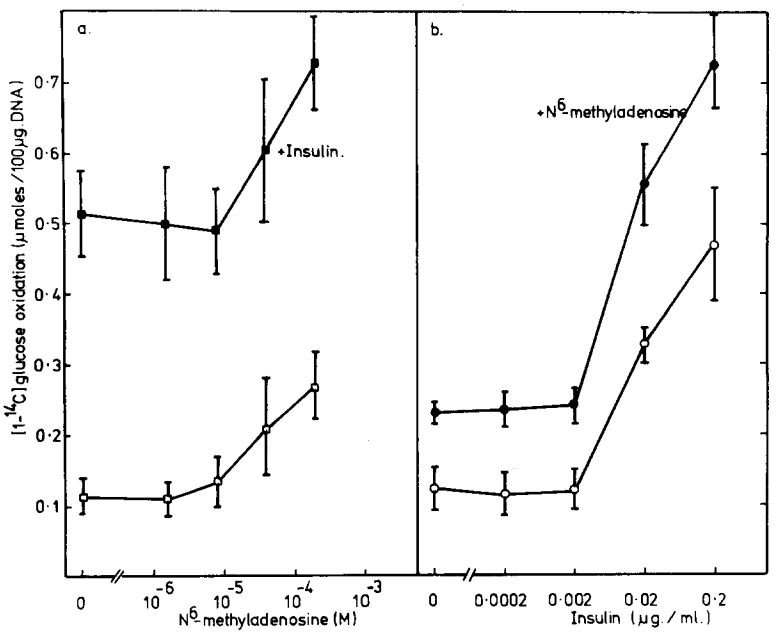

Fig. 6. The additive nature of the actions of insulin and N^6-methyladenosine on [1-^{14}C]glucose oxidation. The conditions employed in the experiments were the same as those described in the legend of Fig. 1. Cells were incubated with [1-^{14}C]glucose (0.25 mM; 0.1 μCi) and adenosine deaminase (2 μg/ml) and, where indicated, N^6-methyladenosine (100 μM) and insulin (0.2 μg/ml) for 1 hr at 37°. The results are the means ± S.E.M. of three paired experiments.

adenosine addition. In spite of this, the status of cyclic AMP with respect to the control of glucose metabolism is far from well understood. Studies on rat adipose tissue or isolated fat cells have shown that lipolytic agents increase glucose oxidation [20–23], while other reports [1, 24] have indicated an inhibition of glucose oxidation elicited by stimulators of cyclic AMP synthesis. Theophylline, an R-site antagonist of adenosine [25] and inhibitor of cyclic nucleotide phosphodiesterase [26], depresses glucose oxidation in fat cells (Fig. 2; [5]). A recent study [27] showed that catecholamines enhance glucose transport via the β-adrenergic receptor. In simplifying this confused picture, a rather paradoxical situation arises in which it appears that agents which stimulate cyclic AMP accumulation through the stimulatory component (N_s) of adenylate cyclase increase glucose transport and oxidation, and agents inhibiting cyclic AMP production via the N_i regulatory protein of adenylate cyclase also stimulate glucose oxidation via stimulation of hexose transport.

N^6-Methyladenosine appears to have two modes of action on glucose oxidation; (1) a weak N_i-mediated effect shared with other R-site agonists, nicotinic acid and prostaglandins of the E series, which is observed as cyclic AMP levels are depressed at N^6-methyladenosine concentrations above 50 μM [8], and (2) the strong stimulatory effect observed at concentrations as low as 20 μM. The second mode of action was independent of cyclic AMP concentration (Table 1) and was not blocked by PIA, nicotinic acid (Table 2) or the R-site antagonist theophylline (Fig. 2) although, in the latter case, a significant lowering of the N^6-methyladenosine dose–response curve was observed. Due to the magnitude of the cyclic AMP-independent effect, the R-site-mediated stimulation of [1-^{14}C]glucose oxidation was masked.

In view of the novelty of the effect of what was previously regarded as a straightforward R-site ligand and the requirement for addition of adenosine deaminase for the manifestation of the N^6-methyladenosine effect (Fig. 1), we were led to consider the possibility that conversion of the adenosine analogue by the enzyme to another molecular species, which in turn is capable of strongly stimulating [1-^{14}C]glucose oxidation, might be responsible for the phenomenon. This possibility was discarded for the reasons outlined previously (see Results), and the prerequisite for adenosine deaminase addition is probably associated with the masking of the N^6-methyladenosine effect by endogenous adenosine. Two obvious possibilities thus arise: (1) either N^6-methyladenosine binds to an external receptor other than the R-site for which adenosine has a higher affinity, thus blocking the action of the analogue, or (2) uptake and internalization of N^6-methyladenosine is a prerequisite for its stimulation of glucose oxidation. Again, it is feasible that endogenous adenosine could occupy the nucleoside transport site thus denying entry into the cell to N^6-methyladenosine. The nucleoside transport inhibitors, dipyridamole and nitrobenzylthioinosine (NBMPR), had little effect on N^6-methyladenosine stimulation of [1-^{14}C]glucose conversion to $^{14}CO_2$ (Table 3), indicating that uptake of the analogue is not important for the manifestation of its effect. However, further work is required to confirm this in view of the documented non-specificity of dipyridamole action and the sparsity of data concerning the action of NBMPR on nucleoside uptake in isolated adipocytes. It is

pertinent that theophylline, which also inhibits adenosine uptake at concentrations as low as 0.5 mM [28], also failed to eliminate the N^6-methyladenosine effect (Fig. 2).

A 15-min exposure of fat cells to N^6-methyladenosine followed by incubation with [1-^{14}C]glucose under conditions facilitating build-up of endogenous adenosine produced a rate of glucose oxidation equivalent to that exhibited when adenosine deaminase activity was present throughout the incubation period (Table 4). Thus, a short exposure of cells to N^6-methyladenosine in the absence of endogenous adenosine was sufficient to "gear-up" the fat cells, even though labeled glucose was added under conditions where the action of the analogue on fat-cell glucose oxidation was presumably blocked.

The flux of glucose carbon through the PPP was stimulated by N^6-methyladenosine as evidenced by the proportionate increase in the conversion to $^{14}CO_2$ of carbon 1 as compared to that of carbon 6 (i.e. increased C_1/C_6 ratio, Table 5). Estimation of the pentose phosphate pathway (PPP) contribution using equation 1 of Katz *et al.* [18] indicates that the percentage of utilized glucose passing through the PPP was increased from 2.9 to 10.5% by N^6-methyladenosine. The contribution of the pentose phosphate pathway was low in comparison with values reported elsewhere [18, 29, 30]. This may be related to the finding that rates of fatty acid synthesis and the flux of glucose through the intimately linked PPP (see below) are low under conditions of stimulated lipolysis [31]. Adenosine deaminase, which enhances glycerol release from fat cells [3], was included throughout, probably explaining the discrepancy. In addition, the summation of the incorporation products of labeled glucose tends to underestimate the total glucose utilized when compared with the value obtained from the quantification of [5-^{3}H]glucose detritiation used here [14], leading to higher estimates of the pentose phosphate shunt contribution.

The extent of glucose uptake over a wide range has been shown to determine the amount of glucose metabolized by the PPP [30]. Although N^6-methyladenosine stimulated glucose transport (Fig. 3), it is uncertain whether this alone accounts for the strong stimulation of [1-^{14}C]glucose oxidation in response to the nucleoside, in view of the increased uptake of 3-*O*-MG elicited by PIA (Fig. 4), a much weaker stimulator of [1-^{14}C]glucose oxidation (Table 2). Since both PIA and N^6-methyladenosine increased 3-*O*-MG uptake into adipocytes, enhancement of hexose transport by adenosine analogues would appear to be an R-site mediated phenomenon.

The flux of glucose through the PPP is regulated to a large degree by the cytoplasmic NADPH/NADP ratio, NADPH being a potent inhibitor of glucose-6-phosphate dehydrogenase, the probable rate-limiting step of the pentose phosphate shunt [19]. That the stimulation by N^6-methyladenosine of [1-^{14}C]glucose oxidation may have involved a mechanism leading to a decrease in NADPH levels is suggested by the finding that PMS, which oxidizes the reduced pyridine nucleotide while strongly stimulating $^{14}CO_2$ production from [1-^{14}C]glucose, greatly reduced the magnitude of the response to N^6-methyladenosine (Fig. 5).

Insulin also increases the C_1/C_6 ratio in fat cells [29, 32, 33] and two hypotheses have been proposed to explain the phenomenon. First, insulin enhances production of hydrogen peroxide in fat cells [34, 35] with activation of a plasma membrane associated NADPH oxidase being implicated as the mechanism responsible for the increased accumulation of the potent oxidizing agent [36]. Destruction of H_2O_2 by glutathione peroxidase is linked to the PPP by the GSH/GSSG and NADPH/NADP$^+$ redox couples [29, 35] with a decrease in NADPH levels de-inhibiting glucose-6-phosphate dehydrogenase and enhancing the flux of glucose through the PPP. Whether N^6-methyladenosine influences this system is unknown. Metabolism of purines stimulates H_2O_2 production as a result of hypoxanthine conversion to uric acid by xanthine oxidase [37]; however, allopurinol, an inhibitor of this enzyme, has no effect on N^6-methyladenosine-stimulated [1-^{14}C]glucose oxidation (unpublished observations).

Second, the enzymatic capacity for fatty acid synthesis is also an important determinate for insulin-stimulated glucose utilization [32, 33]. The amount of glucose uptake is directly related to rates of fatty acid synthesis and flux of glucose through the pentose phosphate shunt [30]. Under conditions of high glucose uptake into cells, ATP levels rise, blocking the citric acid cycle, and citrate is exported from the mitochondria. Outside the mitochondria, citrate is cleaved by the ATP-requiring citrate lyase to acetyl-CoA and oxaloacetate. The oxaloacetate can be reduced to malate and the latter oxidized with NADP$^+$ to pyruvate, which can again enter the mitochondrion. Acetyl groups exported from the mitochondrion as acetyl-CoA can then be carboxylated under the activating influence of citrate to form malonyl-CoA, the precursor of fatty acids. The NADPH formed from oxidation of the maleate provides part of the reducing equivalents needed for fatty acid synthesis. Additional NADPH is available from the pentose phosphate pathway. Utilization of NADPH under conditions facilitating synthesis of fatty acids de-inhibits glucose-6-phosphate dehydrogenase and increases flux of glucose through the pentose phosphate cycle. Insulin not only increases fatty acid synthesis by enhancing glucose uptake but also activates lipogenic enzymes [38].

As shown in Table 5, the incorporation of [^{14}C]glucose into glyceride-fatty acids was strongly enhanced by N^6-methyladenosine, the increase being substantially greater than that of glucose into glyceride-glycerol. Since, as mentioned earlier, experiments were performed in the presence of adenosine deaminase, absence of endogenous adenosine would facilitate breakdown of triglycerides. Because low rates of fatty acid synthesis are associated with conditions of enhanced lipolysis [31], it is possible that the stimulation of [^{14}C]glucose incorporation into fatty acids and, in turn, oxidation of [1-^{14}C]glucose by N^6-methyladenosine, might be related to its R-site related activity as an antilipolytic agent [8]. However, N^6-methyladenosine is much more potent than PIA as a stimulator of [1-^{14}C]glucose conversion to glyceride-fatty acids and $^{14}CO_2$ (unpublished results, Table 2) when added at optimal concentrations under the same conditions,

suggesting an alternative mechanism of action. Whether N^6-methyladenosine has a direct effect on one of the steps of fatty acid synthesis awaits further investigation, although it is worth mentioning that cerulenin, an inhibitor of fatty acid synthetase [39], reduces the increased conversion of [1-^{14}C]glucose to $^{14}CO_2$ elicited by N^6-methyladenosine (unpublished observations).

Although similarities exist between the actions of N^6-methyladenosine and insulin on glucose metabolism in fat cells, stimulation of [1-^{14}C]glucose oxidation by the two agents appears to be additive (Fig. 6) indicating distinct modes of action.

In conclusion, N^6-methyladenosine affects fat-cell glucose metabolism via two distinct mechanisms: (1) a weak R-site-dependent mechanism stimulating hexose transport and, probably, by inhibiting lipolysis enhancing fatty acid synthesis and consequently flux of glucose through the pentose phosphate cycle, and (2) a strong R-site-independent mode of action enhancing [1-^{14}C]glucose oxidation by an unknown mechanism.

Acknowledgements—The research work was partially supported by Grant PNCBNAL 790035 from the Consejo Nacional de Ciencia y Tecnologia (CoNACyT) and by Grant Q-275 from the Robert A. Welch Foundation. J. E. S. gratefully acknowledges the financial aid provided by a Royal Society Fellowship (exchange scheme with the Academia Mexicana) and Secretaria Ejecutiva del Consejo de Estudios de Posgrado, Universidad Nacional Autonoma de Mexico, which supported part of these studies during the author's stay in Mexico.

REFERENCES

1. W. M. Taylor and M. L. Halperin, *Biochem. J.* **178**, 381 (1979).
2. U. Schwabe, P. S. Schonhofer and R. Ebert, *Eur. J. Biochem.* **460**, 537 (1974).
3. J. N. Fain and P. B. Wieser, *J. biol. Chem.* **250**, 1027 (1975).
4. P. B. Wieser and J. N. Fain, *Endocrinology* **96**, 1221 (1975).
5. W. M. Taylor, M. L. Mak and M. L. Halperin, *Proc. natn. Acad. Sci. U.S.A.* **73**, 4359 (1976).
6. J. N. Fain and C. C. Malbon, *Molec. cell. Biochem.* **25**, 143 (1979).
7. C. Londos, D. M. F. Cooper, W. Schlegel and M. Rodbell, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5362 (1978).
8. J. E. Souness and V. Chagoya de Sanchez, *Fedn Eur. Biochem. Soc. Lett.* **125**, 249 (1981).
9. H. A. Krebs and K. Henseleit, *Hoppe-Seyler's Z. physiol. Chem.* **210**, 33 (1932).
10. M. Rodbell, *J. biol. Chem.* **239**, 375 (1964).
11. S. R. Sooranna and E. D. Saggerson, *Fedn Eur. Biochem. Soc. Lett.* **64**, 36 (1976).
12. O. Wieland, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 211. Academic Press, New York (1965).
13. J. Gliemann, K. Osterlind, J. Vinten and S. Gammeltoft, *Biochim. biophys. Acta* **286**, 1 (1972).
14. D. Brown and C. J. Garratt, *Analyt. Biochem.* **61**, 492 (1974).
15. J. Siegel and J. M. Olefsky, *Biochemistry* **19**, 2183 (1980).
16. D. M. F. Cooper and C. Londos, in *Horizons in Biochemistry and Biophysics* (Ed. F. Palmieri), Vol. 6. John Wiley, New York (in press).
17. P. Brajeswar, M. F. Chen and A. R. P. Paterson, *J. med. Chem.* **18**, 968 (1975).
18. J. Katz, B. R. Landau and G. E. Bartsch, *J. biol. Chem.* **241**, 727 (1966).
19. L. V. Eggleston and H. A. Krebs, *Biochem. J.* **138**, 425 (1974).
20. G. F. Cahill, B. Leboeuf and R. B. Flinn, *J. biol. Chem.* **235**, 1246 (1960).
21. G. A. Bray and H. M. Goodman, *J. Lipid Res.* **9**, 714 (1968).
22. D. Hepp, D. R. Challoner and R. H. Williams, *J. biol. Chem.* **243**, 2321 (1968).
23. S. P. Mukherjee, M. D. Yeager, W. S. Lynn and L. Mukherjee, *J. Cell. Biol.* **83**, 251a (1979).
24. M. Rodbell, *J. biol. Chem.* **242**, 5751 (1967).
25. T. Trost and U. Schwabe, *Molec. Pharmac.* **19**, 228 (1981).
26. J. A. Beavo, N. L. Rogers, O. B. Crofford, J. G. Hardman, E. W. Sutherland and E. V. Newman, *Molec. Pharmac.* **6**, 597 (1970).
27. C. Ludvigsen, L. Jarett and J. M. McDonald, *Endocrinology* **106**, 786 (1980).
28. P. D. Rosenblit and D. Levy, *Archs Biochem. Biophys.* **204**, 331 (1980).
29. J. M. May, *Archs Biochem. Biophys.* **207**, 117 (1981).
30. H. Kather, M. Rivera and K. Brand, *Biochem. J.* **128**, 1089 (1972).
31. E. D. Saggerson and E. L. Greenbaum, *Biochem. J.* **119**, 221 (1970).
32. M. P. Czech, *J. clin. Invest.* **57**, 1523 (1976).
33. S. K. Fried, M. Lavau and F. X. Pi-Sunyer, *J. Lipid Res.* **22**, 753 (1981).
34. J. M. May and C. DeHaen, *J. biol. Chem.* **254**, 2214 (1979).
35. S. P. Mukherjee, *Biochem. Pharmac.* **29**, 1239 (1980).
36. S. P. Mukherjee and W. S. Lynn, *Archs Biochem. Biophys.* **184**, 69 (1977).
37. J. I. Davies and M. F. Grahn, *Biochem. Soc. Trans.* **8**, 363 (1980).
38. E. D. Saggerson, S. R. Sooranna and R. D. Harper, *Biochem. Soc. Trans.* **5**, 900 (1977).
39. G. D'Agnolo, I. B. Rosenfield, J. Awaya, S. Omura and P. R. Vagelos, *Biochim. biophys. Acta* **326**, 155 (1973).