The Effect of Guanine Derivatives on Palmitate-1-^{14}C Incorporation into Rat Epididymal Adipose Tissue* (34039)

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Nucleosides, nucleotides, nucleic acids, and homogenates of tissue rich in the above substances inhibit lipolysis in rat epididymal adipose tissue (1–3). A plasma free fatty acid (FFA) lowering action of nucleotides, particularly of guanylic acid, has been reported by Kabal and Ramey (4). The antilipolytic effect of nucleosides has been attributed to a direct inhibition of the lipolytic system through a lowering of intracellular adenosine 3′, 5′-phosphate concentration (3). The possibility remains, however, that the decreased mobilization of FFA in the presence of nucleotides is achieved through increased re-esterification. In the following study we reinvestigated the *in vitro* effect of guanine derivatives on FFA esterification by measuring the incorporation of palmitate-1-^{14}C into the lipid fraction of rat epididymal adipose tissue. Our findings suggest that a direct, glucose-independent stimulation of FFA esterification by these compounds may account for their antilipolytic action.

Methods and Materials. Male Sprague-Dawley rats, weighing 200–300 g, were maintained on Rockland Rat Diet and tap water. Except where otherwise indicated, adipose tissue of *ad libitum*-fed rats was used throughout. Fasted and fasted–refed rats, used in the experiments to assess the role of nutritional state, were either deprived of food for 20–24 hr or refed for 3 days after a 3-day period of fasting. Rats were killed by a blow on the head and exsanguinated. Epididymal adipose tissues were rapidly removed through an abdominal incision and rinsed in a Krebs-Ringer bicarbonate medium (KRB) (5) with half the recommended concentration of Ca^{2+}. The same medium was used for incubation of the adipose tissue fragments. Thirty- to 40-mg fragments from fat pads of each of three rats were pooled to total approximately 100 mg per flask and incubated in 2 ml of medium containing 3% bovine plasma albumin (Armour Fr. V.), 0.1 μCi palmitate-1-^{14}C Na and the appropriate test reagent. Unless otherwise stated, all test reagents were added to a concentration of $2 \times 10^{-3}M$. All nucleotides were added as the Na salt. The FFA concentration of the 3% albumin solution was approximately 0.3 μEq/ml; where a higher concentration of FFA was desired, additional palmitate was complexed with the albumin. After addition of the adipose tissue fragments, flasks were gassed with 95% O_2 – 5% CO_2, capped and incubated with shaking at 37°. Incubation was terminated by rapid chilling of the flasks. Adipose tissue fragments were washed three times in cold saline and homogenized in 5 ml of Dole's extraction mixture (6). After standing for at least 15 min the extract was allowed to separate into two phases by the addition of 3 ml of H_2O and 2 ml of heptane. An aliquot of the upper phase was added to 10 ml of a 0.4% solution of Omnifluor (a blend of 98% PPO and 2% bis-MSB from New England Nuclear) in toluene and counted in a Packard Tri-Carb scintillation spectrometer. A second aliquot was used for FFA titration (6). Labeled FFA were recovered from the alkaline lower phase remaining after titration by the method of Borgstrom (7) and counted as above. Incubating media were extracted, titrated, and counted likewise. When a change in the specific activity of the medium occurred due to dilution with unlabeled FFA during the course of the incubation, mean specific activity was calculated according to Dole(8). The incorporation of Palmitate-1-^{14}C into tissue lipid fraction was estimated from the total lipid radioactivity of the tissue and the specific activity of the medium.

In the experiments designed to study the

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effect of guanine derivatives on glucose utilization by adipose tissue, incubating media contained 5 m*M* of glucose, 200 mg of albumin per 100 ml, 0.25 μCi of glucose-U-^{14}C per flask and the appropriate test reagent. At the end of a 2-hr period of incubation, 0.5 ml of 2 *N* H_2SO_4 and 0.2 ml of hyamine-10X were injected through the stopper, respectively, into the incubating medium and a polypropylene center well, suspended from the rubber stopper. Flasks were shaken for an additional 30 min. $^{14}CO_2$ radioactivity in the center well was counted in a Packard Tri-Carb liquid scintillation counter, total lipid radioactivity was estimated as above.

Purine and pyrimidine derivatives were purchased from P-L Biochemicals, palmitic acid-1-^{14}C from Nuclear Chicago and glucose-U-^{14}C from New England Nuclear Corporation. Prostaglandin E_1 (PGE_1) (U-10 136, Lot No. 8118-JEP-76A) was kindly provided by Dr. John E. Pike of the Upjohn Company.

Results and Discussion. Palmitate-1-^{14}C, taken up by epididymal adipose tissue fragments of fed or fasted–refed rats, was present predominantly in the neutral lipid fraction. Only 10% or less of the lipid radioactivity could be recovered in the tissue FFA fraction. In adipose tissue of fasting rats up to 30% of the label was in the FFA fraction. This probably resulted from lipolysis of newly formed triglycerides, assuming that all of the palmitate-1-^{14}C taken up by the tissue undergoes rapid esterification (8). One could then consider total lipid radioactivity to reflect the rate of FFA esterification, at least in a reasonably qualitative sense.

The incorporation of palmitate-1-^{14}C into adipose tissue lipid was markedly enhanced by the *in vitro* addition of guanosine (G) and its 5′ phosphate esters; to a lesser extent by 2′-deoxyguanosine 5′-phosphate (dGMP); and by guanosine 2′, 3′ cyclic phosphate (G2′, 3′cP) but not at all by guanylic acid [G 3′ (2′) MP] or the free base, guanine (Table I). Of the nucleosides of other bases tested for comparison, a marked stimulation was also obtained with adenosine (A) while uridine (U) and cytidine (C) had only a slight but significant effect. These observations suggest that a purine nucleoside or necleotide with a free OH group in the 2′ and 3′ positions is required for maximal stimulation of palmitate incorporation.

TABLE I. Effect of Purine and Pyrimidine Derivatives on the Incorporation of Palmitate-1-^{14}C into Adipose Tissue Lipid.

Additions[a]	Palmitate-1-^{14}C incorporation (μEq/g/2 hr)	*p*
—	.347 ± .0208[a]	
Guanine	.303 ± .0067	NS
G	1.478 ± .0897	<.001
G 3′ (2′) MP	.375 ± .0126	NS
G 2′, 3′ cP	.547 ± .0347	<.005
dGMP	.600 ± .0108	<.001
GMP	1.643 ± .0401	<.001
GDP	1.276 ± .1048	<.001
GTP	1.016 ± .1162	<.001
A	.913 ± .0278	<.001
U	.553 ± .0482	<.005
C	.495 ± .0302	<.005

[a] All figures are means ± SEM of 3–7 observations.

Further experiments to elucidate the mechanism of this stimulation were carried out using guanosine *in vitro*. Guanosine 5′-phosphate (GMP) was used to obtain a dose-response relationship (Fig. 1) as the solubility of guanosine in a KRB medium did not exceed 2×10^{-3} *M*. The effective concentration range of GMP was 10^{-4} to 10^{-2} *M*; *i.e.*, greater by two orders of magnitude than that reported for the antilipolytic action of adenine derivatives by Kappeler (2). A study of the time course of guanosine stimulation of palmitate incorporation showed it to remain pronounced for periods of incubation of up to 6 hr (Fig. 2).

Experiments employing a high substrate concentration and other known antilipolytic agents; *i.e.*, PGE_1 and nicotinic acid *in vitro* (9) were carried out to test the possibility that the effect of guanosine on palmitate incorporation is secondary to an inhibition of triglyceride breakdown and a consequent increase in the specific activity of a theoretical, small precursor pool (1). Raising the initial palmitate concentration from 0.3 μEq/ml to

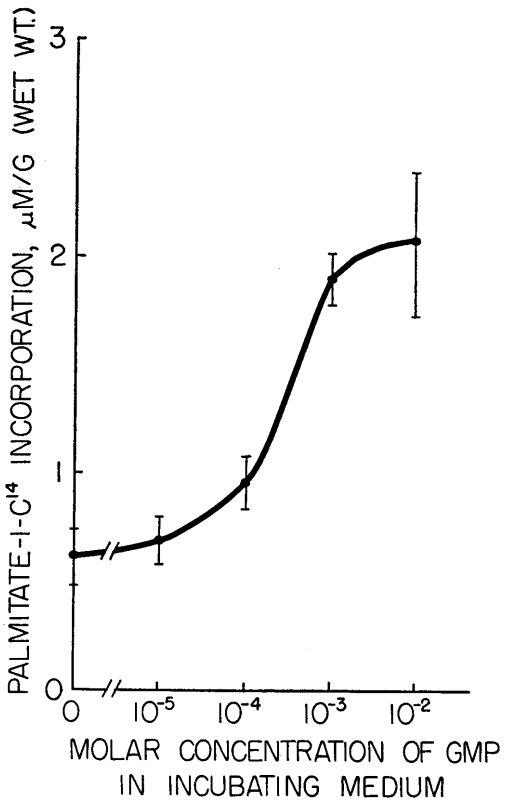

FIG. 1. Effect of increasing concentration of GMP on palmitate-1-^{14}C incorporation into rat epididymal adipose tissue. Each point represents the mean ± SEM of four observations.

1.6 μEq/ml; *i.e.*, to one where no appreciable change in the specific activity of the medium occurs over a 2-hr period of incubation, did not abolish guanosine-induced stimulation of palmitate incorporation (Table II). PGE_1 and nicotinic acid, while reducing the concentration of tissue FFA as well as their release

TABLE II. Palmitate-1-^{14}C Incorporation into Adipose Tissue Lipid. Guanosine Stimulation at Different Substrate Concentrations.

	Palmitate-1-^{14}C incorporation (μEq/g/2 hr)	
	Initial palmitate concentration	
Addition	(0.3 μEq/ml)	(1.6 μEq/ml)
—	.480 ± .023[a]	1.525 ± .179
Guanosine	1.845 ± .103[b]	4.750 ± .324[b]

[a] Mean ± SEM of four observations throughout.
[b] $p < .001$.

TABLE III. Palmitate-1-^{14}C Incorporation into Adipose Tissue Lipid. Effect of G and Antilipolytic Agents.

Additions	Palmitate-1-^{14}C incorporation (μEq/g/2 hr)
—	.765 ± .076[a]
Guanosine	1.430 ± .034[b]
PGE_1, 2.8×10^{-6} *M*	.853 ± .109
PGE_1 + guanosine	1.425 ± .171[c]
Nicotinic acid, 2×10^{-3} *M*	.795 ± .101

[a] Mean ± SEM of four observations throughout.
[b] $p < .001$.
[c] $p < .025$.

into the medium, did not affect the incorporation of palmitate (Table III). Thus, an inhibition of lipolysis, by itself, does not promote an increased incorporation of palmitate into adipose tissue. The absence of net lipolysis in adipose tissue of *ad libitum*-fed rats also lends support to the view that guanosine exerts its effect on esterification directly rather than through a suppression of lipolysis.

TABLE IV. Glucose-U-^{14}C Metabolism of Rat Epididymal Adipose Tissue. *In Vitro* Effect of Guanine Derivatives.

	Glucose-U-^{14}C converted (μmoles/g/2 hr)	
Additions	To CO_2	To lipid
—	.420 ± .045[a]	.753 ± .049
G	.255 ± .036[b]	.458 ± .050[b]
G 3′ (2′) MP	.465 ± .055	.870 ± .062
G 2′, 3′ CP	.710 ± .025[b]	
dGMP	.338 ± .030	.455 ± .021[b]
GMP	.233 ± .033[b]	.395 ± .025[b]

[a] Mean ± SEM of four observations throughout.
[b] $p < .05$.

The above experiments were carried out in a glucose-free incubating medium. Addition of glucose to the medium increased the control but not the guanosine-stimulated palmitate incorporation (Fig. 3). Hence, the magnitude of guanosine-stimulation of palmitate esterification was diminished in the presence of glucose. A similar reduction in the stimulation by guanosine of palmitate incorporation was observed in adipose tissue of fasted–refed

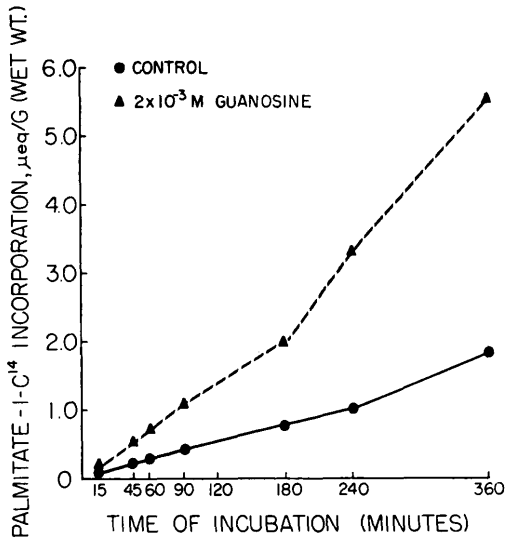

FIG. 2. Effect of length of incubation on palmitate-1-^{14}C incorporation into rat epididymal adipose tissue lipid and its response to guanosine. Each point is the mean of three observations.

rats. In the latter preparation there was a markedly increased control incorporation, resulting from the accumulation of glycogen (11) which is readily converted, upon incubation, to α-glycerophosphate, an essential requirement for fatty acid esterification in adipose tissue (12). An increased intracellular concentration of α-glycerophosphate, an essential requirement for fatty acid esterification in adipose tissue (12). An increased intracellular concentration of α-glycerophosphate would also account for the higher control incorporation observed in the presence of glucose. Control palmitate incorporation was the same in adipose tissue of fasted and *ad libitum*-fed rats, neither of which contains measurable amounts of glycogen (11) and, therefore, only suboptimal quantities of α-glycerophosphate. The slightly reduced guanosine effect in tissues of fasted as compared to *ad libitum*-fed rats is probably due to a partial depletion of enzymes involved in esterification. It is of interest that guanosine-stimulated values are similar for all three types of tissues as well as for tissues incubated in the presence of glucose. This suggests a maximal endogenous capacity for esterification at a given concentration of fatty acid substrate.

The relative ineffectiveness of guanosine in the presence of glucose was investigated by studying its action on glucose metabolism of adipose tissue. Both the oxidation of glucose-U-^{14}C and its conversion to lipid were inhibited by G, GMP, and dGMP; *i.e.*, the same compounds that enhance the esterification of palmitate. One notable exception was G2′, 3′ cP which enhanced glucose utilization as well as esterification. G3′ (2′)MP, again had no effect. The inhibition of glucose utilization by G, GMP, and dGMP would tend to reduce the amount of α-glycerophosphate and acyl CoA available for esterification, thus antagonizing their direct stimulatory action. Since both the oxidation of glucose and its conversion to lipid were inhibited by guanosine and its derivatives, it appears likely that these compounds exert their effect at some early step of glucose utilization such as transport or phosphorylation.

A physiological role of guanine metabolites in the regulation of triglyceride synthesis and plasma FFA levels appears doubtful in the normal carbohydrate-fed animal in which they have two opposing effects; namely, a glucose-independent stimulation of FFA esterification versus an inhibition of glucose utiliz-

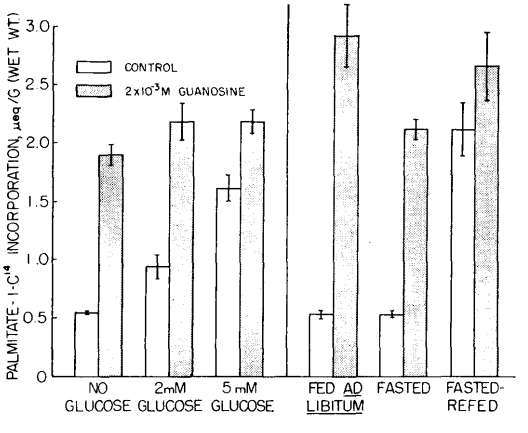

FIG. 3. Effect of the addition of glucose to the incubating medium and the nutritional state of the donor animal on the guanosine-stimulation of palmitate-1-^{14}C incorporation into epididymal adipose tissue lipid. Fasted animals used in the experiments shown on the left.

ation. The glucose-independent stimulation of FFA esterification, may, on the other hand be of importance in states of depressed glucose utilization; *i.e.,* diabetes or prolonged fast. In such states guanine metabolites would not further inhibit glucose utilization in adipose tissue but would accelerate FFA esterification from available substrate and, thereby, tend to lower the elevated plasma level of FFA. Such a mechanism would account for the reported *in vivo* FFA-lowering effect of G3′(2′)MP(4) which, while inactive *in vitro* may be converted to guanosine *in vivo* by liver 5′-nucleotidase (13).

Adenosine and its phosphate esters may be viewed as the more physiological regulators of triglyceride synthesis, insofar as they also stimulate glucose utilization in adipose tissue (2). Thus, while their glucose-independent stimulation of palmitate incorporation is not so great as that of the guanine metabolites, it would be further enhanced by the increased level of α-glycerophosphate.

No definite conclusion regarding the mode of action of guanine derivatives can be reached on the basis of the above results. An increased substrate concentration of either fatty acid or α-glycerophosphate can be definitely excluded. An increased permeability of the cell membrane to palmitate, increased *de novo* synthesis of enzymes, activation of enzymes already present or a co-factor role of guanosine are possibilities consistent with our findings.

The relatively high *in vitro* concentration of guanine derivatives necessary to achieve a stimulation of palmitate incorporation suggests a role as intracellular rather than circulating regulators.

Summary. The incorporation of palmitate-1-^{14}C into rat epididymal adipose tissue lipids was markedly enhanced by the *in vitro* addition of guanosine and its 5′-phosphate esters and to a lesser extent by dGMP, G 2′, 3′ cP, adenosine, uridine, and cytidine. Guanine and G3′(2′)MP were without effect. This stimulation of palmitate incorporation could not be duplicated by employing other known antilipolytic agents, such as nicotinic acid or PGE_1, and persisted under conditions where the specific activity of the incubating medium was kept constant, indicating that the stimulation obtained with guanosine and related compounds was not secondary to an inhibition of lipolysis in adipose tissue. The magnitude of the response to guanosine depended on the glucose concentration of the incubating medium and the nutritional state of the donor animal; little or no stimulation was obtained when esterification was already enhanced by the presence of glucose in the medium or when fat pads of fasted–refed rats were employed. Guanosine and its 5′-phosphate esters inhibited the oxidation of glucose-U-^{14}C as well as its conversion to lipid by epididymal adipose tissue.

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