assay is based on the property of double stranded circular phage λ DNA to be retained on nitrocellulose filters (6). That property was previously used to measure the activity of purified enzymes (7).

In this paper, we describe conditions which allow to measure the ligase activity in cell crude extracts, with a procedure with is fast, sensitive and quantitative. Using this assay, we show that the ligase activity is much more lower in resting lymphocytes than in actively growing cells.

Materials and Methods

Materials : The nitrocellulose filters (25 mm of diameter and 0.45 μm pore) were obtained from Schleicher & Schüll. Leupeptine and antipaine were from Peptide Institute Inc., Tokyo, Japan. Aprotinin was obtained from Laboratoires Choay, Paris, France.

Phage λ $[^{14}C]$DNA was prepared by thermal induction of *E.coli* 159 T^- (λ cIts857 S7). Thymine $[2-^{14}C]$ (specific activity : 19 mCi/mmole) was added immediately after induction. Bacteriophages were purified as described (8). After phenol extraction and dialysis against 2 M NaCl-10 mM Na_3-EDTA, the $[^{14}C]$ labelled DNA was disaggregated by heating at 75°C for 5 min., and cyclised at 50°C for 2 hr (8). Its specific activity is 4.5 mCi/mmole (nucleotides).

Cell cultures: Chinese Hamster Ovary (CHO) cells were grown in Dulbecco's medium supplemented with 5 % fetal calf serum and 5 % horse serum in a 5 % CO_2 humidified atmosphere. The doubling time was about 14 hr. Transformed lymphocytes, strain B Priess (transformed by Epstein Barr virus), were obtained from Dr T. Tursz (Institut Gustave-Roussy). They were grown in suspension in RPMI 1640 medium supplemented with 20 % fetal calf serum, 200 mM glutamine, 30 % glucose and 100 mM Na pyruvate. Their doubling time was about 24 hours.

Lymphocytes : Human peripheral blood lymphocytes were isolated from heparinized venous blood of healthy donors by centrifugation on a Ficoll-Paque (Pharmacia) gradient (9). Purity of the preparations was about 85 %. For stimulation, lymphocytes were suspended ($1X10^6$cells/ml) in RPMI 1640 medium supplemented with 20 % fetal calf serum, 2 mM glutamine and PHA (25 μl/ml of cell suspension). They were maintained at 37°C in a 5 % CO_2 humidified atmosphere. Stimulation was checked by incubating the lymphocytes with $[^3H]$ thymidine (2 μCi /culture) for 2 hours and measuring the amount of incorporated radioactivity.

Determination of ligase activity : Cells were suspended($2.5X10^6$cells/ml) in a buffer containing 20 mM Tris,HCl, 500 mM KCl, 2 mM DTT, pH 7.5. After sonication at 0°C (3 times, 15 sec), Triton X 100 (0,5 % final concentration) and 10 μg/ml of antipaine, leupeptine and aprotinin were added. Ligase reaction was carried out at 37°C in a medium containing 50 mM KCl - 10 mM $MgCl_2$ - 1 mM Na_3-EDTA - 50 mM Tris, HCl pH 7.5 -

cholera toxin but not by pertussis toxin (4). In mouse 3T3 fibroblasts there was an inhibition by 0.1 μM GppNHp of cholera toxin activated adenylate cyclase activity which was reversed by pertussis toxin (7). In contrast, Aktories et al. (8) reported that the inhibitory effect of GTP but not that due to GTPγS on adenylate cyclase activity of rat adipocyte ghosts incubated for 10 min at 25°C with 50 μM forskolin was reversed by pertussis toxin. Similar results were seen by Hildebrandt et al. (9) with respect to adenylate cyclase activity of cyc^- S49 lymphoma cells incubated with 10 mM Mn and 100 μM forskolin. The present results were designed to investigate the effects of pertussis toxin added directly to rat or rabbit adipocytes on GppNHp inhibition of adenylate cyclase activity.

Methods: Adipocytes were isolated by collagenase digestion (10) of the parametrial and omental adipose tissue from 175-200 g female Sprague-Dawley rats of the Charles River CD strain or the parametrial, omental and perirenal adipose tissue of young 3-4 kg female New Zealand white rabbits. The cells were isolated and incubated in Krebs-Ringer phosphate buffer containing 120 mM NaCl, 1.4 mM $CaCl_2$, 5.1 mM KCl, 1.5 mM $MgSO_4$, 10 mM Na_2HPO_4 (pH 7.4) plus 3% fatty acid free bovine albumin (CRG-7 from Armour). Pertussis toxin was purified from a pertussis vaccine concentrate generously provided by the National Institute of Hygiene of Mexico (Secretarià de Solubridad y Asistencia). Purification of the protein was by gel filtration followed by ion exchange chromatography. The material appeared to be purified about 1800-fold based on its ability to affect hamster adipocyte metabolism (2). Adipocytes were incubated for 4 hours in TCM 199 culture medium (Earle's salt, pH 7.4) plus 2% albumin with the gas phase 5% CO_2; 95% O_2 as described by Murayama and Ui (5). Approximately 10 ml of packed cells were incubated in the presence of 14 ml of medium without or with 2 μg/ml of toxin in 50 ml tubes placed on their side in a rotating water bath. The toxin was dissolved in 0.1 M phosphate buffer (pH 7.4) plus 2 M urea at a concentration of 2 mg/ml and added in a volume of 14 μl.

Adipocytes were homogenized using a motor driven Teflon pestle homogenizer with 10 up and down strokes in buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA and 0.25 M sucrose (one volume of packed cells to two volumes of buffer). The homogenates were then centrifuged at 240,000 g•min in an SS34 rotor of a Sorvall RC2B centrifuge. The pellet from this centrifugation was resuspended in homogenization buffer and centrifuged again, and used for assay of adenylate cyclase by a modification of the method of Cooper et al. (11) in a mixture containing 0.2 mM [^{32}P]ATP (50 cpm/pmol), 30 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM cyclic AMP, 0.1% bovine serum albumin, 1 mM creatine phosphate, creatine phosphokinase (10 units/ml), 1 μg/ml of adenosine deaminase, the indicated effectors, and membrane protein (5-10 μg) in a final volume of 100 μl. The incubation period was 6 min at 30°C. The reaction was terminated by the addition of 1 ml of 1% sodium dodecyl sulfate. ^{32}P-Labeled cyclic AMP was purified by sequential chromatography on

Dowex and alumina columns as described by Salomon (12). ^{3}H-Labeled cyclic AMP was added as a recovery standard. Protein was determined by the dye-binding procedure of Bio-Rad Laboratories (Richmond, CA).

(-)Isoproterenol, obtained from Sigma Chemical Company (St. Louis, MO); adenosine deaminase from calf intestine (200 U/mg), Boehringer-Mannheim (Indianapolis, IN); forskolin (7βacetoxy-8,13 epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one), Calbiochem (La Jolla, CA); crude collagenase (Clostridium histolyticum, Lot 4177 CLS11 40C190), Worthington Biochemicals (Freehold, NJ); bovine albumin (CRG-7), Armour Pharmaceutical Company (Chicago, IL); [α-^{32}P]ATP, and ^{3}H-labeled cyclic AMP, ICN (Irvine, CA); Dowex AG50-WX4 (200-400 mesh) and neutral alumina AG7 (100-200 mesh), Bio-Rad Laboratories (Richmond, CA) were used in these studies. Forskolin was dissolved in 95% ethanol at a concentration of 10 mM. This solution was diluted with water to give the desired concentration. Controls contained the same amount of added alcohol.

Results: The incubation of rat adipocytes for 4 hours with pertussis toxin (2 μg/ml) resulted in a 20% decrease in basal adenylate cyclase activity and converted 10 μM GTP from an inhibitor to an activator of basal as well as adenylate cyclase activity stimulated by 0.1 or 1 μM forskolin (Table 1). Similar results were seen with 10 μM GppNHp which inhibited basal adenylate cyclase activity as well as that due to 0.1 or 1 μM

Table 1
Reversal of GTP and GppNHp inhibition of adenylate cyclase activity by incubation of rat adipocytes with pertussis toxin.

Additions	Adenylate Cyclase Activity pmol/min/mg	% Inhibition (-) or Stimulation (+) due to	
		10 μM GTP	10 μM GppNHp
	Control Adipocyte Membranes		
None	71	- 53± 5	-42± 7
Forskolin 0.1 μM	301	- 70± 2	-72± 4
Forskolin 1 μM	778	- 69± 4	-77± 2
	Membranes from Pertussis Toxin-Treated Adipocytes		
None	57	+ 95±30	+39±18
Forskolin 0.1 μM	205	+135±20	- 2± 8
Forskolin 1 μM	645	+ 91±17	-38± 2

Rat adipocytes were incubated for 4 hrs without or with 2 μg/ml of pertussis toxin and the microsomal membranes isolated from the adipocyte homogenates were assayed for adenylate cyclase activity. The values are the means of 4 paired replications and the changes due to guanine nucleotide are the % inhibition (-) or % stimulation (+) as the means ± S.E.

forskolin. There was no inhibition by GppNHp of basal adenylate cyclase activity or that due to 0.1 μM forskolin after pertussis toxin treatment. However, in the presence of 1 μM forskolin only half of the inhibition due to GppNHp was reversed by pertussis toxin (Table 1).

In another series of experiments similar results were seen with 0.1 or 1.0 μM GppNHp (Table 2). The effects of 1 μM GppNHp were identical to those noted with 1 μM GTPγS (Table 2). In these experiments the inhibitory effects of GppNHp or GTPγS on basal adenylate cyclase activity as well as that seen in the presence of 0.1 μM forskolin were reversed by pertussis toxin. However, if the concentration of forskolin was raised to 1 or 10 μM the inhibition due to non-hydrolyzable guanine nucleotide analogs was reduced by 24-66% after pertussis toxin exposure.

Incubation of rabbit adipocytes for 4 hours with 2 μg/ml of pertussis toxin abolished the inhibition by 0.1 or 1 μM GppNHp of

Table 2
Effect of GTPγS and low concentrations of GppNHp on basal and forskolin stimulated rat adipocyte adenylate cyclase.

Additions	Adenylate cyclase activity pmol/min/mg	% inhibition (-) or % stimulation (+) of adenylate cyclase by 10μM GTP Exp. 1	10μM GTP Exp. 2	0.1μM GppNHp Exp. 1	0.1μM GppNHp Exp. 2	1μM GppNHp Exp. 1	1μM GppNHp Exp. 2	1μM GTPγS Exp. 1
				Control adipocyte membranes				
None	75	- 47	- 46	-38	-49	-40	-44	-33
Forskolin 0.1 μM	282	- 62	- 64	-50	-56	-67	-71	-70
Forskolin 1 μM	746	- 72	- 69	-62	-56	-75	-74	-78
Forskolin 10 μM	1385	- 70	- 67	-63	-57	-77	-75	-77
				Membranes from pertussis toxin-treated adipocytes				
None	68	+ 60	+ 64	+15	- 3	+25	+19	+10
Forskolin 0.1 μM	158	+112	+112	- 9	+63	-24	+46	- 6
Forskolin 1 μM	615	+113	+ 49	-15	-37	-35	-43	-25
Forskolin 10 μM	1220	+ 32	+ 39	-32	-25	-52	-41	-45

Rat adipocytes were incubated for 4 hrs without or with 2 μg/ml of pertussis toxin and the microsomal membranes were assayed for adenylate cyclase activity. The values are the means of 2 paired replications. The effects of the added guanine nucleotides are shown as % inhibition (- signs in front of the number) or % stimulation (+ signs in front of the numbers).

forskolin stimulated adenylate cyclase activity (Figure 1). In fact, GppNHp was now an activator of forskolin-stimulated adenylate cyclase activity.

Discussion: Inhibition by GppNHp of forskolin-stimulated rat adipocyte adenylate cyclase is a transient phenomena which only lasts 3-6 min at 30°C and is followed by an enhancement of adenylate cyclase activity (13). The lag period before GppNHp

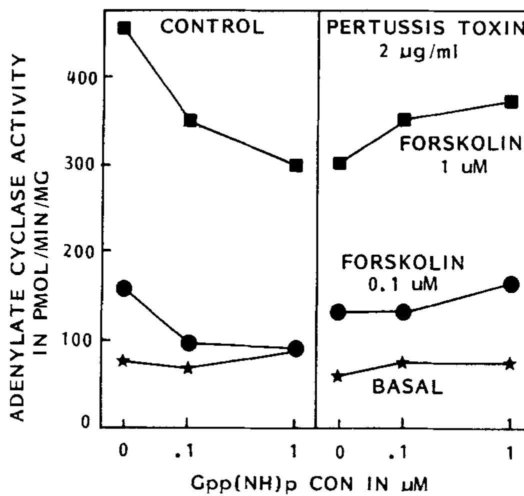

Figure 1
Reversal of inhibitory effect of GppNHp on adenylate cyclase activity after incubation of rabbit adipocytes with pertussis toxin.

Rabbit adipocytes were incubated for 4 hrs without or with 2 μg/ml of pertussis toxin. The microsomal fraction was used for assay of adenylate cyclase. The adenylate cyclase activity in the presence of 10 μM GTP plus 1 μM forskolin was 215 in membranes not exposed to toxin and 500 pmol of cyclic AMP formed per min per mg of protein in those exposed to pertussis toxin. The values are the means of three paired replicates for membranes incubated without (stars), with 0.1 μM forskolin (circles) or 1 μM forskolin (squares) and the indicated concentration of GppNHp.

activates adipocyte adenylate cyclase is abolished by cholera toxin or isoproterenol, but not pertussis toxin (4).

The present results indicate that pertussis toxin abolished the inhibition by GppNHp of basal adenylate cyclase activity as well as that due to a low concentration of forskolin when rat

adipocyte membranes were incubated for 6 min at 30°C. These results are comparable to those of Murayama et al. (8).

In contrast, no effect of pertussis toxin on GTPγS inhibition of rat adipocyte adenylate cyclase activity in the presence of 50 μM forskolin was noted by Aktories et al. (8). Our results also indicate that with a high level of adenylate cyclase activation by forskolin there is a component of GppNHp inhibition of adenylate cyclase activity which is unaffected by pertussis toxin. However, the present results indicate that non-hydrolyzable analogs of GTP have effects which are reversed by pertussis toxin.

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References

1. Gàrcia-Sàinz, J.A. (1981) FEBS Lett. 126, 306-308.
2. Martinez-Olmedo, M.A. and Garcia-Sainz, J.A. (1983) Biochim. Biophys. Acta, in press.
3. Moreno, F.J., Mills, I., Garcia-Sainz, J.A. and Fain, J.N. (1983) J. Biol. Chem., in press.
4. Murayama, T. and Ui, M. (1983) J. Biol. Chem. 258, 3319-3326.
5. Katada, K. and Ui, M. (1982) Proc. Natl. Acad. Sci. USA 79, 3129-3133.
6. Katada, K. and Ui, M. (1982) J. Biol. Chem. 257, 7210-7216.
7. Murayama, T., Katada, T. and Ui, M. (1983) Arch. Biochem. Biolphys. 221, 381-390.
8. Aktories, K., Schultz, G. and Jakobs, K.H. (1983) FEBS Lett. 156, 88-92.
9. Hildebrandt, J.D., Sekura, R.D., Codina, J., Iyengar, R., Manclark, C.R. and Birnbaumer, L. (1983) Nature 302, 706-709.
10. Rodbell, M. (1964) J. Biol. Chem. 239, 375-380.
11. Cooper, D.M.F., Schlegel, W., Lin, M.C. and Rodbell, M. (1979) J. Biol. Chem. 254, 8927-8931.
12. Salomon, Y. (1979) Adv. Cyclic Nucl. Res. 10, 35-55.
13. Hudson, T.H. and Fain, J.N. (1983) J. Biol. Chem. 258, 9755-9761.