

## Guanosine 5'-Triphosphate and Guanosine 5'-[ $\beta$ -Imido]triphosphate Effect a Collision Coupling Mechanism between the Glucagon Receptor and Catalytic Unit of Adenylate Cyclase

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1. GTP, but not p[NH]ppG (guanosine 5'-[ $\beta$ -imido]triphosphate), abolishes the sensitivity of glucagon-stimulated adenylate cyclase to the lipid-phase separations occurring in the outer half of the bilayer in liver plasma membranes from rat. 2. When either GTP or p[NH]ppG alone stimulate adenylate cyclase, the enzyme senses only those lipid-phase separations occurring in the inner half of the bilayer. 3. Trypsin treatment of intact hepatocytes has no effect on the basal, fluoride-, GTP- or p[NH]ppG-stimulated adenylate cyclase activity. However,  $^{125}$ I-labelled-glucagon specific binding decays with a half-life matching that of the decay of glucagon-stimulated adenylate cyclase activity. 4. When GTP or p[NH]ppG are added to assays of glucagon-stimulated activity, the half-life of the trypsin-mediated decay of activity is substantially increased and the decay plots are no longer first-order. 5. Trypsin treatment of purified rat liver plasma membranes abolishes basal and all ligand-stimulated adenylate cyclase activity, and  $^{125}$ I-labelled-glucagon specific binding. 6. Benzyl alcohol activates the GTP- and p[NH]ppG-stimulated activities in an identical fashion, whereas these activities are affected differently when glucagon is present in the assays. 7. We suggest that guanine nucleotides alter the mode of coupling between the receptor and catalytic unit. In the presence of glucagon and GTP, a complex of receptor, catalytic unit and nucleotide regulatory protein occurs as a transient intermediate, releasing a free unstable active catalytic unit. In the presence of p[NH]ppG and glucagon, the transient complex yields a relatively stable complex of the catalytic unit associated with a p[NH]ppG-bound nucleotide-regulatory protein.

Glucagon binds to specific receptors on the external surface of the plasma membrane, leading to the activation of adenylate cyclase on the cytosol surface of the membrane (Birnbaumer, 1973).

We have been able to demonstrate that, in the absence of glucagon, the receptor and catalytic unit of adenylate cyclase are free to migrate laterally in the plane of the bilayer. Upon addition of hormone, the glucagon-receptor complex seeks out and locks on to the catalytic unit of adenylate cyclase, activating it, and forming a multicomponent complex spanning the bilayer membrane (Houslay *et al.*, 1977). This has received strong support from recent experiments in which liver plasma membranes, con-

taining inactivated adenylate cyclase units and functional glucagon receptors, were fused with Freund erythroleukaemia cells possessing active adenylate cyclase but no glucagon receptors. After fusion the active adenylate cyclase could be stimulated by glucagon (Schramm, 1979). This is presumably due to the ability of these components to migrate laterally and interact in the fused-membrane systems.

Evidence has accrued demonstrating in liver plasma membranes from rat and hamster that the uncoupled catalytic unit of adenylate cyclase (i.e. in the absence of glucagon) is only sensitive to lipid-phase separations in the inner half of the bilayer. The coupled activity, in the presence of glucagon, where the receptor and catalytic unit form a multicomponent complex spanning the membrane is sensitive to lipid-phase separations in both bilayer halves

Abbreviation used: p[NH]ppG. guanosine 5'-[ $\beta$ -imido]triphosphate.

(Houslay, 1979; Houslay & Palmer, 1978). Indeed hamster liver cells have been shown to possess the ability to manipulate independently the fluidity of the bilayer halves (Houslay & Palmer, 1978) and this may serve to regulate selectively membrane functions that are asymmetrically disposed in the bilayer.

GTP is believed to be an important physiological modulator of adenylate cyclase activity, being able to activate both the coupled and basal (uncoupled) states of the enzyme (Rodbell *et al.*, 1975). This report investigates the effect that this nucleotide and its analogue, p[NH]ppG, have on the sensitivity of the uncoupled and coupled activities to lipid-phase separations and changes in bilayer fluidity, effected by the neutral local anaesthetic, benzyl alcohol.

### Materials and Methods

Adenylate cyclase assays were carried out as described in detail previously with full precautions to assess linearity in experiments, to keep the pH constant and substrates and ligands saturating (Houslay *et al.*, 1976*a,b*). Arrhenius-plot data were treated as before by least-squares analysis (Houslay & Palmer, 1978). There were no detectable permeability barriers in the membrane preparations (Houslay & Palmer, 1978, 1979). One unit of activity is defined as the transformation of 1  $\mu$ mol of substrate/min.

The preparation of  $^{125}$ I-labelled glucagon and its use in specific binding assays were as described in detail previously (Houslay *et al.*, 1977; Houslay & Palmer, 1979).

Plasma membranes were prepared from the livers of male Sprague-Dawley rats (200–300 g) by a modification (Houslay *et al.*, 1976*a*) of the method of Pilkis *et al.* (1974).

Hepatocytes were isolated from male Sprague-Dawley rats (200–300 g; 24 h starved) and shown to be intact and viable as described previously (Elliott *et al.*, 1976; Craik & Elliott, 1979). Before trypsin treatment cells were resuspended at 3–5 mg dry wt./ml in the presence of 10 mM-lactate under conditions described previously (Smith *et al.*, 1978; Houslay & Elliott, 1979), and preincubated for 15 min at 37°C. Trypsin was used at a final concentration of either 0.1 mg/ml or 0.3 mg/ml in the following procedure. To a 24 ml cell suspension was added 1 ml of trypsin in buffer (Krebs & Henseleit, 1932) containing 2% (w/v) bovine serum albumin, with immediate shaking. The vessels were regularly gassed (every 5 min) with O<sub>2</sub>/CO<sub>2</sub> (19:1), which was essential to ensure a stable adenylate cyclase activity (Houslay & Elliott, 1979). At appropriate time intervals 2.8 ml samples were taken and immediately mixed with 1 ml of ice-cold 1 mM-KHCO<sub>3</sub>, pH 7.2, containing a 50% excess of trypsin inhibi-

tor. The cells were kept for 2–4 min on ice before centrifugation for 10 min at 2500  $g_{av}$  in an MSE Super Minor bench centrifuge at 4°C. Supernatant fluid was removed and the cells were resuspended in 1.5 ml of 1 mM-KHCO<sub>3</sub>, pH 7.2. They were then broken by repeated syringing (Houslay & Elliott, 1979) before centrifugation for 8 min at 14 000  $g_{av}$  in a Jobling 320 microfuge at 4°C. The pellet was resuspended in 0.25 ml of 1 mM-KHCO<sub>3</sub>, pH 7.2, and assays were then performed within 2 h.

For treatment of purified rat liver plasma membranes with trypsin, membranes (2.1 mg of protein/ml, final concentration) were preincubated for 15 min at 10°C in 1 mM-KHCO<sub>3</sub>, pH 7.2, before addition of trypsin (final concentration, 0.064 mg/ml). At time intervals of 0, 4, 8, 12, 16 and 20 min portions of 300  $\mu$ l were removed and vortex-mixed with 150  $\mu$ l of trypsin inhibitor (0.32 mg/ml) (150% excess) in ice-cold 1 mM-KHCO<sub>3</sub>, pH 7.2. Assays were then performed within 1 h. In control experiments in the absence of trypsin it was ascertained that the basal and all ligand-stimulated adenylate cyclase activities remained constant over this time period, as did  $^{125}$ I-labelled-glucagon specific binding.

Membranes were treated with 0.4 M-NaCl in a 'cocktail' containing (final concentrations) 20 mM-2-mercaptoethanol, 40 mM-Tris/HCl, final pH 7.4, and 3 mg of plasma membranes/ml. They were then incubated for 45 min at 0°C before being centrifuged at 300 000  $g_{av}$  for 30 min. The pelleted material was resuspended in 1 mM-KHCO<sub>3</sub>, pH 7.2, and washed twice with 1 ml of the same by centrifugation at 14 000  $g_{av}$  for 5 min at 4°C, before final resuspension in the same buffer.

Protein concentration was determined by a modification (Houslay & Palmer, 1978) of the method of Goa (1953).

Glucagon was a kind gift from Dr. W. W. Bromer of Eli Lilly and Co., Indianapolis, IN, U.S.A. Lactate, phosphocreatine, creatine phosphokinase, trypsin (type III from bovine pancreas) and trypsin inhibitor (type 1-S from soya bean) were from Sigma, Kingston upon Thames, Surrey, U.K. Cyclic AMP, ATP, GTP (sodium salt), p[NH]ppG, triethanolamine hydrochloride and collagenase were from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Benzyl alcohol was from Hopkin and Williams, Chadwell Heath, Essex, U.K. All other chemicals were of AnalaR quality from BDH Chemicals, Poole, Dorset, U.K.

### Results

*Effect of guanine nucleotides on the glucagon-stimulated adenylate cyclase activity of rat and hamster liver plasma membranes.*

The addition of GTP (100  $\mu$ M) to assays of the glucagon-stimulated activity of rat liver plasma

membranes caused a dramatic change in the form of the Arrhenius plots. These normally exhibited a well-defined break at around 28°C (Houslay *et al.*, 1976a), but the presence of GTP abolished this, yielding a linear plot (Fig. 1, Table 1). The activation caused by GTP is most marked at temperatures greater than 28°C, and negligible around 4°C (Fig. 1). This situation contrasts strongly with that found if the analogue, p[NH]ppG (100 μM), is added to assays of glucagon-stimulated activity. Arrhenius plots in this instance remain non-linear with a well-defined break at around 28°C (Fig. 1).

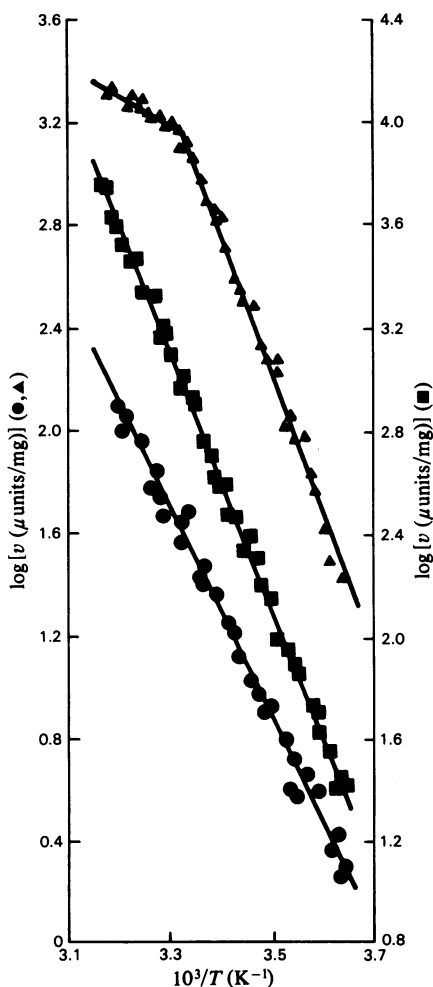


Fig. 1. Effects of GTP and p[NH]ppG on Arrhenius plots of rat liver plasma-membrane adenylate cyclase activity in the coupled and uncoupled states

▲, Glucagon (1 μM) + p[NH]ppG (100 μM); ■, glucagon (1 μM) + GTP (100 μM); ●, GTP (100 μM) alone. Note that the two y-axes have the same units, but cover a slightly different range to separate out the results for the glucagon-plus-p[NH]ppG-stimulated and glucagon-plus-GTP-stimulated activities.

Table 1. Forms of the Arrhenius plots of GTP- and p[NH]ppG-stimulated adenylate cyclase activity in the presence and absence of glucagon. Errors in parentheses are 95% confidence limits, and others are ±s.d. (see Houslay & Palmer, 1978).

Animal source	Ligand	No. of plots	Break point (°C)		Activation energy (kJ/mol)	
			Upper	Lower	Above upper break	Below upper break
Rat	GTP	3	—	—	—	—
	GTP + glucagon	6	—	—	79.4 ± 10.5	—
	p[NH]ppG (from Houslay <i>et al.</i> , 1976a)	3	—	—	80.2 ± 6.3	—
	p[NH]ppG + glucagon	4	28.5 (27.1–29.6)	—	—	93.7 (89.5–98.5)
Hamster	GTP	3	26.0 (24.5–27.5)	—	51.2 (46.2–56.8)	71.8 (65.3–77.2)
	GTP + glucagon	3	26.1 (25.2–27.1)	—	76.0 (73.2–79.2)	63.4 (60.4–66.0)
	p[NH]ppG	4	25.7 (25.1–26.8)	—	64.3 (60.3–68.3)	110.5 (100.0–115.5)
	p[NH]ppG + glucagon	3	26.1 (25.4–28.9)	12.5 (10.4–14.3)	80.7 (78.1–84.0)	62.5 (60.2–63.0)

The effect with p[NH]ppG on glucagon-stimulated activity confirms our earlier observation (Houslay *et al.*, 1976a). However, in the study of Houslay *et al.* (1976a), negligible stimulation of the glucagon-stimulated activity could be achieved with p[NH]ppG. In our present investigations, with a wide variety of membrane preparations, we could reproducibly achieve a 2.76 ( $\pm 0.66$ )-fold activation ( $n = 15$ ) of the glucagon-stimulated activity with p[NH]ppG at 30°C. Time courses for such experiments were linear over 20 min, with no perceptible lag time ( $< 1$  min). We are at a loss to explain such a discrepancy unless those early batches of p[NH]ppG contained a contaminant.

When GTP (100  $\mu\text{M}$ ) alone (Fig. 1 and Table 1) or p[NH]ppG (100  $\mu\text{M}$ ) alone (Houslay *et al.*, 1976a) were present, Arrhenius plots of adenylate cyclase activity were linear over the entire temperature range.

Arrhenius plots of the glucagon-stimulated adenylate cyclase activity of hamster liver plasma membranes exhibit two well-defined breaks at 28 and 13°C (Houslay & Palmer, 1978). The addition of GTP to these assays abolishes the 13°C break, and leaves a single break occurring at 26°C (Fig. 2 and Table 1). Interestingly the activation energy for the reaction increases above 26°C, whereas in the absence of GTP it actually decreases (Fig. 2 and Table 1; Houslay & Palmer, 1978).

The addition of p[NH]ppG to assays of the glucagon-stimulated adenylate cyclase yielded Arrhenius plots with two well-defined break points at 26 and 13°C (Fig. 2 and Table 1). In contrast with the Arrhenius plot obtained when glucagon alone was present, the activation energy for the reaction was actually greater at temperatures above 26°C than it was immediately below (Fig. 2 and Table 1). This situation parallels that when GTP was added to such assays.

Arrhenius plots of the adenylate cyclase activity of hamster liver plasma membranes, in the presence of either GTP or p[NH]ppG alone, exhibited a single break at 26°C, with activation energies above 26°C being less than that exhibited below (Fig. 2 and Table 1).

Extreme care was taken to assess the presence of significant lag times for the onset of p[NH]ppG activation. For example, 20-point time courses over 28 min at 4°C failed to indicate lag times greater than 1 min, which would be in accord with other workers (Rodbell *et al.*, 1975) for this system.

#### *Ligand-stimulated adenylate cyclase activity of trypsin-treated intact hepatocytes*

Isolated hepatocytes possess adenylate cyclase activity that can be stimulated by a number of ligands: GTP (100  $\mu\text{M}$ ) 2.25 ( $\pm 0.2$ )-fold; p[NH]ppG (100  $\mu\text{M}$ ) 3.3 ( $\pm 0.6$ )-fold; NaF (15 mM) 15.2

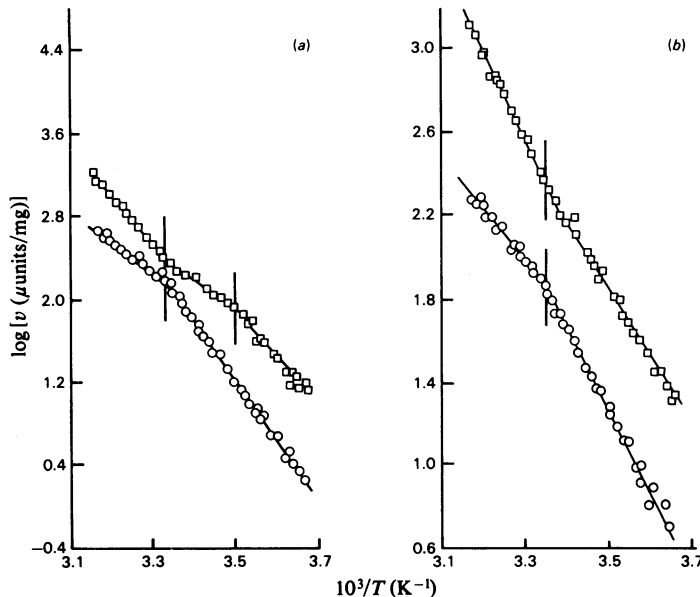


Fig. 2. Effects of GTP and p[NH]ppG on Arrhenius plots of hamster liver plasma-membrane adenylate cyclase activity in the coupled and uncoupled states

(a) Glucagon (1  $\mu\text{M}$ ) + p[NH]ppG (100  $\mu\text{M}$ ) ( $\square$ ) and p[NH]ppG (100  $\mu\text{M}$ ) alone ( $\circ$ ). (b) Glucagon (1  $\mu\text{M}$ ) + GTP (100  $\mu\text{M}$ ) ( $\square$ ) and GTP (100  $\mu\text{M}$ ) alone ( $\circ$ ).

Table 2. Decay of adenylate cyclase activity in rat liver plasma membranes treated with trypsin

Half-lives for trypsin-mediated losses of ligand-stimulated adenylate cyclase activity in rat liver plasma membranes are given as a ratio of the loss of glucagon-stimulated activity, ( $7.7 \pm 2.0$  min) at  $10^\circ\text{C}$ . Half-lives were estimated from the slopes of regression plots of time courses of inactivation (see the Materials and Methods section). Four separate experiments were performed on three plasma-membrane preparations. Errors are  $\pm$  s.e.m. ( $n = 4$ )

Activity	Ratio of half-lives
Glucagon-stimulated adenylate cyclase (GSAC)	(1)
$^{125}\text{I}$ -labelled-glucagon specific binding	$1.05 \pm 0.35$
Glucagon-plus-GTP-stimulated adenylate cyclase (GSAC + GTP)	$1.21 \pm 0.15$
Glucagon-plus-p[NH]ppG-stimulated adenylate cyclase (GSAC + p[NH]ppG)	$1.24 \pm 0.10$
GTP-stimulated adenylate cyclase (GTP)	$1.19 \pm 0.04$
p[NH]ppG-stimulated adenylate cyclase (p[NH]ppG)	$1.13 \pm 0.06$
Fluoride-stimulated adenylate cyclase	$1.29 \pm 0.12$
Basal adenylate cyclase (B)	$1.62 \pm 0.12$
GSAC - B	$1.05 \pm 0.04$
(GSAC + GTP) - GTP	$1.16 \pm 0.10$
(GSAC + p[NH]ppG) - p[NH]ppG	$1.15 \pm 0.06$

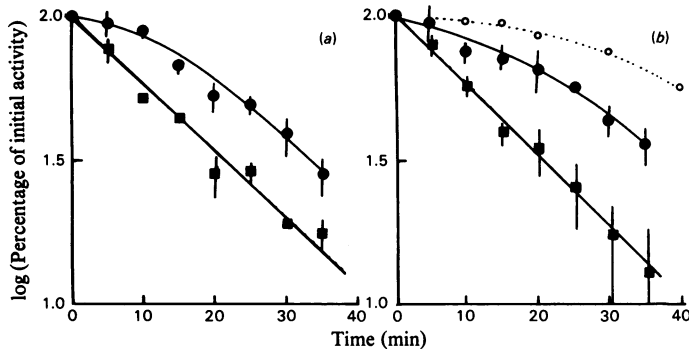


Fig. 3. Trypsin-mediated loss in the glucagon-binding and glucagon-stimulated adenylate cyclase activities of intact rat hepatocytes

(a) Glucagon-stimulated adenylate cyclase activity (■) and GTP-plus-glucagon-stimulated adenylate cyclase activity (●). (b)  $^{125}\text{I}$ -labelled-glucagon specific binding (■) and p[NH]ppG-plus-glucagon-stimulated adenylate cyclase activity (●). The upper broken curve is the decay estimated from tangents to the curves found in Fig. 4(c). See the Results section. Unless otherwise specified the activities given were derived from the amount of cyclic AMP produced in 10 min. Assays were in duplicate, and these data are combined from two experiments with errors as  $\pm$  s.d. The concentration of glucagon was  $1 \mu\text{M}$  and that of the guanine nucleotides  $100 \mu\text{M}$ .

( $\pm 2$ )-fold; glucagon ( $1 \mu\text{M}$ )  $22.2 (\pm 4.2)$ -fold; glucagon ( $1 \mu\text{M}$ ) plus GTP ( $100 \mu\text{M}$ )  $28.9 (\pm 1.8)$ -fold and glucagon ( $1 \mu\text{M}$ ) plus p[NH]ppG ( $100 \mu\text{M}$ )  $44.1 (\pm 6.8)$ -fold; three preparations were assayed for adenylate cyclase in duplicate in each case (errors are  $\pm$  s.e.m. with  $n = 3$ ).

Treatment of intact hepatocytes with trypsin at either  $0.1 \text{ mg/ml}$  or  $0.3 \text{ mg/ml}$  under the conditions described in the Materials and Methods section had no significant effect on the basal, fluoride-, GTP- and p[NH]ppG-stimulated activities. These all remained within 10% of their original value over the time course of the experiment (estimated half-lives  $> 100$  min).

This was not found to be true for either  $^{125}\text{I}$ -labelled-glucagon specific binding to the glucagon receptor or glucagon-stimulated adenylate cyclase activities (Fig. 3), which all progressively decayed in the presence of trypsin.  $^{125}\text{I}$ -labelled-glucagon specific binding to receptors and the glucagon-stimulated activity decayed exponentially, producing linear semi-log plots over a 90% decay of binding or activity, exhibiting identical half-lives (Fig. 3).

The addition of GTP or p[NH]ppG had a profound effect on the form of these plots. The decay of activity now appeared not to be first-order, but the semi-log plots were displaced to the right and curve concave downwards (Fig. 3). This gave an apparent

reduced sensitivity to trypsin action, with double the estimated half-life of the glucagon-stimulated activity. Subtraction of the rates of p[NH]ppG-stimulated activity alone from those of adenylate cyclase activity in the presence of glucagon and p[NH]ppG had little effect on the form of the plots. The same was true for GTP and GTP plus glucagon. This may well be because the activity in the presence of GTP or p[NH]ppG alone is very small relative to that when glucagon is present also. The ratio of the half-lives for decay of these activities (shown in Fig. 3) relative to glucagon-stimulated adenylate cyclase were;  $^{125}\text{I}$ -labelled-glucagon specific binding,  $1.01 \pm 0.03$  (4); GTP + glucagon-stimulated adenylate cyclase  $1.92 \pm 0.15$  (8); p[NH]ppG + glucagon-stimulated adenylate cyclase  $2.57 \pm 0.32$  (6) or  $3.96 \pm 0.4$  for the data from the broken-line plot (see Fig. 3b for details). In these cases errors are given as  $\pm$ S.E.M. with the numbers of separate experiments in parentheses. For each individual experiment samples were taken at eight time points for duplicate assays of all the activities tested. The decay of glucagon-stimulated adenylate cyclase activity exhibited a half-life of  $16.8 \pm 2.3$  min at 0.1 mg of trypsin/ml and  $6.4 \pm 1.0$  min at 0.3 mg of trypsin/ml. When trypsin was not present, all these activities remained stable over the time course of the experiment.

Estimations of adenylate cyclase activity in these experiments were achieved by a 10 min stopped assay for comparative purposes. However, a critical investigation of the linearity of cyclic AMP production by the membrane fraction of trypsin-treated hepatocytes was also undertaken. Fig. 4 details a

typical experiment where cells were treated for various time periods with 0.3 mg of trypsin/ml. These samples were then assayed for glucagon-stimulated adenylate cyclase activity in the presence or absence of either GTP or p[NH]ppG. In all cases the control assays yielded linear time courses over a period of 18 min. Progressive exposure to trypsin under these conditions caused a loss in ligand-stimulated activity as detailed above, and the rates of cyclic AMP production in the presence of glucagon alone or glucagon plus GTP were linear in both cases (Figs. 4a and 4b). This was clearly not true when glucagon plus p[NH]ppG were the stimulating ligands, for, as the time of exposure to trypsin progressed, the rates of cyclic AMP production in the isolated membrane fraction became increasingly non-linear (Fig. 4c), accelerating over the time course. Clearly our comparative exercise in Fig. 3 underestimates considerably the true potential activity in the case of residual activity able to be stimulated by glucagon plus p[NH]ppG. Thus we expect that the curves in Fig. 3 would be in reality displaced further to the right, and of a shallower gradient (see Fig. 3).

#### *Treatment of purified rat liver plasma membranes with trypsin*

Trypsin treatment of purified rat liver plasma membranes caused the decay of all ligand-stimulated adenylate cyclase activities as well as basal activity (Table 2). The decays of basal, fluoride-, GTP- and p[NH]ppG-stimulated adenylate cyclase activities were first-order and under the conditions

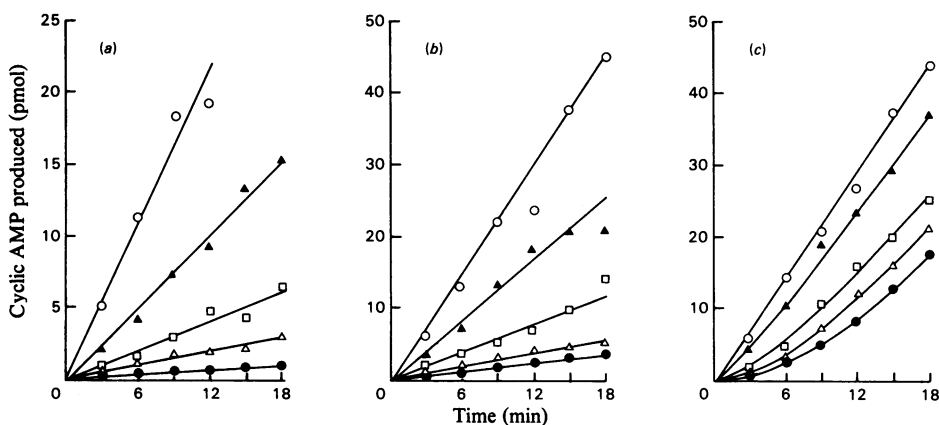


Fig. 4. Time courses of ligand-stimulated cyclic AMP production by a membrane fraction from trypsin-treated rat hepatocytes

Rat hepatocytes were treated with 0.3 mg of trypsin/ml at  $37^\circ\text{C}$  as detailed in the Materials and Methods section for zero (○), 10 min (▲), 20 min (□), 30 min (△) and 40 min (●). Membranes were then used for adenylate cyclase assay in the presence of (a) glucagon ( $1\ \mu\text{M}$ ) alone, (b) glucagon ( $1\ \mu\text{M}$ ) + GTP ( $100\ \mu\text{M}$ ) and (c) glucagon ( $1\ \mu\text{M}$ ) + p[NH]ppG ( $100\ \mu\text{M}$ ). The amount of cyclic AMP produced in a  $50\ \mu\text{l}$  sample from the assay 'cocktail' (see Houslay *et al.*, 1976a) at various time intervals is given.

used exhibited similar half-lives (Table 2). Greater than 95% of the activity was abolished during the incubation. The decays of  $^{125}\text{I}$ -labelled-glucagon specific binding and glucagon-stimulated adenylate cyclase activity were first-order, exhibiting similar half-lives which were shorter than those of the basal activity (Table 2). When GTP or p[NH]ppG was included in assays of glucagon-stimulated adenylate cyclase, the plots were apparently linear, with half-lives of a slightly longer duration than those found with glucagon alone in the assays (Table 2).

*Action of benzyl alcohol on guanine nucleotide-stimulated adenylate cyclase*

The neutral local anaesthetic benzyl alcohol augmented adenylate cyclase activity in the presence of guanine nucleotides irrespective of whether glucagon was present or not (Figs. 5a and 5b). Maximum stimulation was seen at concentrations of benzyl alcohol between 50 and 70 mM, above which these activities were progressively inhibited by further additions of benzyl alcohol. Such changes in activity were reversed upon washing or dilution.

The concentration-dependent effects of benzyl alcohol on adenylate cyclase activity in the presence of GTP or p[NH]ppG alone were effectively identical (Fig. 5a), achieving a 2.7-fold activation at 50 mM-benzyl alcohol. This was not found to be the case for these ligands when glucagon was also present in the assays. In this instance the p[NH]ppG-plus-

glucagon-stimulated activity was activated some 2.5-fold in the presence of 60 mM-benzyl alcohol, whereas that of GTP plus glucagon was only raised 1.6-fold (Fig. 5b). The sensitivity of glucagon-stimulated activity alone (Dipple & Houslay, 1978) was very different from when guanine nucleotides were also present in the assay (Fig. 5b).

*Treatment of purified rat liver plasma membranes with high NaCl concentrations*

Treatment of rat liver plasma membranes with high NaCl concentrations (0.4 M) sufficient to release peripheral proteins from these membranes (R. J. Marchmont & M. D. Houslay, unpublished observations) had no significant effect on the ability of GTP or p[NH]ppG to augment the glucagon-stimulated adenylate cyclase activity of the preparation. In native membranes GTP (100  $\mu\text{M}$ ) enhanced the glucagon-stimulated activity 2.36 ( $\pm 0.69$ )-fold ( $n = 12$ ) and in NaCl-treated membranes 2.56 ( $\pm 0.19$ )-fold ( $n = 4$ ). In native membranes p[NH]ppG (100  $\mu\text{M}$ ) enhanced the glucagon-stimulated activity some 2.76 ( $\pm 0.66$ )-fold ( $n = 15$ ) and in NaCl-treated membranes 2.81 ( $\pm 0.33$ )-fold ( $n = 4$ ). Glucagon (1  $\mu\text{M}$ ) stimulated the basal rate some 15–30-fold, and in these experiments we used eight different membrane preparations for assessing the native activity, and two for the NaCl-treated activity. The errors are given as  $\pm$ s.d., with the numbers of individual experiments in parentheses.

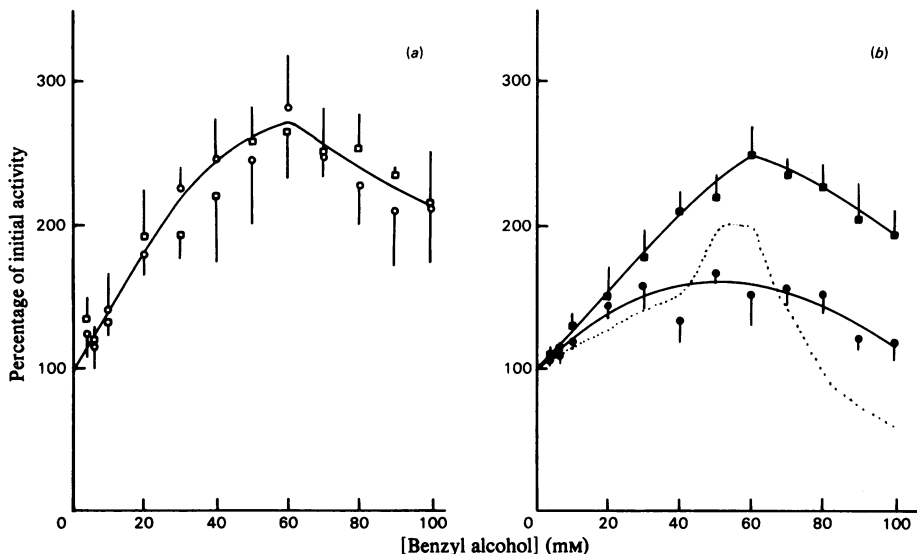


Fig. 5. Augmentation by benzyl alcohol of GTP- and p[NH]ppG-stimulated adenylate cyclase activity in the coupled and uncoupled states

Rat liver plasma membranes were used as before (Dipple & Houslay, 1978). (a) GTP (100  $\mu\text{M}$ ) alone (O); p[NH]ppG (100  $\mu\text{M}$ ) alone (□). (b) GTP (100  $\mu\text{M}$ ) + glucagon (1  $\mu\text{M}$ ) (●); p[NH]ppG (100  $\mu\text{M}$ ) + glucagon (1  $\mu\text{M}$ ) (■). The broken line is the result obtained with glucagon alone (from Dipple & Houslay, 1978).

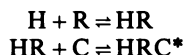
## Discussion

In rat liver plasma membranes a lipid-phase separation occurs at 28°C (Houslay *et al.*, 1976a; Sauerheber *et al.*, 1977), which is apparently localized in the external half of the bilayer (Houslay *et al.*, 1976c; Houslay, 1979). This lipid-phase separation is only experienced by adenylate cyclase when it is physically coupled to the glucagon receptor, an event that is achieved by glucagon itself or its antagonist des-1-histidine-glucagon (Houslay *et al.*, 1977). When the catalytic unit of adenylate cyclase is not associated with the receptor, as with the various uncoupled activities of adenylate cyclase, then it only experiences lipid-phase separations that occur in the inner half of the bilayer. This is amply demonstrated in hamster liver plasma membranes, where a phase separation occurs at 26°C in the inner half of the bilayer and at 13°C in the outer half of the bilayer (Houslay & Palmer, 1978).

Our results (Figs. 1 and 2) with Arrhenius plots of GTP- and p[NH]ppG-stimulated adenylate cyclase activity support this, as they are linear for rat liver plasma membranes and exhibit a single break at 26°C for hamster liver plasma membranes. It is believed that these guanine nucleotides regulate basal activity by either direct action on the catalytic unit or through a nucleotide-regulatory protein associated with it (Welton *et al.*, 1977; Lad *et al.*, 1977; Iyengar *et al.*, 1979a). We presume then that these units are localized in the inner half of the bilayer.

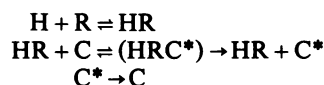
When GTP is added to assays of glucagon-stimulated adenylate cyclase, Arrhenius plots of this activity clearly demonstrate the loss of sensitivity to the lipid-phase separation that occurs in the outer half of the bilayer (Figs. 1 and 2 and Table 1). This could be due to a 'weakening' of the physical interaction between the receptor and catalytic unit achieved by GTP as it enhances enzyme activity. Alternatively we would like to suggest that it reflects a change in the mode of coupling between the receptor and catalytic unit.

The mobile receptor model, obeyed in the presence of glucagon alone (Houslay *et al.*, 1977), can be represented schematically as:



where H is glucagon, R is the glucagon receptor, C is the catalytic unit in its basal state and HRC\* is the active multicomponent complex spanning the bilayer that accumulates as a consequence of glucagon binding to the receptor. We suggest that in the presence of GTP this mechanism changes to a collision-coupling mechanism similar to the type proposed for the activation of turkey erythrocyte adenylate cyclase by  $\beta$ -receptors in the presence of p[NH]ppG (Tolkovsky & Levitzki, 1978a,b). In this

case we envisage that the mechanism may be represented by:



where the active complex (HRC\*) does not accumulate as the end product of the sequence, but occurs as a transient intermediate rapidly giving rise to C\*, a free active form of the catalytic unit. The activity of C\* then decays to the basal-level C. Arrhenius plots of the activity of C\* which would be produced in the presence of glucagon plus GTP would then only reflect those lipid-phase separations occurring in the inner half of the bilayer.

If this model is obeyed, then an interesting consequence is that a single occupied receptor (HR) could, within its lifetime, activate more than one catalytic unit (see Tolkovsky & Levitzki, 1978a,b). This contrasts with the mobile receptor model where a single occupied receptor can activate but a single catalytic unit. A way of testing such an hypothesis would be to alter the R:C stoichiometry by reducing the concentration of glucagon receptors.

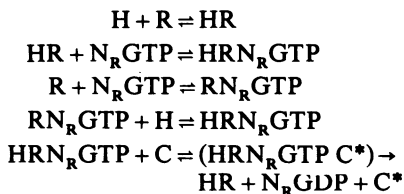
Trypsin has been used to demonstrate the asymmetry of the glucagon receptor and catalytic unit of adenylate cyclase in intact fat-cells (see Birnbaumer, 1973). In this work we have demonstrated quite clearly that in intact rat hepatocytes, basal adenylate cyclase activity and that stimulated by fluoride, GTP and p[NH]ppG are quite unaffected by externally applied trypsin under conditions where <sup>125</sup>I-labelled-glucagon specific binding and glucagon-stimulated adenylate cyclase activity is abolished. However, when a plasma-membrane preparation is used that has no permeability barriers (Houslay & Palmer, 1979), then all of these activities, as well as binding, are lost, indicating their susceptibility to degradation by trypsin. This demonstrates the asymmetric location of these functions and that we can lower glucagon receptor concentration without affecting the catalytic unit concentration.

As would be predicted from the mobile receptor model, the decay of <sup>125</sup>I-labelled-glucagon specific binding parallels the decay of glucagon-stimulated adenylate cyclase activity under saturating glucagon conditions (Fig. 3). The linear decay plot of <sup>125</sup>I-labelled-glucagon specific binding suggests that, if spare receptors do exist (see Houslay *et al.*, 1977), then they are similarly sensitive to trypsin treatment. However, if GTP is added to assays of glucagon-stimulated adenylate cyclase activity, then the half-life for decay is dramatically increased by almost 2-fold (Fig. 3). This apparent reduced sensitivity to trypsin treatment is most readily explained by the proposed collision-coupling mechanism, where a single occupied receptor can activate more than one catalytic unit. Indeed one would predict that the



decay plot should remain horizontal (at the 100% level) before decreasing with a slope equal to that of the decay of <sup>125</sup>I-labelled-glucagon specific binding. The width of this horizontal section would depend upon the number of catalytic units that a single occupied receptor could activate and upon the initial concentration ratio of receptors and catalytic units. Clearly the number of catalytic units able to be activated by a single occupied receptor depends on a number of functions, such as the dissociation constant for the glucagon-receptor complex, the lifetime of the activated catalytic unit, lateral diffusion constants and the possibility that the occupied receptor may be released from (HRC\*) in a desensitized state. That a curve is obtained rather than a horizontal section followed by a linear decay is undoubtedly due to the fact that the dissociation constant for HR + C ⇌ (HRC\*) is finite, and influenced by a decrease in receptor concentration.

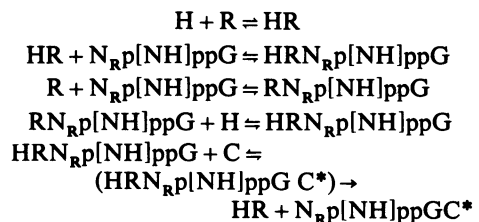
Welton *et al.* (1977) and Iyengar *et al.* (1979a) have produced strong evidence to support the existence of physically distinct guanine nucleotide regulatory sites. One appears to be associated with the catalytic unit and be responsible for modulating the basal activity, and a second separate protein is responsible for modulating coupling. We can, in the presence of GTP, envisage some (or all) of the following equilibria to ensue:



where N<sub>R</sub> is the guanine nucleotide-regulating unit affecting coupling, and (HRN<sub>R</sub>GTP C\*) does not accumulate, but is a transient intermediate. Presumably the breakdown of (HRN<sub>R</sub>GTP C\*) to its constituents would lead to the hydrolysis of the associated GTP to GDP (see Iyengar *et al.*, 1979b).

The trypsin experiments also demonstrate quite clearly that p[NH]ppG markedly reduced the trypsin-sensitivity of glucagon-stimulated activity (Fig. 3). This could not have been predicted from the Arrhenius plots of glucagon-plus-p[NH]ppG-stimulated adenylate cyclase, which clearly demonstrate a sensitivity to lipid-phase separations occurring in both bilayer halves (Figs. 1 and 2; Houslay *et al.*, 1976a). To account for this we would like to suggest that the guanine nucleotide regulatory protein (N<sub>R</sub>) associated with this coupling process is a distinct transmembrane protein. That it is not released by high-salt treatment indicates that it is not a peripheral protein. Also the transient complex formed of (HRN<sub>R</sub>p[NH]ppG C\*) will decay to give a persistently activated complex of N<sub>R</sub>p[NH]ppG C\*.

Such a postulate would allow for the activity being sensitive to lipid-phase separations in both halves of the bilayer. It has been noted by many workers (see e.g. Rodbell *et al.*, 1975; Iyengar *et al.*, 1979a,b) that treatment with glucagon and p[NH]ppG leads to a persistently activated state of the enzyme. We can see that, at the high concentrations of ligands used, time courses of cyclic AMP production by hepatocyte membranes in the presence of glucagon and p[NH]ppG ensued with little perceptible lag time (Fig. 4c). However, as receptor numbers were progressively depleted by trypsin action (from 100% to less than 10%), time courses of cyclic AMP production in the presence of these ligands became increasingly non-linear, with distinctly accelerating rates. This would be expected if N<sub>R</sub>p[NH]ppG C\* accumulated as a relatively stable complex, and a single occupied receptor could lead to such an activation of an increasing number of catalytic units over the time course. By implication we must define the nucleotide-regulatory protein (N<sub>R</sub>) as not being irreversibly bound (if at all) to the receptor, because, if this had been the case, then the HR released would have been unable to achieve the activation of further catalytic units. Indeed treatment of plasma membranes with detergent (Welton *et al.*, 1977) liberated 90% of those proteins binding <sup>3</sup>H-labelled p[NH]ppG, for which 10% were associated with the catalytic unit of adenylate cyclase and the rest appeared to form a single population with a K<sub>D</sub> for p[NH]ppG similar to the K<sub>a</sub> for activation of glucagon-stimulated adenylate cyclase by p[NH]ppG (Rodbell *et al.*, 1971; Iyengar *et al.*, 1979a). If these proteins are indeed N<sub>R</sub> then they are found distinct from and in excess of the glucagon receptors as our model would imply. Thus in the presence of glucagon and pp[NH]pG we envisage:



where N<sub>R</sub>p[NH]ppG is the transmembrane nucleotide regulatory protein involved in coupling with bound p[NH]ppG. The species in parenthesis exists as a transient intermediate and does not accumulate.

The data obtained for benzyl alcohol action give further support to our proposals. The similarity in dose response of the GTP- and p[NH]ppG-activated states is presumably because they act in a similar fashion on the regulatory protein associated with the catalytic unit, where one might expect to see the same protein complex responding in a similar

fashion to changes in bilayer fluidity. However, when glucagon alone, glucagon plus GTP and glucagon plus p[NH]ppG are the stimulating ligands, we have proposed the existence of different complexes in each case. One might well expect that these forms would respond differently to changes in bilayer fluidity, which would also no doubt affect the various rate constants involved in the very different processes leading to the formation of the active complexes.

The presence of fundamentally different complexes in these states may also explain why in Arrhenius plots of glucagon-stimulated adenylate cyclase activity in hamster liver plasma membranes the activation energy increased above the 26°C break when guanine nucleotides were added, but not when glucagon was present alone.

We note that the response of the GTP- and p[NH]ppG-stimulated activities to benzyl alcohol does not parallel that of fluoride (Dipple & Houslay, 1978) in exhibiting a marked inhibition with 10 mM benzyl alcohol. This could mean that fluoride does not act in an analogous fashion to these nucleotides, which is consistent with the conclusions of Johnson *et al.* (1975), who suggested that they lead to very different active configurations of the enzyme.

We believe that our results demonstrate that GTP and p[NH]ppG alter the mechanism of coupling induced by the hormone glucagon, and give rise to fundamentally different complexes. The most significant points of this are that a single occupied receptor can activate a number of catalytic units and that, in the presence of GTP, the physiological effector, the physical properties of the external half of the bilayer will not regulate adenylate cyclase activity, although we should bear in mind that the viscosity of both halves of the bilayer will influence the rates of free lateral diffusion of the receptor, nucleotide regulatory unit and catalytic unit which is fundamental to these models. As GTP has been demonstrated to attain concentrations of approx. 0.6 mM in the liver cell (Pogson *et al.*, 1979), it is likely that this state will be physiologically relevant.

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