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# COMPARISON OF THE EPINEPHRINE-MEDIATED ACTIVATION OF ADENYLATE CYCLASE IN PLASMA MEMBRANES FROM LIVER AND ASCITES HEPATOMAS OF RATS

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## SUMMARY

(1) The apparent [$^3$H]epinephrine binding parameters of plasma membranes from rat liver and ascites hepatomas such as AH-7974, AH-371A and AH-130, as measured by equilibrium dialysis and/or Millipore filtration, were almost similar to each other. The epinephrine binding sites in the plasma membranes were heterogeneous ($\alpha$-, $\beta$-receptors and non-specific sites), but the pattern of these binding sites in the liver membranes appeared almost similar to that in the hepatoma membranes.

2. The $\beta$-receptors seemed to be specifically involved in the epinephrine-mediated activation of adenylate cyclase of the liver membranes. In spite of the presence of almost similar $\beta$-receptors and adenylate cyclase, the adenylate cyclase of hepatoma membranes was found to be less sensitive to the epinephrine-mediated activation.

3. GTP alone was found to activate adenylate cyclase of liver and hepatoma membranes to some extents when the concentration of ATP was lower (0.3 mM). When GTP was added with epinephrine, a marked, synergistic activation of adenylate cyclase was observed in liver plasma membranes, but not in hepatoma ones.

4. The synergistic activation of adenylate cyclase by epinephrine plus GTP showed a characteristic kinetic feature, reaching a maximal peak within 1 min or so after mixing.

5. Binding of [$^3$H]epinephrine to liver membranes proceeded monophasically in the absence of GTP, while it proceeded biphasically in the presence of GTP, showing the retardation of binding at some earlier stages. GTP added at the time of binding equilibrium induced the temporary release of bound epinephrine from the $\beta$-receptors. The GTP-induced temporary release of bound epinephrine, occuring within 4–5 min after the addition of GTP, was less marked in the hepatoma membranes as compared with the liver membranes.

6. Possible impairment of the GTP-dependent coupling mechanism in the receptor-adenylate cyclase system of hepatoma plasma membranes was suggested.

## INTRODUCTION

The mammalian liver is one of the target organs of epinephrine. The presence of epinephrine receptors as well as the epinephrine-mediated activation of adenylate cyclase in the plasma membranes of the liver have been reported [1–6]. In addition to the epinephrine-mediated metabolic regulations such as the stimulation of glycogenolysis, the inhibition of glycogen synthesis, the induction of glyconeogenesis and so on [7–11], epinephrine [12–14] or cyclic AMP [15–21] is known to control the cellular proliferation. The tumor cells, in contrast to the normal ones, appear to be less sensitive to the growth control by epinephrine [12–14].

The results of earlier investigations on the receptor-adenylate cyclase system in liver and hepatomas were, however, not always consistent, in part because different biological systems were used for assaying adenylate cyclase (plasma membranes, microsomes, homogenates, and so on) and in part because the hepatoma cells used were of different malignancy. According to Emmelot and Bos [22], the adenylate cyclase activity of plasma membranes of rat hepatoma 484A cells is lower than that of the rat liver and is not stimulated by epinephrine, while the enzyme activity of plasma membranes of well-differentiated mouse hepatoma 147042 cells is similar to that of mouse liver and is stimulated by epinephrine.

In the present study, the epinephrine receptors and the epinephrine-mediated activation of adenylate cyclase were compared among plasma membranes prepared from rat liver and ascites hepatomas such as AH-130, AH-371A and AH-7974.

## MATERIALS AND METHODS

*Preparation of plasma membranes.* Plasma membranes of the liver were prepared from male, Wistar rats weighing 120–160 g either by the method of Berman et al. [23], as has been modified by Yamamoto and Terayama [24], in the isotonic medium or by the method of Ray [25] in the hypotonic one. The plasma membrane fractions sedimented at the interface between sucrose layers of $d$ (density) = 1.13 and 1.16 and at the interface between sucrose layers of $d$ = 1.16 and 1.18, were collected and they were referred to as $PM_0$ and $PM_1$, respectively. The plasma membranes of ascites hepatomas were prepared by the method of Emmelot and Bos [26], as has been modified by Shimizu [27], from AH-371A, AH-7974 and AH-130 cells. These ascites hepatoma cells were harvested from the peritoneal cavities of Donryu strain rats weighing 120–160 g several days after inoculation. The hepatoma plasma membranes sedimented at the interface between the sucrose layers of $d$ = 1.16 and 1.18 (corresponding to $PM_1$) were used in the present study.

Yields of $PM_0$ and $PM_1$ from the liver homogenate in the isotonic medium were 0.2 and 0.4 mg protein per g wet liver, respectively, while that of $PM_1$ from the liver homogenate in the hypotonic medium was about 1.0 mg protein per g wet liver. Usually, plasma membranes ($PM_1$) of 0.6–1.0 mg protein were obtained from 1 ml of packed ascites hepatoma cells. Electron-microscopical observations on the plasma membrane preparations from rat liver revealed that $PM_1$ was rich in adherent double-membranes with tight junctions or desmosomes, while $PM_0$ was rich in small vesicles. The contamination of mitochondria or other subcellular organelles appeared negligibly small.

*Analysis of epinephrine binding sites in plasma membranes by equilibrium dialysis.* Freshly prepared plasma membranes were suspended in 0.25 M sucrose/10 mM $MgCl_2$/25 mM KCl/50 mM Tris · HCl buffer, pH 7.4, at the concentration of about 1 mg protein per ml. 1 ml of the membrane suspension in cellophane tubing (Visking; an inflated diameter of 6.4 mm) was dialyzed against 5 ml of the same medium containing various concentrations of [$^3$H]epinephrine, with gentle shaking at 4 °C for 24 h. Preliminary experiments have shown that dialysis equilibrium is attained within 24 h under the present experimental conditions. After dialysis, aliquots of the inner and outer liquids were assayed for radioactivity and another aliquot of the inner liquid was assayed for protein. Concentrations (mol/l) of free and bound [$^3$H]-epinephrine were calculated from radioactivity of the outer liquid and from a difference in radioactivity between the inner and the outer liquids, respectively. $K_d$ (M) (dissociation constant) and $N$ (mol/g protein) (number of binding sites) were obtained by the least square method from the Linweaver-Burk plots of the binding.

*Analysis of epinephrine binding sites by Millipore filtration.* Plasma membranes (about 1 mg protein/ml) in 300 μl of 20 mM Tris · HCl buffer, pH 7.4, containing [$^3$H]epinephrine at various concentrations were incubated at 37 °C for 15 min except when otherwise specified. After incubation, a 50 μl aliquot of it was quickly filtered on a Millipore disk (HAWP 304 FO), which was then washed with 10 ml of the ice-cold Tris buffer (20 mM, pH 7.4). The above procedures were accomplished within 15 s. In order to prevent the adsorption of [$^3$H]epinephrine on a Millipore disk, the disks, which had been soaked for 30 min in the ice-cold Tris buffer containing 1 mM unlabelled epinephrine, were used. [$^3$H]Epinephrine bound to plasma membranes was assayed by measuring radioactivity retained on the Millipore disk.

*Assay of adenylate cyclase.* Plasma membranes of about 1 mg protein were suspended in 1 ml of the standard reaction mixture, containing 3 mM ATP, 10 mM theophylline, 5 mM phosphoenolpyruvate, 10 μg pyruvate kinase, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 20 mM Tris · HCl buffer, pH 7.4, according to the method of Rall and Sutherland [28]. When the effects of hormones or other chemicals were to be tested, they were added into the above reaction mixture at concentrations as indicated in the text. The whole reaction mixture was incubated at 37 °C for 15 min except when otherwise specified, heated in a boiling water bath for 3 min to stop the reaction, and then centrifuged at 10 000 rev./min for 15 min. A 10 μl aliquot of the supernatant was used for the assay of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) by the method of Gilman [29], using the cyclic AMP-binding protein (the cyclic AMP binding capacity of 0.28–0.30 pmol cyclic AMP per μg protein) partially purified from bovine heart muscle according to the method of Miyamoto et al. [30].

*Assays of radioactivity and protein.* Radioactivities of the outer and inner liquids after equilibrium dialysis were counted in a liquid scintillation spectrometer (ALOKA LSC-651) after samples had been oxidized with 0.1 ml of 70 % $HClO_4$ and 0.2 ml of 30 % $H_2O_2$ at 70 °C for 2–4 h. PPO/toluene/ethylcellosolve was used as the scintillation fluid. The radioactivity trapped in a Millipore disk was also counted in a liquid scintillation spectrometer after the disk had been dissolved in 1 ml of ethylcellosolve by heating at 70 °C for 2 h, using PPO/POPOP/toluene/ethylcellosolve as the scintillation fluid. Protein was assayed by the method of Lowry et al. [31] with bovine serum albumin as standard.

*Chemicals and biochemicals.* Cyclic adenosine 3′, 5′-monophosphate (cyclic AMP), cyclic guanosine 3′,5′-monophosphate (cyclic GMP), adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), 5′-guanylylimidodiphosphate (GMP-PNP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), phosphoenolpyruvate and pyruvate kinase were purchased from the Boehringer-Mannheim, Germany. DL-Epinephrine and theophylline were purchased from the Tokyo Chem., Industry, Japan. Phentolamine and propranolol were donated by the Research Laboratories of the Yoshitomi Pharmaceutical Co., Japan. All the above chemicals were of the reagent grade or chromatographically pure. [$^3$H]Epinephrine (DL-[7-$^3$H]-epinephrine; 12.0–14.3 Ci/mmol) and cyclic [$^3$H]AMP (cyclic [8-$^3$H]adenosine 3′,5′-monophosphate; 27.5 Ci/mmol) were purchased from the Radiochemical Center, G. B. and they were of more than 95 % radiochemical purity. The [$^3$H]epinephrine was used without dilution with unlabelled epinephrine when the binding assays were carried out at $10^{-7}$ M or lower, but it was diluted with unlabelled epinephrine when used at higher concentrations.

## RESULTS

(1) *Epinephrine binding parameters of plasma membranes of liver and ascites hepatomas as determined by equilibrium dialysis and by Millipore filtration.* The plasma membrane fractions, $PM_0$ and $PM_1$, were prepared from the rat liver and ascites hepatomas such as AH-7974, AH371A and AH-130, by the various methods as described in Materials and Methods. The binding of [$^3$H]epinephrine to these plasma membrane preparations was investigated by equilibrium dialysis and Millipore filtration as described in Materials and Methods.

Some of the results of these binding experiments are shown in Fig. 1, in which the amount of [$^3$H]epinephrine bound to the plasma membranes is plotted against the concentration of free [$^3$H]epinephrine. The binding of [$^3$H]epinephrine to these plasma membranes appears to reach a saturation level at about $4 \cdot 10^{-7}$–$5 \cdot 10^{-7}$ M of free [$^3$H]epinephrine. However, upon increasing the concentration of [$^3$H]epinephrine further, the binding of apparently non-specific nature tends to increase. In Fig. 2 are shown some of the Lineweaver-Burk plots of [$^3$H]epinephrine binding in a range of epinephrine concentrations lower than $4 \cdot 10^{-7}$ M. The apparent binding parameters ($N$ and $K_d$) thus obtained are summarized in Table I. The binding parameters seem to vary to some extents from one batch of plasma membranes to another. If we take this fact into considerations, it seems reasonable to assume that no appreciable difference in the apparent binding parameters ($N$ and $K_d$) may exist among liver and hepatoma plasma membranes without regards to the membrane fractions, the preparation methods or the assay methods. In the following experiments, the binding of [$^3$H]epinephrine was assayed by Millipore filtration, using plasma membrane $PM_1$ ($d = 1.16$–$1.18$) prepared from the homogenates of the liver and ascites hepatomas in the hypotonic medium [25, 27]. It should be noted that the fluctuations of binding assays under the present experimental conditions were usually less than $\pm 5$ %.

(2) *Effects of unlabelled epinephrine, and α- and β-blockers on the over-all binding of [$^3$H]epinephrine to plasma membranes of rat liver and ascites hepatomas.* The simultaneous addition fo $10^{-6}$, $10^{-5}$ and $10^{-4}$ M epinephrine with [$^3$H]-epinephrine ($10^{-7}$ M) reduced the binding of [$^3$H]epinephrine to the plasma mem-

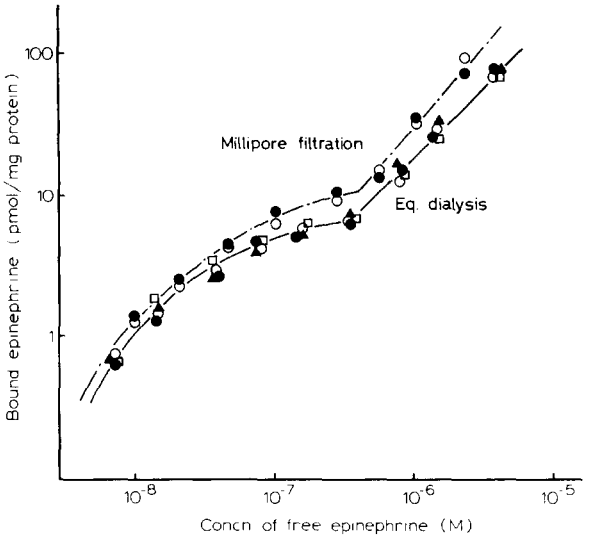


Fig. 1. Binding of [$^3$H]epinephrine to plasma membranes prepared from liver and AH-130. Plasma membranes were prepared by various methods as described in Materials and Methods. In case of equilibrium dialysis, membranes equivalent to 1.0–1.2 mg protein were suspended in 1 ml of 0.25 M sucrose/10 mM $MgCl_2$/25 mM KCl/50 mM Tris · HCl, pH 7.5, and the suspension was dialyzed at 4 °C for 24 h against 5 ml of the same medium containing [$^3$H]epinephrine (12.0–14.3 Ci/mmol) at various concentrations (the specific activity of [$^3$H]epinephrine was reduced adequately when used at $10^{-6}$ M or higher concentrations). In case of Millipore filtration, plasma membranes equivalent to 0.3–0.4 mg protein were suspended in 0.3 ml of 20 mM Tris · HCl, pH 7.4, containing [$^3$H]epinephrine at various concentrations and the suspensions were incubated at 37 °C for 15 min. The bound and free [$^3$H]epinephrine were assayed as described in Materials and Methods. The solid line indicates the binding of [$^3$H]epinephrine as determined by equilibrium dialysis, while the broken line indicates the binding as determined by Millipore filtration. Each point is a mean of duplicate determinations in a single experiment using the same batch of plasma membranes. ○, liver $PM_1$ (hypotonic) [25]; ●, AH-130 $PM_1$ (hypotonic) [26, 27]; □, liver $PM_0$ (isotonic) [23, 24], and ▲, liver $PM_1$ (isotonic) [23, 24].

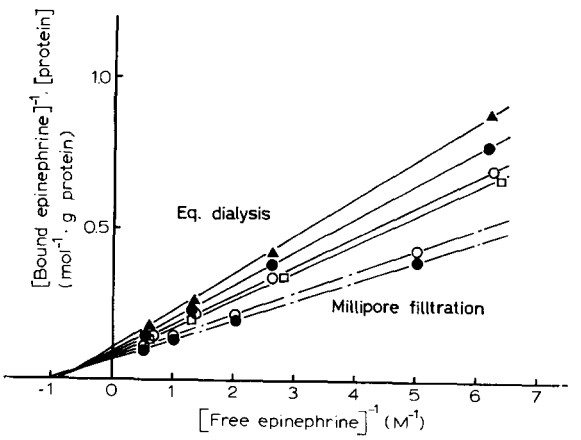


Fig. 2. Lineweaver-Burk plots of binding of [$^3$H]epinephrine to plasma membranes from liver and AH-130. Analyses of binding of [$^3$H]epinephrine to plasma membranes were carried out as described in Fig. 1. Solid lines indicate data obtained by equilibrium dialysis and broken lines indicate those obtained by Millipore filtration. ○, liver $PM_1$ (hypotonic) [25]; ●, AH-130 $PM_1$ (hypotonic) [26, 27]; □, liver $PM_0$ (isotonic) [23, 24]; and ▲, liver $PM_1$ (isotonic) [23, 24].

TABLE I

[$^3$H]EPINEPHRINE BINDING PARAMETERS OF PLASMA MEMBRANES PREPARED FROM LIVER AND ASCITES HEPATOMAS BY DIFFERENT METHODS AS ASSAYED BY EQUILIBRIUM DIALYSIS AND MILLIPORE FILTRATION

Binding assays were carried out by equilibrium dialysis and Millipore filtration as described in Materials and Methods. Preparation methods are classified according to the tonicity of homogenizing media. Both the isotonic [23, 24] and hypotonic [25] media were used for preparation of liver membranes and only the hypotonic medium [26, 27] was used for preparation of hepatoma membranes. $K_d$ is an apparent dissociation constant of the epinephrine-ligand complexes and $N$ is the number of binding sites per g protein of plasma membranes.

| Assay method | Plasma membranes (No. of expts.) | Preparation method | Binding parameters | |
|---|---|---|---|---|
| | | | $K_d$ (nM) | $N$ (nmol/g protein) |
| Equilibrium dialysis | Liver $PM_1$ (6) | Isotonic | 121±13* | 11.2±0.7* |
| Equilibrium dialysis | Liver $PM_0$ (2) | Isotonic | 94±12** | 12.9±3.1** |
| Equilibrium dialysis | Liver $PM_1$ (2) | Hypotonic | 100±30** | 12.5±1.1** |
| Equilibrium dialysis | AH-371A (1) | Hypotonic | 118 | 10.0 |
| Equilibrium dialysis | AH-7974 (1) | Hypotonic | 110 | 8.3 |
| Equilibrium dialysis | AH-130 (1) | Hypotonic | 110 | 15.4 |
| Millipore | Liver $PM_1$ (2) | Hypotonic | 109±21** | 13.4±0.7** |
| Millipore | AH-130 (1) | Hypotonic | 92 | 14.7 |

* Mean±standard error.
** Mean±average deviation.

branes of liver or AH-130 similarly by about 40, 50 and 65 %, respectively.

Propranolol (β-adrenergic antagonist) or phentolamine (α-adrenergic antagonist) was added at various concentrations into the suspension of plasma membranes from liver and AH-130 simultaneously with [$^3$H]epinephrine ($10^{-7}$ M) (without preincubation), or the plasma membranes were preincubated with each of these blockers at various concentrations at 0–4 °C for 3 h, and then [$^3$H]epinephrine ($10^{-7}$ M) was added (with preincubation). The binding of [$^3$H] epinephrine was assayed by Millipore filtration after the incubation at 37 °C for 15 min in both cases.

As shown in Fig. 3, both propranolol and phentolamine inhibited the epinephrine-binding dose dependently. No significant difference in the patterns of inhibition by these blockers was observed between the liver and AH-130, although the inhibition was always larger in the case of with preincubation than in the case of without preincubation.

(3) *Effects of the α- and β-blockers on the epinephrine-mediated activation of adenylate cyclase in plasma membranes of rat liver.* As shown in Table II, the adenylate cyclase of liver plasma membranes, which had been preincubated (0 °C, 3h) with propranolol ($10^{-4}$ M), was not activated by epinephrine ($10^{-5}$ M). On the other hand, the similar treatment of the plasma membranes with phentolamine ($10^{-4}$ M) did not effect the epinephrine-mediated activation of the adenylate cyclase.

Fig. 4 shows the dose dependency in the inhibition of epinephrine-mediated activation of adenylate cyclase by propranolol. The inhibition of adenylate cyclase by propranolol is larger in the case of with preincubation than in the case of without preincubation.

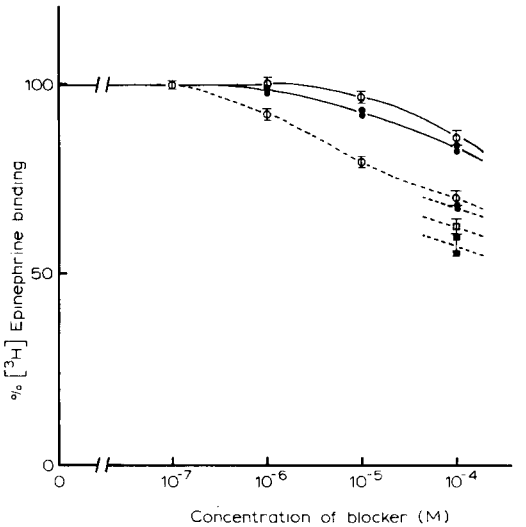


Fig. 3. Effects of propranolol and phentolamine on the binding of [$^3$H]epinephrine to plasma membranes from liver and AH-130. Plasma membranes ($PM_1$) were prepared from liver and AH-130 in the hypotonic medium [25–27]. In the case of without preincubation (solid lines), aliquots of plasma membrane suspension in water, propranolol or phentolamine solution in water, Tris · HCl buffer (pH 7.4) and [$^3$H]epinephrine solution in water were mixed so that the final concentrations of Tris buffer and [$^3$H]epinephrine may become 20 mM and $10^{-7}$ M, respectively, but the final concentration of propranolol or phentolamine may become as indicated in the abscissa ($10^{-7}$–$10^{-4}$ M). The whole mixture of a final volume of 0.3 ml was incubated at 37 °C for 15 min, and [$^3$H]epinephrine bound to plasma membranes was assayed by Millipore filtration as described in Materials and Methods. In case of with preincubation (dotted lines), plasma membranes, Tris buffer and propranolol or phentolamine were mixed first and preincubated at 4 °C for 3 h prior to the addition of [$^3$H]epinephrine. The following procedures were the same as those in the case of without preincubation. ○, indicates the effect of propranolol on the binding of [$^3$H]epinephrine to liver plasma membranes (mean ± average deviation from two experiments, each with duplicate determinations); ●, indicates the effect of propranolol on the binding of [$^3$H]epinephrine to AH-130 plasma membranes (single experiment with duplicate determinations); □, indicates the effect of phentolamine (only at $10^{-4}$ M) on the binding to liver membranes (mean ± average deviation from two experiments, each with duplicate determinations); and ■, indicates the effect of phentolamine (only at $10^{-4}$ M) on the binding of AH-130 membranes (a single experiment with duplicate determinations).

(4) *Effect of epinephrine at various concentrations on the adenylate cyclase activity of plasma membranes of rat liver and ascites hepatomas.* As shown in Fig. 5, the adenylate cyclase activity of plasma membranes of these ascites hepatomas was not stimulated by epinephrine at $10^{-7}$ or $10^{-6}$ M in contrast to the considerable stimulation of the liver enzyme by epinephrine at these concentrations. It should be noted that the basal activity of adenylate cyclase in the hepatoma membranes was almost similar to that in the liver membranes.

In parallel experiments we tested the effects of NaF and prostaglandin $E_1$ on the adenylate cyclase activity of liver and hepatoma membranes. The results summarized in Table III indicate that the prostaglandin $E_1$-mediated activation of adenylate cyclase occurs only in liver membranes but not in hepatoma ones, while the NaF-mediated activation of the enzyme occurs similarly in both liver and hepatoma membranes.

TABLE II
EFFECTS OF THE α- AND β-ADRENERGIC BLOCKING AGENTS ON THE EPINEPHRINE-MEDIATED ACTIVATION OF ADENYLATE CYCLASE IN THE PLASMA MEMBRANES OF THE RAT LIVER

The plasma membranes, $PM_1$ (0.93 mg protein), prepared from rat liver in the hypotonic medium [25] were preincubated in 1 ml of the reaction mixture [28] containing phentolamine or propranolol (each at $10^{-4}$ M) at 4 °C for 3 h. After the addition of epinephrine in 10 μl (final concentration of $10^{-5}$ M), the whole reaction mixtures were incubated at 37 °C for 15 min and cyclic AMP thus formed was assayed. Results are the mean ± average deviation from two experiments, each with duplicate determinations.

| Adrenergic blocker | Adenylate cyclase activity (nmol of cyclic AMP formed/mg protein per h) | |
|---|---|---|
| | With epinephrine | Without epinephrine |
| Phentolamine | 2.93 ± 0.14 | 2.00 ± 0.01 |
| Propranolol | 2.01 ± 0.11 | 2.04 ± 0.03 |
| None | 2.88 ± 0.17 | 2.11 ± 0.10 |

(5) *Effect of GTP on the epinephrine-mediated activation of adenylate cyclase in plasma membranes of liver and ascites hepatomas.* In order to elucidate the mechanisms for the lack of epinephrine-mediated activation of adenylate cyclase in hepatoma plasma membranes, the effect of GTP on the receptor-adenylate cyclase system in liver and hepatoma plasma membranes was investigated, since GTP has been reported to be required for the hormone-mediated activation of adenylate cyclase [32–39], especially under low ATP concentrations. ATP at higher concentrations can serve not only as the enzyme substrate but also as a substitute for GTP.

As shown in Fig. 6, the adenylate cyclase activity of plasma membranes of rat liver, as assayed in the presence of ATP of 0.3 mM instead of 3 mM, was not stimulated by epinephrine at concentrations up to $10^{-6}$ M. Epinephrine at $10^{-5}$M stimulated only slightly. Addition of GTP ($5 \cdot 10^{-5}$ M) recovered the sensitivity of epinephrine-mediated activation of the adenylate cyclase of liver plasma membranes. On the other hand, the adenylate cyclase of plasma membranes of ascites hepatomas remained insensitive to the epinephrine-mediated activation without regards to the presence or the absence of GTP as also shown in Fig. 6. It should be noted that the basal activity of adenylate cyclase (in the absence of epinephrine) is almost similarly stimulated by GTP ($5 \cdot 10^{-5}$ M) in both liver and hepatoma plasma membranes.

As shown in Fig. 7, the adenylate cyclase of liver membranes seems to be synergistically activated by epinephrine and GTP at concentrations higher than $10^{-7}$ M, while no synergism seems to exist between the effects of epinephrine and GTP in the case of AH-130 plasma membranes.

(6) *Kinetics of cyclic AMP-formation by plasma membranes of rat liver in the presence of epinephrine, GTP, or epinephrine plus GTP.* Kinetics of cyclic AMP-formation by plasma membranes of rat liver were investigated in the presence of GTP ($6 \cdot 10^{-5}$ M), epinephrine ($10^{-5}$ M), or GTP ($6 \cdot 10^{-5}$ M) plus epinephrine ($10^{-5}$ M). The concentration of ATP was 0.3 mM throughout these experiments. As shown in Fig. 8, the cyclic AMP formation appears to proceed in a biphasic fashion in the presence of epinephrine plus GTP. The earliest and most rapid reaction

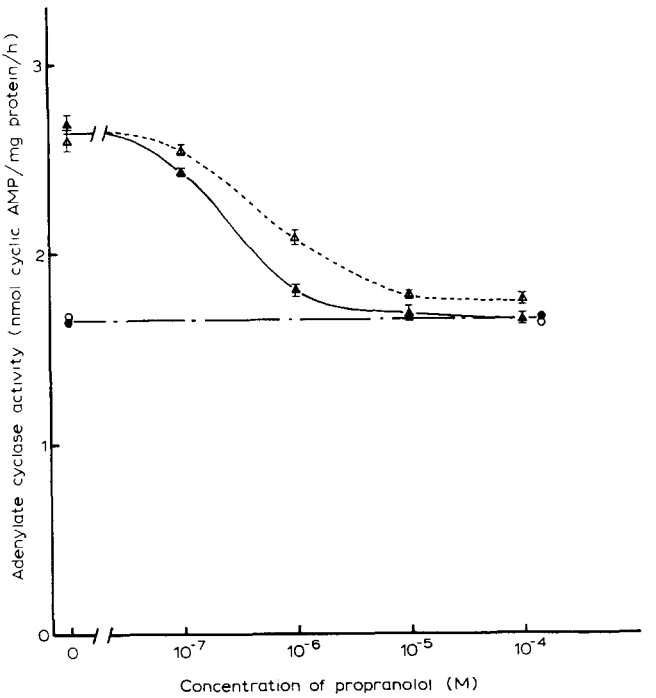


Fig. 4. Effect of propranolol on the epinephrine-mediated activation of adenylate cyclase in plasma membranes of liver. Plasma membranes ($PM_1$) of rat liver were preincubated with propranolol at various concentrations ($10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$ M) or without propranolol at 0 °C for 3 h. Aliquots of the preincubated suspension with or without propranolol, and the concentrated reaction mixture containing epinephrine and propranolol were mixed so that the final concentration of each component may become the same with that in the standard reaction mixture for the assay of adenylate cyclase, and the final concentration of propranolol may become equal to the propranolol concentration in the preincubation mixture in the case of preincubation with propranolol or may become 0, $10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$ M in the case of preincubation without propranolol. The final concentration of epinephrine was made either $10^{-5}$ or 0 M. The whole mixture was incubated at 37 °C for 15 min, and cyclic AMP thus formed was measured. ▲ and △, indicate the effect of propranolol on the epinephrine-mediated activation of adenylate cyclase in the plasma membranes preincubated with or without propranolol, respectively. The data are expressed as mean±average deviation from two experiments, each with duplicate determinations; ● and ○, indicate the effect of propranolol on the basal activity (no epinephrine) of the plasma membranes preincubated with and without propranolol, respectively (single determinations).

lasts only for the first few minutes of incubation and then the second and less rapid reaction seems to follow after the intermediary sluggish phase. On the other hand, the cyclic AMP formation seems to proceed monophasically in the presence of only one of these effectors or in the absence of them, apparently lacking the earliest and most marked stimulation, which seems to be ascribed to the synergistic effect of GTP plus epinephrine. Thus, the dotted line obtained by subtracting the individual effect of epinephrine and GTP from the effect of epinephrine plus GTP (in Fig. 8) corresponds to the synergistic effect of epinephrine plus GTP, which reaches the maximum within 1 min.

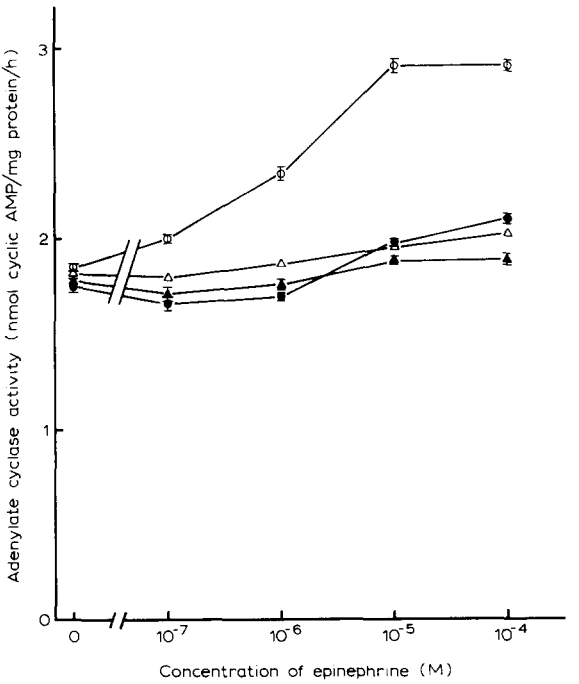


Fig. 5. Comparison of the epinephrine-mediated activation of adenylate cyclase in plasma membranes from liver and ascites hepatomas. Plasma membranes ($PM_1$) prepared from liver and ascites hepatomas such as AH-371A, AH-130 and AH-7974 in the hypotonic medium were incubated in the standard reaction mixture at 37 °C for 15 min in the presence of epinephrine at various concentrations as indicated in the abscissa. Each reaction mixture contained $PM_1$ equivalent to 0.9–1.3 mg protein. The data for plasma membranes of liver (○), AH-371A (●) and AH-7974 (▲) are from two experiments, each with duplicate determinations (mean ± average deviation), while the data for plasma membranes of AH-130 (△) are from two experiments, each with single determinations.

TABLE III

EFFECTS OF NaF AND PROSTAGLANDIN $E_1$ ON THE ACTIVITY OF ADENYLATE CYCLASE OF PLASMA MEMBRANES FROM LIVER AND HEPATOMAS

Plasma membranes in the standard reaction mixture for assaying adenylate cyclase were incubated at 37 °C for 15 min, and cyclic AMP thus formed was assayed as described in Materials and Methods. Concentrations of NaF and prostaglandin $E_1$ added to the reaction mixture were $10^{-2}$ and $10^{-5}$ M, respectively. Adenylate cyclase activities (nmol cyclic AMP/mg protein per h) in the table are mean ± S.E. from 3–4 determinations.

| Plasma membranes | Adenylate cyclase activities | | |
|---|---|---|---|
| | None | NaF | Prostaglandin $E_1$ |
| Liver | 1.84±0.01 | 7.51±0.04 | 2.65±0.05 |
| AH-371A | 1.75±0.03 | 6.50±0.02 | 1.65±0.04 |
| AH-7974 | 1.78±0.02 | 7.20±0.06 | 1.88±0.03 |
| AH-130 | 1.82±0.01 | 7.65±0.01 | 1.94±0.01 |

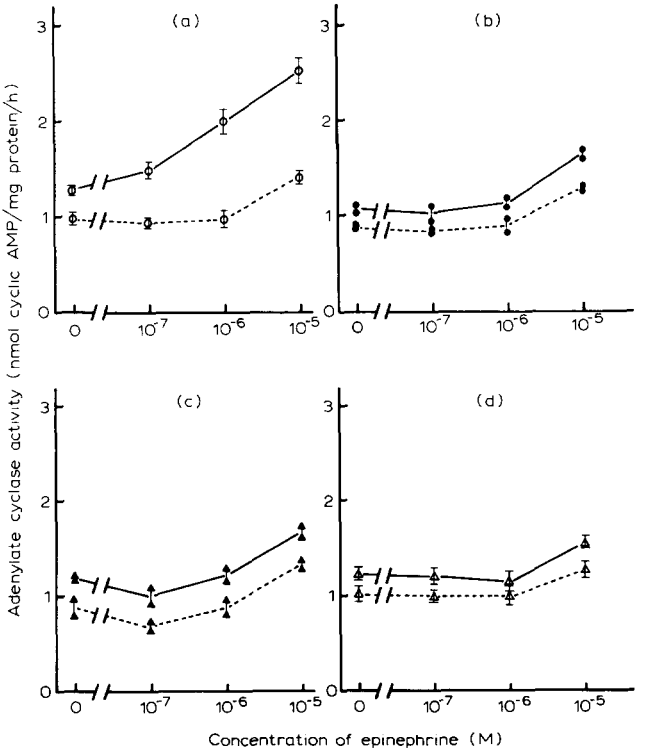


Fig. 6. Effect of GTP on the epinephrine-mediated activation of adenylate cyclase in plasma membranes of liver and ascites hepatomas as assayed at a low concentration of ATP (0.3 mM). Plasma membranes from liver and hepatomas such as AH-371A, AH-130 and AH-7974 were incubated at 37 °C for 15 min in the reaction mixture (for assaying adenylate cyclase) containing 0.3 mM ATP and epinephrine at various concentrations (0, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M), with or without fortification with $5 \cdot 10^{-5}$ M GTP. The other procedures were the same as described in the preceding figures (Figs. 4 and 5). —, with $5 \cdot 10^{-5}$ M GTP; - - - -, without GTP. a, liver plasma membranes (four determinations: mean ± S.E.); b, AH-371A plasma membranes (two determinations); c, AH-7974 plasma membranes (two determinations), and d, AH-130 plasma membranes (three determinations: mean ± S.E.).

Addition of GTP ($6 \cdot 10^{-5}$ M) to the reaction mixtures with or without epinephrine ($10^{-5}$ M) at 10 min after the onset of incubation, when the formation of cyclic AMP nearly reached the plateau level, stimulated the formation of cyclic AMP. Here again a much higher initial rate was observed in the presence of epinephrine than in the absence of it.

(7) *Effect of GTP on kinetics of [$^3$H]epinephrine binding to plasma membranes of rat liver.* As shown in Fig. 9, the binding of [$^3$H]epinephrine (at $10^{-7}$ M) to liver plasma membranes proceeds monophasically in the absence of GTP, reaching a plateau level at about 10 min of incubation at 37 °C, while it proceeds biphasically in the presence of GTP ($10^{-4}$ M). In both cases, however, the final level of the binding was similar. A difference curve between the kinetics in the presence of GTP and that in the absence of GTP (the dotted line in Fig. 9) indicates that the GTP effect retarding

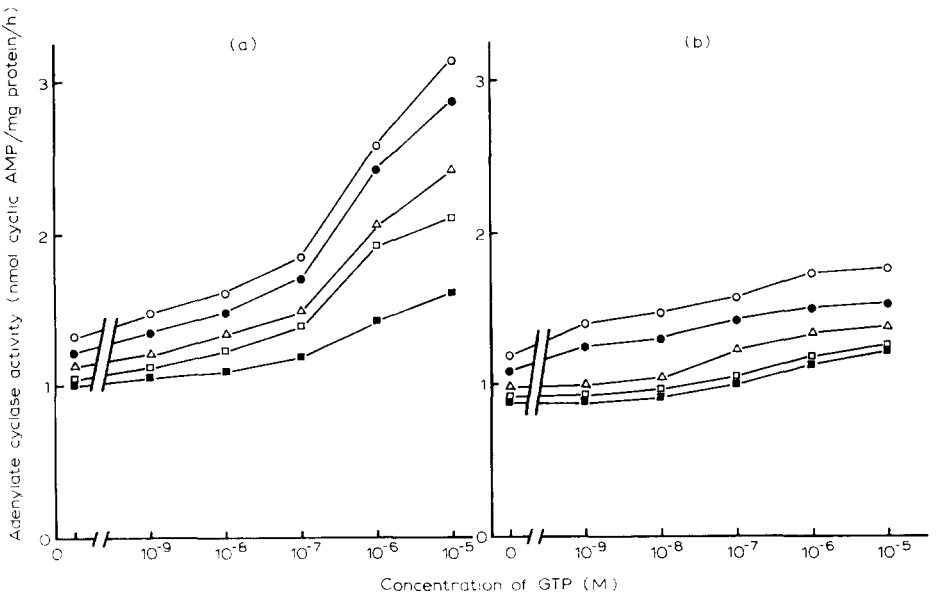


Fig. 7. The synergistic activation of adenylate cyclase in plasma membranes of rat liver by epinephrine and GTP in contrast to the non-synergistic activation of the enzyme in plasma membranes of AH-130. Plasma membranes of liver (a) and AH-130 (b), equivalent to 1.0 and 1.1 mg protein, respectively, were incubated at 37 °C for 15 min in the reaction mixture for the assay of adenylate cyclase containing ATP (0.3 mM), GTP (at various concentrations as indicated in the abscissa) and epinephrine (at 0–$10^{-4}$ M), and amounts of cyclic AMP formed were assayed as described in Materials and Methods. ■, □, △, ● and ○, indicate the enzyme activity at the epinephrine concentration of 0, $10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$ M, respectively. The data are from single determinations.

the epinephrine binding is only temporary and the maximal retardation is seen several minutes after the onset of incubation.

When GTP was added to the plasma membrane suspension 10 or 25 min after the onset of incubation with [$^3$H]epinephrine, the rapid release of bound [$^3$H]epinephrine from the plasma membranes was observed as also illustrated in Fig. 9. Under these conditions, about 40 % of the bound [$^3$H]epinephrine were released at the maximum. It should be noted, however, that the GTP induced release of bound [$^3$H]epinephrine is only temporary and soon followed by the rebinding process, returning back to the original binding level 25 min after the addition of GTP. This release-and-rebinding process of epinephrine seems to be related to the above-described retardation of epinephrine binding in the presence of GTP. If GTP was added again 15 or 25 min after the first addition of GTP, the release of bound [$^3$H]-epinephrine took place within a few minutes but to much less extents as compared with the release after the first addition of GTP.

As shown in Fig. 10, the binding of [$^3$H]epinephrine ($10^{-7}$ M) to liver plasma membranes preincubated with propranolol ($10^{-4}$ M) proceeds similarly to that to liver membranes preincubated without propranolol but the final level of binding in the former was lower than that in the latter. Addition of GTP ($10^{-4}$ M) at 10 min after the onset of incubation with [$^3$H]epinephrine induced the temporary release of

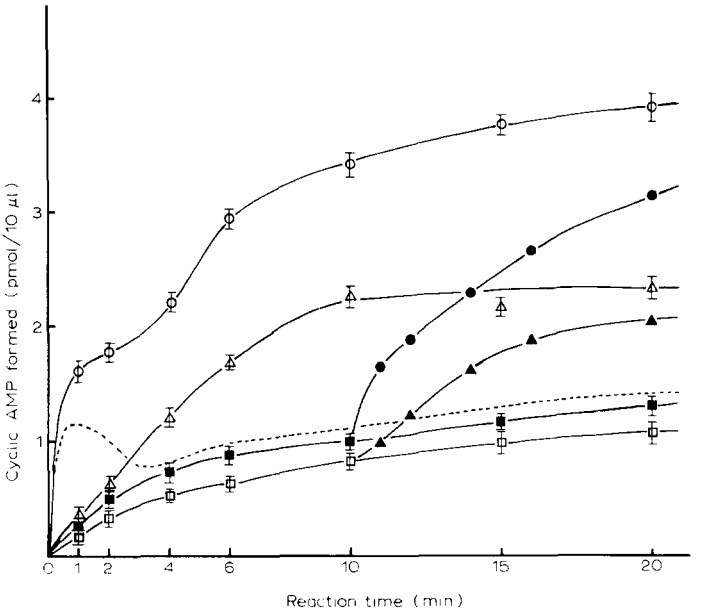


Fig. 8. Kinetics of cyclic AMP formation by liver plasma membranes and effects of epinephrine, GTP and epinephrine plus GTP on it. Liver plasma membranes equivalent to 1.1 mg protein were incubated at 37 °C for various intervals of time in the reaction mixture containing 0.3 mM ATP, with or without fortification by GTP ($6 \cdot 10^{-5}$ M), epinephrine ($10^{-5}$ M), or GTP ($6 \cdot 10^{-5}$ M) plus epinephrine ($10^{-5}$ M). In parallel experiments, GTP was added at 10 min of incubation to the reaction mixtures containing none of the effectors or containing only epinephrine, and the formation of cyclic AMP thereafter was followed. □, ■, △ and ○, indicate no effector, only epinephrine, only GTP and epinephrine plus GTP, respectively. These data are mean ± average deviation from two experiments, each with duplicate determinations. ▲ and ●, indicate GTP addition at 10 min to the reaction mixture containing no effector and that to the reaction mixture containing only epinephrine, respectively. The data are from duplicate determinations of one experiment. The dotted line corresponds to (epinephrine plus GTP) − (GTP only) − (epinephrine only) + (no effector) and may represent the synergistic effect of epinephrine plus GTP.

bound [$^3$H]epinephrine in both cases, but the extent of release at the maximum was much larger in the absence of propranolol than in the presence of it. These results may suggest that the GTP-induced temporary release of bound epinephrine is specifically related to the $\beta$-receptors.

In Fig. 11a are shown the kinetics of [$^3$H]epinephrine binding to hepatoma (AH-130, AH-7974) plasma membranes as compared with that to liver plasma membranes. No appreciable difference was detected among these binding kinetics. However, if GTP ($10^{-4}$ M) is added 10 min after the onset of incubation, the temporary release of bound epinephrine following the addition of GTP was found to be less marked in hepatoma membranes as compared with liver ones. The temporary release of bound epinephrine at the maximum (as measured 4 min after the addition of GTP) is dependent on the concentration of GTP. As shown in Fig. 11b, liver plasma membranes are much more sensitive to the GTP-induced temporary release of bound epinephrine than hepatoma membranes and the significant release of epinephrine is detectable at $10^{-6}$ M GTP.

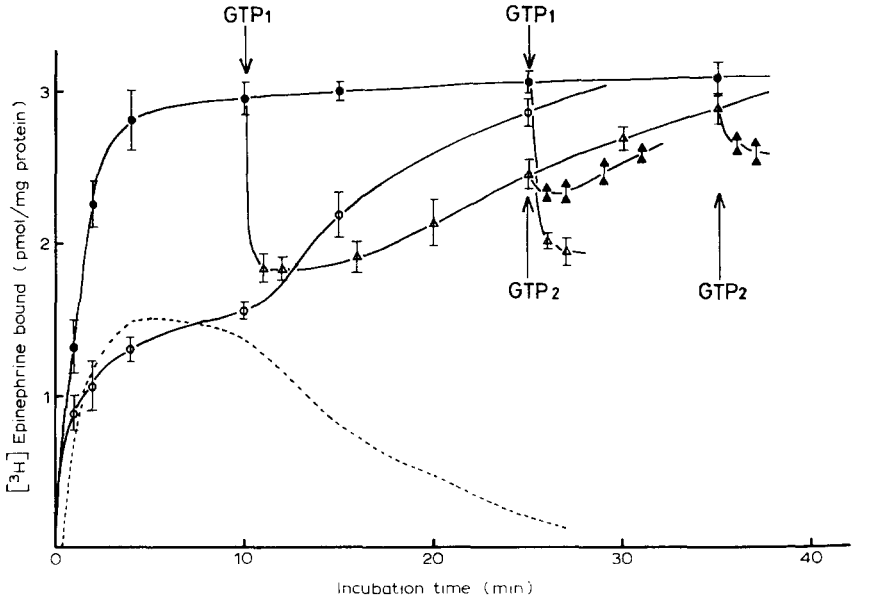


Fig. 9. Retardation of [$^3$H]epinephrine binding to liver plasma membranes by GTP and GTP-induced temporary release of bound epinephrine from membranes. Rat liver plasma membranes (0.4 mg protein) were incubated with $10^{-7}$ M [$^3$H]epinephrine in 0.3 ml of 20 mM Tris · HCl buffer, pH 7.4, at 37 °C for various intervals of time in the presence or the absence of GTP ($10^{-4}$ M) to compare the kinetics of [$^3$H]epinephrine binding. In parallel experiments, using the same batch of plasma membranes, the first and the second additions of GTP were carried out at time points indicated in the figure ($GTP_1$, $GTP_2$) and the level of [$^3$H]epinephrine bound to plasma membranes was followed thereafter. ○ and ●, indicate [$^3$H]epinephrine binding to plasma membranes in the presence (I) and the absence (II) of GTP, respectively. △ and ▲, indicate [$^3$H]epinephrine binding after the first (III) and the second (IV) addition of GTP, respectively. Duplicate determinations were carried out at each time point in each experiment, and the experiments were repeated three times in I and III, two times in II and once in IV. Data are expressed as mean ±S.E. (I and III), mean ±average deviation (II), and observed value (IV). The dotted line in this figure corresponds to the difference between I and II, representing the retardation effect of GTP on [$^3$H]epinephrine binding.

## DISCUSSION

In the present study, we have found that plasma membranes prepared from rat liver and some ascites hepatomas such as AH-7974, AH-371A and AH-130 contain specific binding sites for [$^3$H]epinephrine. No appreciable differences in the number of specific binding sites as well as in the apparent dissociation constant for the epinephrine-ligand complexes were detected among plasma membranes prepared from these cells. The number of specific binding sites for [$^3$H]epinephrine was about 10–15 nmol/g protein, which seems to be very small (only one-hundredth) as compared with the corresponding value reported on the liver microsomes [40]. Plasma membranes may be included in the microsomal fraction together with fragments from endoplasmic reticula when tissues were homogenized in a motor-driven homogenizer. Therefore, the microsomes thus prepared may contain epinephrine receptors of a plasma membrane origin and larger amounts of catecholamine *O*-methyltransferase of an endoplasmic reticulum origin [40, 41]. Liver and ascites hepatoma plasma

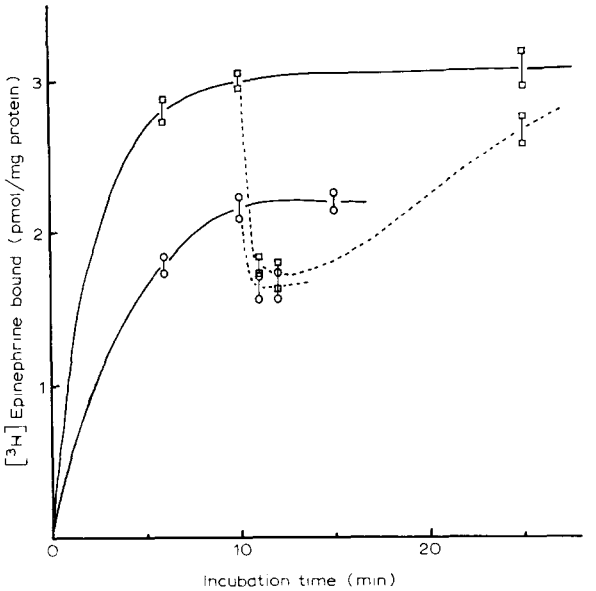


Fig. 10. Effects of propranolol on the binding of [$^3$H]epinephrine to plasma membranes of rat liver and on the GTP-induced temporary release of bound epinephrine. Liver plasma membranes (0.4 mg protein) preincubated at 0 °C for 3 h with or without $10^{-4}$ M propranolol were then incubated with [$^3$H]epinephrine ($10^{-7}$ M) in 0.3 ml of 20 mM Tris · HCl buffer, pH 7.4, containing $10^{-4}$ M propranolol or no propranolol at 37 °C for various intervals of time, and amounts of [$^3$H]epinephrine bound to the membranes were measured by Millipore filtration. In parallel experiments, GTP (final concentration of $10^{-4}$ M) was added to the incubation mixtures with or without propranolol 10 min after the onset of incubation and the levels of bound epinephrine thereafter were followed similarly. ○—○ and □—□, indicate the binding of [$^3$H]epinephrine to the membranes in the presence and the absence of propranolol, respectively. ○- - - -○ and □- - - -□, indicate the GTP-induced temporary release of bound epinephrine in the presence and the absence of propranolol, respectively. The data are observed values of duplicate determinations in one experiment.

membranes seem to contain both α- and β-receptors and the binding of [$^3$H]epinephrine to the membranes was inhibited by simultaneous or previous addition of phentolamine (α-blocker) and propranolol (β-blocker). The portion of β-receptors in the bulk of specific binding sites in the plasma membranes seemed to be about 30–40 % in liver and hepatomas as judged from the pattern of inhibition of [$^3$H]-epinephrine binding by these blockers.

The epinephrine-mediated activation of adenylate cyclase in liver plasma membranes was found to be specifically related to the β-receptors. In spite of the similarity of the β-receptors as well as of the basal adenylate cyclase activity in liver and hepatoma plasma membranes, the adenylate cyclase of hepatoma membranes was found less sensitive to the epinephrine-mediated activation as compared with that of liver ones. In the present study, we have found that the epinephrine-mediated activation of adenylate cyclase of liver plasma membranes requires GTP when the concentration of ATP is lowered (0.3 mM) in accordance with the results of earlier investigators [32–39]. Moreover, we have revealed that GTP and epinephrine activate synergistically the adenylate cyclase of liver plasma membranes, but not the adenylate

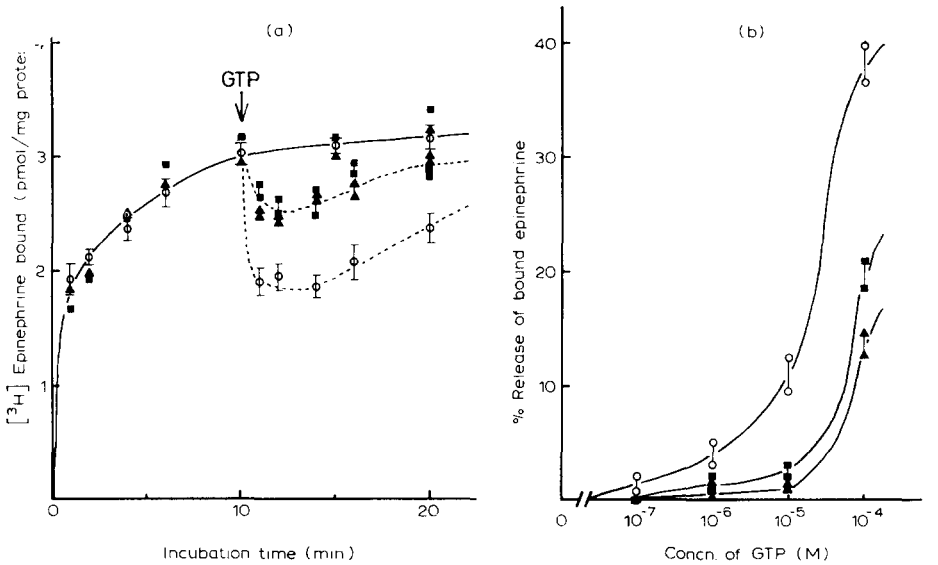


Fig. 11. Comparison of the GTP-induced temporary release of bound epinephrine between liver and hepatoma plasma membranes. (a) Kinetics of [$^3$H]epinephrine binding and GTP-induced temporary release of bound epinephrine. Plasma membranes of liver (○), AH-130 (▲) and AH-7974 (■) were incubated with [$^3$H]epinephrine ($10^{-7}$ M) at 37 °C. In parallel experiments using the same batch of plasma membranes, GTP ($10^{-4}$ M) was added at 10 min of incubation, and levels of [$^3$H]epinephrine bound to membranes were followed as described earlier. Data are expressed as mean ±S.E. (from four experiments, each with duplicate determinations) in the case of liver plasma membranes. Data for the hepatoma plasma membranes are duplicate determinations in one experiment. —, [$^3$H]-epinephrine binding kinetics in the absence of GTP; - - - -, kinetics of GTP-induced temporary release of bound [$^3$H]epinephrine. (b) Dependency of the temporary release of bound epinephrine on GTP concentrations. In experiments similar to the above, the concentration of GTP added at 10 min of incubation was varied ($10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$ M), and bound epinephrine levels were measured before (at 10 min of incubation) and 4 min after the addition of GTP. Ordinate: percent release of bound epinephrine; abscissa: GTP concentration. Data are observed values of duplicate determinations in one experiment.

cyclase of hepatoma plasma membranes. These findings seem to suggest that the lack of epinephrine-mediated activation of adenylate cyclase in hepatoma membranes may be ascribed to the impairment in the GTP-dependent coupling mechanism in the receptor-adenylate cyclase system.

The kinetic investigations on the binding or the release of [$^3$H]epinephrine to or from plasma membranes in the presence or the absence of GTP revealed some interesting facts which seem to be useful in elucidating the mechanism of the GTP effect. No appreciable difference in the kinetics of [$^3$H]epinephrine binding was observed between liver and hepatoma plasma membranes when GTP was absent. In the presence of GTP the binding of [$^3$H]epinephrine was retarded in a characteristic fashion at some earlier stages of binding. If GTP was added at a time when the binding of [$^3$H]epinephrine was almost completed (binding equilibrium), the temporary release of bound [$^3$H]epinephrine was observed. This GTP-induced temporary release of bound epinephrine seems to be specific to the $\beta$-receptors because it becomes markedly reduced when plasma membranes preincubated with propranolol were used.

Both the GTP-induced temporary release of bound epinephrine and the characteristic retardation of epinephrine binding by GTP seem to be closely related phenomena and they may suggest that GTP induces some dramatic changes in the receptor-adenylate cyclase system in membranes, resulting in the temporary loss of epinephrine binding to $\beta$-receptors. It seems interesting that the extent of GTP ($10^{-4}$ M)-induced temporary release of bound epinephrine at the maximum (4 min after the addition of GTP) is much smaller (about 17 %) in hepatoma plasma membranes as compared with liver ones (about 40 %). This difference may not be ascribed to the $\beta$-receptors themselves, because the $\beta$-receptor content seems to be similar to each other between liver and hepatoma plasma membranes as has been discussed earlier. Furthermore, the present study has revealed that the temporary release of bound epinephrine is dependent on the GTP concentration, and the sensitivity of liver plasma membranes to GTP is much higher than that of hepatoma ones.

The mechanism for the GTP-induced temporary release of bound epinephrine is now under investigations in our laboratory. The results of experiments so far accumulated seem to suggest that either one of GTPase, guanylate cyclase and GTP-dependent kinase reactions may not be involved in this phenomenon (in preparation), and therefore the possibility that the transient change of conformation of some key components in the receptor-adenylate cyclase system itself or in the plasma membranes surrounding it may occur after the addition of GTP seems to be worthwhile to be tested. Anyway, the synergistic activation of adenylate cyclase by GTP and epinephrine appears to occur in parallel to, or more exactly speaking, prior to the temporary release of bound epinephrine. The synergistic actiavtion effect reaches a peak within a very short time (1 min or less) as has been shown in the present paper. After this, the synergistic effect seems to be reduced a little and then become steady. Such features have been confirmed repeatedly and might be interpreted as reflecting the transient change in the conformation of key component(s) as discussed above, although the final conclusion on the mechanism of action of GTP should be waited until more elaborate experiments will be accomplished.

Our findings in the present study indicating the impairment of the GTP-dependent coupling mechanism in the receptor-adenylate cyclase system in the hepatoma plasma membranes may suggest that these ascites hepatoma cells are insensitive to the epinephrine-mediated regulation of metabolisms under the conditions of physiological concentrations of ATP and GTP and seem to be in accord with the reported insensitiveness of the hepatoma cells to the growth control by epinephrine [12–14].

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