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HORMONE ACTION AT THE MEMBRANE LEVEL

I. PROPERTIES OF ADENYL CYCLASE IN ISOLATED PLASMA MEMBRANES OF RAT LIVER

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SUMMARY

Membrane bound adenyl cyclase in isolated rat liver plasma membranes exhibits a requirement for Mg^{2+} and has optimal activity near pH 8.0. The optimal concentration of ATP is approx. 0.3 mM at a Mg^{2+} concentration of 1.0 mM. Ca^{2+} at a concentration range of 0.03–0.3 mM stimulates the basal activity of this enzyme. The enzyme activity is abolished by 1 min heating at 100°. Detergents such as Triton X-100 and sodium dodecyl sulfate alter the membrane structure and slightly enhance the enzyme actively, whereas sodium deoxycholate slightly inhibits the enzyme.

Glucagon and epinephrine stimulate whereas insulin inhibits adenyl cyclase activity. When studied under identical conditions on the same membrane preparation the glucagon stimulation is seen earlier and at lower hormone concentration ($1 \cdot 10^{-6}$ M) than is the epinephrine stimulation which requires 10^{-5} M hormone. The inhibition by insulin is seen at $1 \cdot 10^{-5}$ M or greater. The stimulation by glucagon is inhibited by $1 \cdot 10^{-4}$–$1 \cdot 10^{-5}$ M Ca^{2+} but the stimulation by epinephrine is enhanced by $1 \cdot 10^{-4}$–$1 \cdot 10^{-5}$ M Ca^{2+}. Moreover, epinephrine enhances, whereas glucagon inhibits the binding of Ca^{2+} to the membrane.

Studies on the combined effects of the hormones show that the stimulation by glucagon *plus* epinephrine is not additive and that insulin antagonizes the glucagon stimulation of adenyl cyclase.

Na^{+} inhibits the basal activity of adenyl cyclase but K^{+} stimulates the enzyme. F^{-} inhibits the enzyme.

These results point to a complex interplay of hormones and metal ions with the membrane bound adenyl cyclase.

INTRODUCTION

The biological significance of the enzyme adenyl cyclase is becoming increasingly important. Current thinking in the field suggests that certain hormones act as primary

Abbreviation: PCMB, *p*-chloromercuribenzoate.

messengers which stimulate the membrane bound adenyl cyclase to produce cyclic AMP which acts as a second messenger[1-4]. Cyclic AMP activates a number of kinases which in turn affect the activity of certain enzymes[2,5-11]. This process is thought to be important in the control of the metabolic machinery of the cell.

The localization of adenyl cyclase in the erythrocyte ghosts[12,13,30] and in fat cell ghosts[14,15] has recently been reported. MARINETTI *et al.*[16] have demonstrated the localization of adenyl cyclase in the plasma membrane of rat liver.

The membrane bound adenyl cyclase of the frog erythrocyte has been partially purified by ROSEN AND ROSEN[18]. The soluble adenyl cyclase of *Escherichia coli* has been purified by TAO AND LIPMANN[19].

POHL *et al.*[20] and BIRNBAUMER AND RODBELL[21] reported the glucagon stimulation of adenyl cyclase in isolated rat liver plasma membranes and the glucagon and epinephrine stimulation of adenyl cyclase in fat cell ghosts. MARINETTI *et al.*[16] found that adenyl cyclase of isolated plasma membranes of rat liver is stimulated by glucagon and epinephrine.

In this paper we report the properties of the plasma membrane bound adenyl cyclase. In a subsequent paper[22] data on the binding of epinephrine and glucagon to isolated plasma membranes will be given and discussed in terms of receptor proteins in the membrane and their relationship to adenyl cyclase activity.

METHODS AND REAGENTS

The methods of preparing the rat liver plasma membranes and the adenyl cyclase assay* have been reported earlier[16,23]. In all experiments controls containing all the reagents except membrane were incubated and analyzed under identical conditions as the system containing membrane so that any non-enzymatic formation of cyclic AMP or any cyclic AMP in the [^{3}H]ATP (uniformly labeled) could be ascertained and subtracted from the experimental values.

[^{3}H]ATP (uniformly labeled) was obtained from New England Nuclear Corp. Crystalline pork glucagon was a gift from Lilly Research Laboratories. L-Epinephrine was purchased from Sigma. ATP, ADP and AMP were purchased from P-L Laboratories. $^{45}CaCl_2$ was obtained from ICN Corp.

RESULTS

Having previously established that the enzyme adenyl cyclase is localized in the rat liver plasma membrane[16] experiments were carried out to determine the optimum conditions for the activity of the enzyme and its activation by hormones.

The data in Fig. 1 shows that the optimum membrane protein concentration is about 60–70 μg. The decline in activity at higher membrane levels may be due to membrane aggregation. It is recognized that this optimum enzyme activity prevails for a fixed amount of ATP, Mg^{2+}, Ca^{2+} and for a specified pH.

The effect of ATP concentration on adenyl cyclase activity is given in Fig. 2.

* The validity of the two-dimensional chromatographic assay for cyclic AMP is based on the following: (a) co-chromatography with authentic 3′,5′-cyclic AMP, (b) the hydrolysis of cyclic AMP to 5′-AMP by brain phosphodiesterase and (c) the complete separation of cyclic AMP from ATP, ADP, 5′-AMP, adenosine, adenine, hypoxanthine, inosine, inosinic acid, ribose and ribose phosphate.

At an added concentration of 1 mM Mg^{2+} and 85 μg of membrane protein the level of ATP needed to attain maximum velocity is about 0.3 mM. The K_m under these experimental conditions is approx. 0.1 mM with respect to ATP. This value agrees with that obtained by ROSEN AND ROSEN[18] for the partially purified erythrocyte and adenyl cyclase.

The effect of time of incubation on the total activity and specific activity of adenyl cyclase is shown in Fig. 3. The total amount of cyclic AMP formed levels off after about 10 min under these experimental conditions of 0.3 mM ATP and 1.0 mM Mg^{2+}. However, the relative specific activity of the enzyme is highest in the early

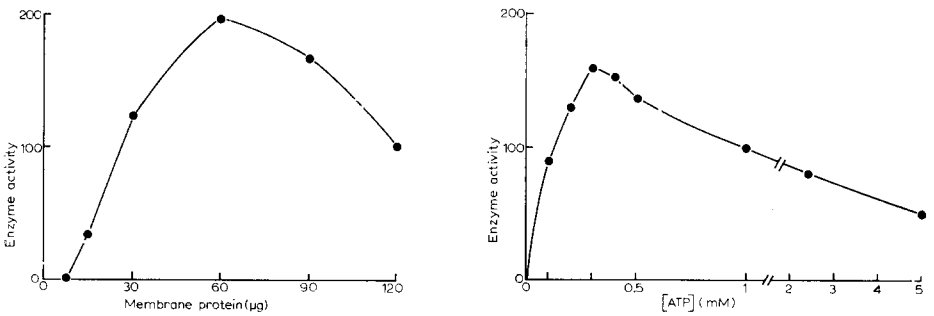

Fig. 1. Adenyl cyclase activity as a function of membrane protein concentration. The incubation system contained 0.3 mM ATP containing 0.7 μC of [^{3}H]ATP, 20 mM Tris buffer (pH 7.5), 1 mM $MgCl_2$, and different amounts of membrane. Incubation was carried out at 37° for 10 min. Aliquots were removed for the assay of cyclic AMP. The activity of adenyl cyclase is given as nmoles/mg protein per h.

Fig. 2. Adenyl cyclase activity as a function of ATP concentration. The incubation system contained 85.0 μg of membrane protein, 20 mM Tris buffer (pH 7.5), 1 mM $MgCl_2$ and different amounts of ATP containing constant specific activity of [^{3}H]ATP. Incubation was carried out at 37° for 5 min. Aliquots were removed for assay of cyclic AMP. Enzyme activity as nmoles/mg protein per h.

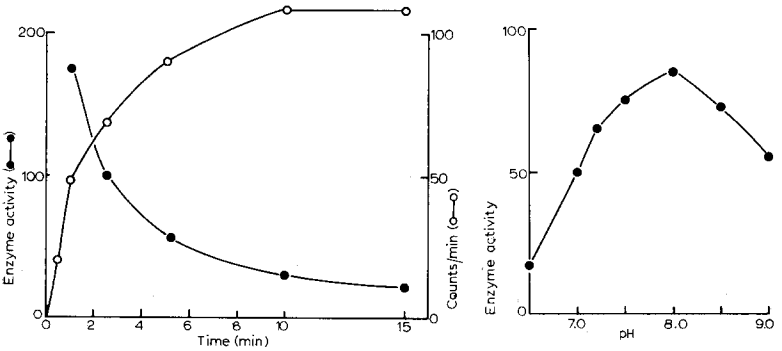

Fig. 3. Adenyl cyclase activity as a function of time of incubation. The system contained 95 μg of membrane protein and ATP, $MgCl_2$ and buffer as given in Fig. 1. ●—●, enzyme activity as nmoles/mg protein per h; ○—○, amount of cyclic AMP formed as counts/min.

Fig. 4. The effect of pH on the adenyl cyclase activity. The system contained 80 μg of membrane protein, 0.3 mM ATP, 1 mM $MgCl_2$ and 20 mM buffer. For pH 7.0–9.0 Tris buffer was used. For pH 6.5, phosphate buffer was used. Incubation was carried out at 37° for 10 min. Enzyme activity as nmoles/mg protein per h.

time period. The drop in specific activity is related in part to the concomitant drop in ATP concentration because of an active membrane ATPase. An analysis of ATP during these experiments has shown that 90 % is converted to ADP, AMP and other products within 10 min but the major product is ADP.

The effect of pH on the enzyme is shown in Fig. 4. The optimum pH under conditions of 0.3 mM ATP and 1.0 mM Mg^{2+} is about 8.0. This value is nearly the same as the pH optimum for the erythrocyte adenyl cyclase[18] but is lower than the pH optimum of about 9.0 for the soluble *Escherichia coli* adenyl cyclase[19].

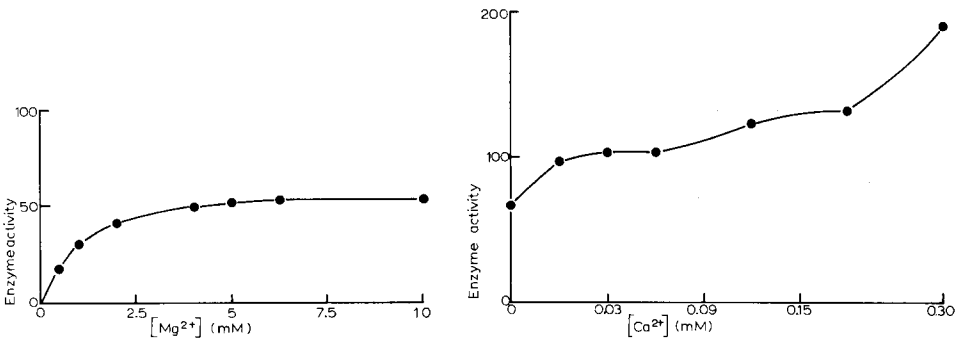

Fig. 5. Effect of Mg^{2+} concentration on adenyl cyclase activity. The system contained 80 μg of membrane protein, 2.5 mM ATP and 20 mM Tris buffer (pH 7.5). The $MgCl_2$ concentration was varied over the range indicated. Incubation was carried out at 37° for 10 min. Enzyme activity as nmoles/mg protein per h.

Fig. 6. Effect of Ca^{2+} concentration on adenyl cyclase activity. The system contained 85.0 μg of membrane protein, 0.3 mM ATP, 1.0 mM $MgCl_2$ and 20 mM Tris buffer (pH 7.5). Incubation was carried out at 37° for 10 min. Enzyme activity as nmoles/mg protein per h.

The effect of Mg^{2+} on adenyl cyclase is shown in Fig. 5. In the presence of added 2.5 mM ATP, the optimal Mg^{2+} concentration is between 4 and 5 mM. The effect of Ca^{2+} on adenyl cyclase is shown in Fig. 6. The stimulation of the enzyme by Ca^{2+} is seen over the concentration range $3 \cdot 10^{-4}$–$3 \cdot 10^{-5}$ M. However, without added Ca^{2+} there is appreciable enzyme activity but without added Mg^{2+} there is essentially no enzyme activity. The latter shows the absolute requirement for Mg^{2+} and supports the concept that a magnesium–ATP complex is the actual substrate.

The effects of combinations of Mg^{2+} and Ca^{2+} are shown in Table I. Ca^{2+} enhances the enzyme activity even in the presence of Mg^{2+}. This effect of Ca^{2+} was more pronounced at higher levels of Ca^{2+}. In this experiment the added ATP was 5 mM. This higher level of ATP was used in order to maintain the concentration of ATP in the presence of the divalent cations.

The effects of Na^+ and K^+, and F^- are seen in Table II. Na^+ at 50 mM inhibited the enzyme whereas K^+ at 50 mM gave a moderate stimulation. The K^+ stimulation prevailed even in the presence of Na^+. F^- inhibited the enzyme. We have repeatedly observed an inhibition of adenyl cyclase by F^-. The Na^+ and K^+ effects are similar to those reported by BIRNBAUMER *et al.*[24] but the F^- effect is different since others[18,24,25] have reported a stimulation of adenyl cyclase by F^-. On the other hand TAO AND LIPMANN[19] have recently shown a strong inhibition of *E. coli* adenyl cyclase by F^-. The reason for this different F^- effect is not clear but may be related to the different

experimental conditions (possibly to the age and state of dispersion of the membrane and to the concentrations of Mg^{2+} and Ca^{2+}). Moreover, since NaF was used part of the inhibition may be due to Na^+ ions.

TABLE I

EFFECT OF Ca^{2+} AND Mg^{2+} ON ADENYL CYCLASE ACTIVITY

The system contained 5 mM ATP, 20 mM Tris buffer (pH 7.5), and plasma membranes. Incubation was carried out for 10 min at 37°. Data are averages of duplicate analyses.

Additions	*Adenyl cyclase activity (nmoles cyclic AMP/mg protein per h)*
Mg^{2+} (2 mM)	30
Mg^{2+} (2 mM) + Ca^{2+} (2 mM)	45
Mg^{2+} (4 mM)	40
Mg^{2+} (4 mM) + Ca^{2+} (4 mM)	60
Mg^{2+} (8 mM)	50
Mg^{2+} (8 mM) + Ca^{2+} (4 mM)	94
Mg^{2+} (8 mM) + Ca^{2+} (8 mM)	95

TABLE II

THE EFFECT OF Na^+, K^+ AND F^- ON ADENYL CYCLASE ACTIVITY

Data are averages ± S.D. of 3 separate analyses except for Na^+ (150 mM) where one analysis is represented. The system contained 0.3 mM ATP, 1 mM Mg^{2+}, 20 mM Tris buffer (pH 7.4) and 60–80 μg of membrane protein. Incubation was done at 37° for 10 min.

Additions	*Adenyl cyclase activity (nmoles cyclic AMP/mg protein per h)*
Control	75 ± 5
Na^+ (50 mM)	46.6 ± 6
Na^+ (150 mM)	20.8
K^+ (50 mM)	90.2 ± 4.8
Na^+ (50 mM) + K^+ (50 mM)	86.6 ± 2.9
F^- (10 mM)	42.2 ± 2

TABLE III

THE EFFECT OF PRODUCTS OF ATP BREAKDOWN ON ADENYL CYCLASE ACTIVITY

Data are averages ± S.D. of 3 separate analyses. The incubation system contained 0.3 mM ATP, 1 mM Mg^{2+}, 0.2 mM Tris buffer (pH 7.4), and 60–80 μg of membrane protein. Incubation was carried out at 37° for 10 min.

Additions	*Adenyl cyclase activity (nmoles cyclic AMP/mg protein per h)*
None – control	70 ± 5
P_i (0.5 mM)	82 ± 3.2
P_i (2.0 mM)	31 ± 5
ADP (0.5 mM)	70 ± 5
P_i (0.5 mM) + ADP (0.5 mM)	67.9 ± 7
ADP (1.25 mM)	28.6 ± 3.5
ADP (2.5 mM)	38 ± 3
ADP (2.5 mM) + P_i (2 mM)	20 ± 5

The analysis of ATP, ADP and AMP in the presence and absence of Na^+ and K^+ showed that in all systems the major amount of ATP was hydrolyzed within 10 min but Na^+ maintained a much higher level of ADP and AMP than did K^+. This may be due to an inhibition of 5'-nucleotidase by Na^+.

The effects of P_i and ADP on adenyl cyclase are given in Table III. P_i alone gave a slight stimulation. ADP at 0.5 mM had no effect but at 2.5 mM gave an appreciable inhibition. These experiments were carried out to see if the addition of P_i and ADP could inhibit the ATPase and thereby lead to a more sustained level of ATP which might increase the path *via* adenyl cyclase. The data are not clear on this point since P_i stimulated adenyl cyclase but ADP inhibited.

The action of detergents on adenyl cyclase is given in Table IV. Triton X-100 and sodium dodecyl sulfate gave a slight stimulation whereas deoxycholate gave a small inhibition of the enzyme. All these detergents cause "solubilization of the membrane" but the membrane particles so obtained are heterogeneous and still have very high molecular weights[27]. It is obvious that alteration of the membrane structure by detergents does not necessarily inhibit the enzyme, but rather can lead to a more active enzyme. However, a large macromolecular state of the cyclase system is believed to be necessary for hormone sensitivity as will be pointed out later.

TABLE IV

EFFECT OF DETERGENTS ON ADENYL CYCLASE ACTIVITY

Data are averages of duplicate analyses. Incubation carried out as in Table II.

Additions	*Adenyl cyclase activity (nmoles cyclic AMP/mg protein per h)*
Control	50
Triton X-100 (0.2%)	57
Deoxycholate (0.2%)	40
Sodium dodecyl sulfate (0.2%)	55

TABLE V

THE EFFECT OF Ca^{2+} ON THE GLUCAGON AND EPINEPHRINE STIMULATION OF ADENYL CYCLASE

Data are averages of duplicate analyses. The system contained 80 μg membrane protein, 0.3 mM ATP, 1.0 mM $MgCl_2$, 20 mM Tris buffer (pH 7.5). Incubation was carried out for 10 min at 37°.

System	*Adenyl cyclase activity (nmoles cyclic AMP/mg protein per h)*
Control	75
+ glucagon ($1 \cdot 10^{-6}$ M)	120
+ glucagon ($1 \cdot 10^{-6}$ M) + Ca^{2+} ($1 \cdot 10^{-5}$ M)	95
+ glucagon ($1 \cdot 10^{-6}$ M) + Ca^{2+} ($0.5 \cdot 10^{-4}$ M)	100
+ glucagon ($1 \cdot 10^{-6}$ M) + Ca^{2+} ($1 \cdot 10^{-4}$ M)	110
+ glucagon ($1 \cdot 10^{-6}$ M) + Ca^{2+} ($1 \cdot 10^{-3}$ M)	135
+ epinephrine ($1 \cdot 10^{-5}$ M)	115
+ epinephrine ($1 \cdot 10^{-5}$ M) + Ca^{2+} ($1 \cdot 10^{-5}$ M)	117
+ epinephrine ($1 \cdot 10^{-5}$ M) + Ca^{2+} ($0.5 \cdot 10^{-4}$ M)	120
+ epinephrine ($1 \cdot 10^{-5}$ M) + Ca^{2+} ($1 \cdot 10^{-4}$ M)	125
+ epinephrine ($1 \cdot 10^{-5}$ M) + Ca^{2+} ($1 \cdot 10^{-3}$ M)	140

The effects of Ca^{2+} on the glucagon and epinephrine stimulation of adenyl cyclase are shown in Table V. At levels below $5 \cdot 10^{-4}$ mM, Ca^{2+} inhibited the glucagon stimulation of the enzyme, but at 10^{-3} mM, Ca^{2+} enhanced the glucagon effect.

The effect of Ca^{2+} on the epinephrine stimulation of adenyl cyclase is considerably different since at all levels ($1 \cdot 10^{-3}$–$1 \cdot 10^{-5}$ M) Ca^{2+} enhanced the epinephrine stimulation of adenyl cyclase. This was not the case with glucagon These results point to a complex interplay of cations and hormones with the membrane. In contrast to our findings, BAR AND HECHTER[28] reported Ca^{2+} was not required for the glucagon and epinephrine stimulation of adenyl cyclase in fat cell ghosts but was required for ACTH stimulation[29]. BIRNBAUMER *et al.*[24] report that 0.1 mM Ca^{2+} inhibits the ACTH-stimulated adenyl cyclase in fat cells but they state that complete removal of Ca^{2+} from fat cell ghosts with a calcium chelator (EGTA) causes a loss of the ACTH-stimulated activity but not the glucagon and epinephrine stimulation of adenyl cyclase. They conclude that Ca^{2+} may be necessary for the interaction of ACTH with a receptor protein.

The effects of Ca^{2+} on adenyl cyclase and its response to hormones may be different in different tissues and may be dependent not only on the absolute amount of Ca^{2+} but on its relation to the Mg^{2+} concentration and on the state of dispersion of the membrane system.

TABLE VI

THE EFFECT OF GLUCAGON, EPINEPHRINE AND INSULIN ON ADENYL CYCLASE ACTIVITY

Data are averages ± S.D. of 4 separate analyses. Incubation carried out as in Table V.

Additions	*Adenyl cyclase activity (nmoles cyclic AMP/mg protein per h)*
Control	75 ± 5
Glucagon ($1 \cdot 10^{-6}$ M)	113.6 ± 6.5
Epinephrine ($1 \cdot 10^{-5}$ M)	99.2 ± 4.4
Insulin ($1 \cdot 10^{-5}$ M)	62.7 ± 4.5
Glucagon ($1 \cdot 10^{-6}$ M) + epinephrine ($1 \cdot 10^{-5}$ M)	130 ± 10
Glucagon ($1 \cdot 10^{-6}$ M) + insulin ($1 \cdot 10^{-5}$ M)	83.3 ± 4.1

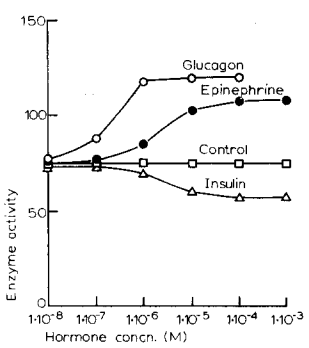

Fig. 7. Dose response curves showing the effect of glucagon, epinephrine, and insulin on adenyl cyclase activity. The incubation system contained 85.0 μg of membrane protein, 0.3 mM ATP, 1.0 mM $MgCl_2$, 20.0 mM Tris buffer (pH 7.5). Incubation was carried out at 37° for 10 min. □—□, control; △—△, with insulin; ●—●, with epinephrine; ○—○, with glucagon.

TABLE VII

THE EFFECT OF HORMONES ON THE UPTAKE OF $^{45}Ca^{2+}$ BY PLASMA MEMBRANES

Incubation carried out as in Table V. Each tube contained 5 μC of $^{45}Ca^{2+}$. The final Ca^{2+} concentration was $1 \cdot 10^{-5}$ M. Tris buffer (pH 7.4) was used at a final concentration of 20 mM.

Additions	*Ca^{2+} bound (pmoles/mg protein)*
Control	300
Glucagon ($1 \cdot 10^{-6}$ M)	34
Glucagon ($1 \cdot 10^{-6}$ M) + insulin ($1 \cdot 10^{-5}$ M)	20
Epinephrine ($1 \cdot 10^{-5}$ M)	1300
Epinephrine ($1 \cdot 10^{-5}$ M) + glucagon ($1 \cdot 10^{-6}$ M)	1284

TABLE VIII

EFFECT OF PCMB ON ADENYL CYCLASE ACTIVITY

Data are averages of two experiments. The incubation system contained 1 mM ATP, 4 mM $MgCl_2$ and 40 mM Tris buffer (pH 7.4). Incubation was carried out for 15 min at 37°.

System	*Adenyl cyclase activity (nmoles/mg protein per h)*
Control	40.6
PCMB ($5 \cdot 10^{-5}$ M)	40.3
PCMB ($1 \cdot 10^{-4}$ M)	63

The interplay of hormones was examined and the data are shown in Table VI. The usual stimulation by glucagon and epinephrine alone are seen. Insulin gave a small inhibition of adenyl cyclase activity and also neutralized to a large extent the glucagon stimulation. In contrast to this effect of insulin on glucagon, the adenyl cyclase activity was enhanced by epinephrine *plus* glucagon and was greater than with each hormone alone, but the combined effects are not additive. Similar non-additive effects of these hormones were also reported by BIRNBAUMER AND RODBELL[21] for the fat cell adenyl cyclase.

The dose response of the hormones on adenyl cyclase is shown in Fig. 7. The stimulation by glucagon and epinephrine is seen to reach a plateau at the higher hormone concentration. The glucagon stimulation is seen at $1 \cdot 10^{-6}$ M whereas $1 \cdot 10^{-5}$ M epinephrine is needed for its stimulation of adenyl cyclase. The inhibition by insulin begins at approx. $1 \cdot 10^{-5}$ M.

In order to elucidate the interplay of hormones with Ca^{2+}, a study was carried out to see how glucagon and epinephrine influence the binding of Ca^{2+} to the membrane. This data is shown in Table VII. Glucagon inhibited Ca^{2+} binding and insulin had little effect on this action of glucagon. On the other hand epinephrine stimulated Ca^{2+} binding and glucagon was not able to diminish this action of epinephrine. These studies were done at low levels of Ca^{2+}. It will be recalled in Table V that low levels of Ca^{2+} inhibited the glucagon stimulation of adenyl cyclase but enhanced the stimulation of this enzyme by epinephrine. These data suggest that there is a different mode of interaction of these hormones with Ca^{2+}. In the next paper we will present evidence that the hormones bind to specific receptor proteins on the membrane. It is

apparent that the membrane system is complex and that a subtle and delicate interplay exists between the hormones, receptor proteins, ions, and adenyl cyclase.

The effect of *p*-chloromercuribenzoate (PCMB) on adenyl cyclase activity is given in Table VIII. At $1 \cdot 10^{-5}$ M PCMB no effect was seen but at $1 \cdot 10^{-4}$ M PCMB an enhancement of the enzyme activity occurred. The relationship of this effect of PCMB and its effect on hormone binding will be discussed in the subsequent paper[22].

DISCUSSION

The mechanism of action of hormones at the molecular level represents a challenging problem and frontier area in modern biology. Certain hormones such as epinephrine, glucagon and insulin appear to act at the membrane level although their mode of action remains obscure. The stimulation of cyclic AMP production by glucagon and epinephrine in liver systems coupled with the finding that adenyl cyclase is localized in the plasma membrane and is stimulated *in vitro* by these hormones has led to a general mechanism of action of these hormones. This mechanism, proposed by SUTHERLAND and co-workers[1-3] considers hormones to be primary messengers which act on the membrane to stimulate the membrane bound adenyl cyclase. This leads to increased levels of cyclic AMP which acts as a second messenger. However, this mechanism does not state how the hormones bring about the activation of this enzyme. Do the hormones act directly with the adenyl cyclase or do they bind to specific receptors which then bring about a stimulation of the enzyme by some allosteric modification of the membrane structure? How does the coupling take place between the receptor and the adenyl cyclase?

POHL *et al.*[20] have reported the stimulation of adenyl cyclase in isolated liver plasma membranes by glucagon but not by epinephrine. The former finding is significant since glucagon stimulates adenyl cyclase *in vivo*. However, the latter finding is not in accord with *in vivo* and *in vitro* studies which show that epinephrine also stimulates adenyl cyclase. MARINETTI *et al.*[16] showed the localization of adenyl cyclase in the plasma membrane of rat liver and the activation of this membrane bound enzyme in isolated plasma membranes by both glucagon and epinephrine. BIRNBAUMER AND RODBELL[21] later reported the stimulation of adenyl cyclase of fat cell plasma membrane by both glucagon and epinephrine. These workers carried out a study on the properties of the fat cell adenyl cyclase and postulated the existence of hormone receptors in the membrane. However no data was given relating to the binding of hormones to the membrane. Epinephrine binding to the isolated liver plasma membrane was recently published by MARINETTI *et al.*[16].

This present paper gives the properties of the liver cell plasma membrane adenyl cyclase and its stimulation by glucagon and epinephrine and its inhibition by insulin. The pH optimum of liver adenyl cyclase is 8.0. A similar pH optimum has been reported for the frog erythrocyte adenyl cyclase[18]. Although we found the optimum concentration of ATP to be 0.3 mM, the significance of this value is difficult to assess since the isolated liver cell plasma membranes (and the fat cell membrane and erythrocyte membrane) contains other enzymes which act on ATP and on ADP and AMP. These enzymes are ATPase, myokinase; ATP pyrophosphohydrolase and 5'-nucleotidase. Hence the actual concentration of ATP added to the membranes is very rapidly lowered by the active ATPase and partially regenerated by the myokinase.

BIRNBAUMER AND RODBELL[21] and BAR AND HECHTER[29] attempted to circumvent this problem by adding an ATP regenerating enzyme to their systems. However, unless the ATP levels are actually measured at different time intervals, the true concentration of ATP remains unknown. Moreover in membrane systems the effective concentration of substrates, and ions and the actual pH in the region of the enzyme can differ greatly from their values in the bulk phase. In spite of these uncertainties one can demonstrate an "apparent" optimum pH and "apparent" optimal levels of ATP, Mg^{2+} and Ca^{2+} in the isolated membrane systems under specified experimental conditions. The biological significance of these results can only be speculative at present. It seems clear, however, that a complex interplay exists between hormones, metal cations and adenyl cyclase. Data in the next paper will demonstrate that the hormones bind to receptor proteins in the membrane and that hormone binding to the plasma membrane is highly specific when compared to other cell fractions. The binding of the hormones to the receptor proteins does not necessarily mean that this is directly related to their biological action or to their stimulation of adenyl cyclase although it favors such a hypothesis.

The column fractionation of sonicated and detergent treated membranes showed two peaks for adenyl cyclase. A similar finding was reported by ROSEN AND ROSEN[18] for frog erythrocyte membrane bound adenyl cyclase. This raises the question whether different adenyl cyclases respond specifically to certain hormones.

ADDENDUM (Received April 27th, 1970)

BITENSKY *et al.*[31,32] have provided evidence for the existence of two discrete membrane bound hepatic adenyl cyclases in the adult male rat. One adenyl cyclase system responds to glucagon and the other responds to epinephrine. These adenyl cyclase systems also vary separately as a function of age, sex and steroid hormone levels. REIK *et al.*[33] have recently provided electron microscopic data suggesting the preferential localization of isoproterenol-sensitive adenyl cyclase in the parenchymal cells of rat liver and the preferential localization of the glucagon-sensitive adenyl cyclase in the reticuloendothelial cells.

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REFERENCES

1 E. W. SUTHERLAND, I. OYE AND R. W. BUTCHER, *Recent Progr. Hormone Res.*, 21 (1965) 623.
2 E. W. SUTHERLAND AND G. A. ROBISON, *Pharmacol. Rev.*, 18 (1966) 145.
3 G. A. ROBISON, R. W. BUTCHER AND E. W. SUTHERLAND, *New Engl. J. Med.*, 276 (1967) 187.
4 J. ORLOFF AND J. HENDLER, *Am. J. Med.*, 42 (1967) 757.
5 E. W. SUTHERLAND AND T. W. RALL, *Pharmacol. Rev.*, 12 (1960) 265.
6 N. HAUGAARD AND M. E. HESS, *Pharmacol. Rev.*, 17 (1965) 27.
7 D. B. STONE AND T. E. MANSOUR, *Mol. Pharmacol.*, 3 (1967) 161.
8 J. S. BISHOP AND J. LARNER, *Biochim. Biophys. Acta*, 171 (1969) 374.
9 D. A. WELSH, J. P. PERKINS AND E. G. KREBS, *J. Biol. Chem.*, 243 (1968) 3763.
10 T. A. LANGON, *Science*, 162 (1968) 579.

11 J. F. KNO AND P. GREENGARD, *J. Biol. Chem.*, 244 (1969) 3417.
12 P. R. DAVOREN AND E. W. SUTHERLAND, *J. Biol. Chem.*, 238 (1963) 3009.
13 I. OYE AND E. W. SUTHERLAND, *Biochim. Biophys. Acta*, 127 (1966) 347.
14 M. RODBELL, *J. Biol. Chem.*, 242 (1967) 5744.
15 M. RODBELL, A. B. JONES, G. E. CHIAPPE DE CINGOLANE AND L. BIRNBAUMER, *Recent Progr. Hormone Res.*, 24 (1968) 215.
16 G. V. MARINETTI, T. K. RAY AND V. TOMASI, *Biochem. Biophys. Res. Commun.*, 36 (1969) 185.
17 D. W. MCKEEL AND L. JARETT, *Federation Proc.*, 28 (1969) 879.
18 O. ROSEN AND S. M. ROSEN, *Arch. Biochem. Biophys.*, 131 (1969) 449.
19 M. TAO AND F. LIPMANN, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 86.
20 S. L. POHL, L. BIRNBAUMER AND M. RODBELL, *Science*, 164 (1969) 566.
21 L. BIRNBAUMER AND M. RODBELL, *J. Biol. Chem.*, 244 (1969) 3477.
22 V. TOMASI, S. KORETZ, T. K. RAY, J. DUNNICK AND G. V. MARINETTI, *Biochim. Biophys. Acta*, 211 (1970) 31.
23 T. K. RAY, *Biochim. Biophys. Acta*, 196 (1970) 1.
24 L. BIRNBAUMER, S. L. POHL AND M. RODBELL, *J. Biol. Chem.*, 244 (1969) 3468.
25 E. W. SUTHERLAND, T. W. RALL AND J. MENON, *J. Biol. Chem.*, 237 (1962) 1220.
26 P. E. CRYER, L. JARETT AND D. M. KIPNES, *Biochim. Biophys. Acta*, 177 (1969) 586.
27 T. K. RAY, V. TOMASI AND G. V. MARINETTI, *Federation Proc.*, 28 (1969) 891.
28 H. P. BAR AND O. HECHTER, *Proc. Natl. Acad. Sci. U.S.*, 1969, in the press.
29 H. P. BAR AND O. HECHTER, *Biochem. Biophys. Res. Commun.*, 35 (1969) 681.
30 P. R. DAVOREN AND E. W. SUTHERLAND, *J. Biol. Chem.*, 238 (1963) 3016.
31 M. W. BITENSKY, V. RUSSELL AND W. ROBERTSON, *Biochem. Biophys. Res. Commun.*, 31 (1968) 706.
32 M. W. BITENSKY, V. RUSSELL AND M. BLANCO, *Endocrinology*, 86 (1970) 154.
33 L. REIK, G. L. PETZOLD, J. A. HIGGINS, P. GREENGARD AND R. J. BARNETT, *Science*, 168 (1970) 382.