sarcomas, two reticulum cell sarcomas, two anaplastic sarcomas, and two osteogenic sarcomas. Finally, since infectious SV40 was not recovered from the cells of two lymphosarcomas, three anaplastic sarcomas, and three osteosarcomas when grown in culture for 1 week in the absence of GMK indicator cells, it appears that the SV40 T antigen is not an expression of a recurrent infectious process, but rather, it is a manifestation of the oncogenic state.

The above data indicate that, under appropriate experimental conditions (*9*) relating both to the host (species, age) and to the viral agent (dosage, route of inoculation), the DNA virus SV40 can induce leukemia, lymphoma, and osteosarcoma in addition to the anaplastic sarcoma with which it is already known to be associated. These findings do not support the view held by some investigators that viruses which induce experimental leukemia, lymphoma, and osteosarcoma are always of the RNA type (*1, 10*). It should be of great interest, therefore, to determine whether other oncogenic DNA viruses (polyoma virus, adenoviruses) can induce, under comparable experimental conditions, hematopoietic, lymphoreticular, and osteomesenchymal malignant neoplasms. This may well prove to be the case, since evidence has been presented which suggests that polyoma virus may rarely cause osteosarcoma in the mouse (*11*), and that herpes DNA viruses can induce avian (*2*) and simian (*3*) lymphomatous proliferations. Furthermore, there is some evidence suggesting that a herpes-type virus, the Epstein-Barr agent (*12*), may be related etiologically to Burkitt's lymphoma, a neoplasm that affects predominantly children living in Africa (*13*).

Although a decision on the issues raised should await the results of further inquiry, we can now state with assurance that, for the first time, a DNA virus other than a member of the herpes group has been implicated in the experimental induction of leukemia, lymphoma, and osteosarcoma. It is evident, therefore, that, in attempting to isolate and identify viral agents possibly involved in analogous diseases of man, attention should be directed toward DNA viruses.

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7. Antiserum to SV40 was derived from rabbits immunized with strain HDCS-DHK of SV40 (Pitman-Moore). It had a neutralization titer in vitro 1 : 1280, against 100 $TCID_{50}$ of SV40. Normal rabbit control serum failed when diluted 1 : 4 to neutralize 100 $TCID_{50}$ of SV40. Both types of serums were inactivated at 56°C for 30 minutes before use. The virus-serum mixtures were incubated at 37°C for 2 hours prior to inoculation. Both rabbit serums (VR-3002, VR-3000) were purchased from Grand Island Biological Co.
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Hormone-Calcium Interactions with the Plasma Membrane of Rat Liver Cells

Abstract. *The binding constants and the number of binding sites for insulin, glucagon, epinephrine, cyclic adenosine monophosphate, and calcium ions for the plasma membrane of rat liver were determined by Scatchard plots. The plots are biphasic or multiphasic, an indication of at least two types of binding sites for each ligand. At least three types of binding sites were found for insulin. In the concentration range of 10^{-6} to 10^{-8} molar, glucagon, epinephrine, and hydrocortisone increased calcium ion binding to the plasma membrane, whereas insulin decreased this binding. At hormone concentrations of 10^{-6} to 10^{-7} molar, glucagon was the most effective in increasing calcium binding, but at a hormone concentration of 10^{-8} molar, hydrocortisone was the most effective in stimulating calcium binding. Adenosine triphosphate reversed the effect of insulin and inhibited the effect of the other hormones. These studies suggest a relation between hormones and calcium with respect to membrane structure and function.*

Certain hormones, such as glucagon, epinephrine, and insulin, probably exert at least part of their physiological efects at the level of the cell plasma membrane (*1–4*). Rasmussen (*5*) has proposed a model to integrate the relation between hormone, calcium ion, and cyclic adenosine monophosphate (cyclic AMP). In this model, certain hormones act to stimulate the membrane-bound adenylate cyclase that produces cyclic AMP from adenosine triphosphate (ATP). The cyclic AMP then activates protein kinases in the cytoplasm which, in turn, phosphorylate several enzymes or contractile proteins within the cell to modulate the activities of these macromolecules. A second controlling influence in the cell is postulated to be the calcium ion (Ca^{2+}). The mobilization of calcium from intracellular pools, or its influx from extracellular fluid, are thought to occur by hormonal or electrical stimulation of the cell plasma membrane or by an action of cyclic AMP on intracellular membranes. The increased intracellular concentration of Ca^{2+} may induce other enzyme reactions within the cell, or it may act as a negative feedback control on adenylate cyclase. Calcium pumps are also considered to play a role in the active efflux of calcium from the cell. Rasmussen (*5*) suggests

that certain hormones interact with their receptors in the membrane to simultaneously increase calcium permeability and adenylate cyclase activation as has been shown in many experiments in which excitation of cells is followed by a rise in concentration of cyclic AMP. In some systems this rise is accompanied by an increased uptake of Ca^{2+} into the cell or a requirement for Ca^{2+} in the external medium.

Hormones may have more than one action on cell membranes. They may activate membrane-bound adenylate cyclase, as has been demonstrated in isolated plasma membranes from liver (*6–10*), and in isolated fat cells (*11*). The effect of insulin on glucose transport in muscle and fat cells is well established (*1–4*). However, insulin does not penetrate the fat cell (*12*) yet it produces other effects in the cell which are independent of glucose transport. Apparently, insulin has at least two independent actions on fat cells.

The binding of insulin, glucagon, and epinephrine to isolated plasma membranes of the rat liver or to isolated fat cells has recently been reported (*6, 9, 13–22*). We demonstrate here that hormones bind to plasma membranes and can influence the binding of Ca^{2+} to isolated plasma membranes. These effects are independent of the adenylate cyclase system, and they suggest a relation between potentially important hormones and calcium with respect to membrane structure and function.

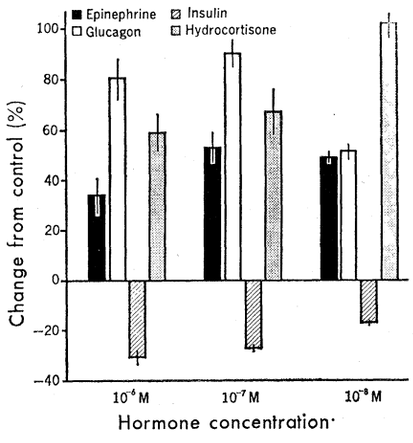

Fig. 1. The effect of hormones on the binding of calcium ions to the plasma membrane. Membranes (60 to 70 μg of protein) were incubated with 0.001*M* $CaCl_2$ (containing 0.5 μc of ^{45}Ca) for 10 minutes at 37°C in a total volume of 1.0 ml containing 0.1*M* tris buffer, *p*H 7.5, and varying amounts of hormone. The binding of ^{45}Ca was determined as described in Table 1.

Plasma membranes of rat liver were prepared by the method of Ray (*23*) and washed with ethylenediaminetetraacetate (EDTA) and 50 m*M* tris(hydroxymethyl)aminomethane (*p*H 7.4) before use. The [^{131}I]insulin (Insulin 131-H Imusay, specific activity, 287 mc/mg; Abbott) was purified by column chromatography on Sephadex G-10; this labeled insulin contains less than 1 atom of iodine per insulin molecule. The [^{125}I]insulin (specific activity, 110 mc/mg) with 1 atom of iodine per insulin molecule was prepared by the method of Izzo *et al.* (*24*). The [^{131}I]-glucagon (specific activity, 114 mc/mg) (*25*) contained approximately 1 atom of iodine per molecule of glucagon, and was homogeneous after Sephadex G-10 chromatography. The specific activities of other labeled hormones used is as follows: DL-7-[^{3}H]epinephrine, 7 to 14 c/mmole (New England Nuclear); $^{45}CaCl_2$, 6 mc per milligram of calcium (I.C.N.); [^{3}H]cyclic AMP, 24 c/mmole (New England Nuclear). The hydrocortisone was unlabeled (Nutritional Biochemical). Protein was determined, throughout the experiments, by the method of Lowry *et al.* (*26*).

Scatchard plots (*27*) of the bound hormones, cyclic AMP, and calcium were used to determine the binding constants and the number of binding sites per milligram of membrane protein. The results of these analyses are shown in Table 1. All plots were biphasic, an indication of at least two types of binding sites. The high affinity sites are believed to represent the physiologically important sites. The low affinity sites probably represent nonspecific binding. The lowest hormone concentration was that for insulin (10^{-9} to $10^{-11}M$). The high affinity site was saturated at about $10^{-9}M$ insulin and had a binding constant of $10^{10}\,M^{-1}$. A dissociation constant of $6.7 \times 10^{-11}M$ has been calculated for insulin binding to crude liver membranes (*19*).

The binding constants for cyclic AMP and calcium ions are much lower than those for the hormones, and the number of binding sites are much greater. This is not unexpected for calcium since Ca^{2+} can bind to a number of ionized anionic functional groups on the membrane (carboxyl and phosphate groups). The binding of cyclic AMP was weak and could not be observed by the Millipore filtration method.

The binding of calcium to isolated plasma membranes has the following characteristics (*28*): (i) an optimum *p*H of 7.8 to 7.9; (ii) saturation kinetics where equilibrium is attained after 10 to 20 minutes; (iii) a binding that is inhibited by EDTA and Mg^{2+} ions, but not by Na^{1+} or K^{1+}; (iv) a binding that is decreased by prior treatment of the membrane with Pronase, trypsin, neuraminidase, and phospholipase C, but increased by treatment of the membrane with phospholipase D.

The effect of hormones on binding of calcium to the membrane is shown in Fig. 1. Epinephrine, glucagon, and

Table 1. Binding of glucagon, insulin, epinephrine, cycle AMP, and Ca^{+2} to the plasma membrane. Binding of glucagon and insulin was determined by incubating membranes (60 to 70 μg of protein) for 10 minutes at 37°C in 1 ml of 0.1*M* tris(hydroxymethyl)-aminomethane buffer (tris), *p*H 7.5. Glucagon (0.5 to 6.0 μc) was tested over the range 10^{-5} to $10^{-8}M$. Insulin (0.5 to 0.05 μc) was used from 10^{-5} to $10^{-11}M$. After 10 minutes, 2 ml of ice-cold 0.1*M* acetic acid were added, and the membranes were centrifuged at 2800 rev/min for 15 minutes. The supernatant was discarded, and the membrane pellet was washed twice with 2 ml of 0.1*M* acetic acid. The pellet was dissolved in 1 ml of solubilizer (NCS, Nuclear-Chicago), and was then diluted with 10 ml of Brays scintillation solution. The binding of epinephrine and calcium was determined by incubating membranes (60 to 70 μg of protein) for 10 minutes at 37°C in 1 ml of 0.1*M* tris buffer, *p*H 7.5. Epinephrine (0.6 μc) was tested at concentrations of 10^{-5} to $10^{-8}M$, and calcium (0.5 μc) was tested at concentrations of 10^{-3} to $10^{-7}M$. After 10 minutes, the membrane suspension was filtered through HA 0.45 μm Millipore filter disks (diameter, 25 mm), and washed two times with 5 ml of tris buffer. The filter disks were placed in counting vials containing 10 ml of Brays solution, and the radioactivity was measured (Packard liquid-scintillation spectrometer). Binding of cyclic AMP (10^{-4} to $10^{-8}M$, containing 0.01 μc of cyclic AMP) was measured by equilibrium dialysis as no binding was detected by the Millipore procedure. The K_2 for cyclic AMP was very small and is not included in the table.

Order	Association constant (M^{-1})		Binding sites (picomoles per milligram of membrane protein)
	Glucagon		
K_1	9.4×10^8	n_1	50
K_2	3.1×10^4	n_2	1300
	Insulin		
K_1	1×10^{10}	n_1	0.1
K_2	4.1×10^6	n_2	56
K_3	1.3×10^5	n_3	1270
	Epinephrine		
K_1	1.8×10^7	n_1	13
K_2	1.4×10^5	n_2	162
	Ca^{2+}		
K_1	4.0×10^3	n_1	22,000
K_2	3.1×10^2	n_2	126,000
	Cyclic AMP		
K_1	7.3×10^4	n_1	41,000

hydrocortisone at concentrations of 10^{-6} to $10^{-8}M$, increased Ca^{2+} binding whereas insulin decreased Ca^{2+} binding. At hormone concentrations of 10^{-6} to $10^{-7}M$, glucagon had the greatest effect, but at $10^{-8}M$, hydrocortisone exerted the greatest effect. Glucagon and epinephrine both stimulated binding most at $10^{-7}M$, but the stimulation by hydrocortisone increases with decreasing hormone concentration. With hydrocortisone, a hydrophobic hormone, the physical state of dispersion may influence its effect on the membrane.

Adenosine triphosphate at a concentration of 0.3 mM abolished the effect of insulin, and decreased the effect of the other hormones.

Inasmuch as the concentration of each hormone is very small, the hormone effects cannot be stoichiometrically related to the Ca^{2+} binding. In these systems we calculate that at $10^{-8}M$ hydrocortisone, one molecule of hormone leads to a binding of 3000 atoms of calcium. This postulates that the hormones act catalytically or that they induce conformational changes over a large segment of the membrane, modifying the binding of many calcium ions.

The effect of insulin opposite to that of the other hormones provides additional evidence for the antagonistic action of insulin with glucagon and epinephrine. Insulin antagonizes the glucagon stimulation of adenylate cyclase in isolated plasma membranes of rat liver (*8, 10*).

With respect to the model of Rasmussen (*5*), our studies suggest that the hormone effects on calcium binding may also be related to calcium transport through the membrane. We demonstrated that hormones such as glucagon, epinephrine, and insulin have two independent effects on the membrane, one being to either activate or inhibit adenylate cyclase activity, and the other being to stimulate or inhibit calcium binding. Although the concentration of hormones that is required to influence calcium binding is higher than the physiological concentration (insulin and glucagon have concentrations in portal blood of 10^{-9} to $10^{-10}M$), the effects may have physiological significance as the isolated membranes might have lower biological sensitivity than do membranes on intact cells.

Inasmuch as the amount of calcium bound to the membrane influences the permeability of the membrane, our studies also may suggest that certain hormones influence membrane permeability by controlling the amount of bound metal ions. Thus, insulin may make the cell membrane more permeable to certain substrates by decreasing the amount of bound calcium ions.

The significance of the ATP effect on modifying the calcium binding due to hormones is not clear. Whether this is related to the chelating property of ATP, to ATP altering the membrane structure, or indirectly to the cyclic AMP formed from ATP is not known. In the membrane systems we used, cyclic AMP is bound very weakly to the membrane and stimulates Ca^{2+} binding only at very high concentrations (10^{-3} to $10^{-5}M$) of cyclic AMP.

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Norepinephrine and Dopamine: Assay by Mass Fragmentography in the Picomole Range

Abstract. *Gas chromatography–mass spectrometry makes possible the simultaneous measurement of norepinephrine and dopamine in concentrations of 0.1-milligram tissue samples. Specificity of the assay is confirmed both by the retention time of the compound and by the mass to charge ratio of the fragments recorded. The sensitivity is of the order of 0.5 picomole, and linearity of the response is maintained up to at least 200 picomoles.*

A number of simple biochemical models of adrenergic neurons predict that small pools of catecholamines are preferentially released by nerve impulses (*1*). Since only circumstantial evidence supports this prediction, these pools must now be identified, characterized, and measured if these multicompartment models are to guide future investigation on the molecular nature of the mechanisms regulating adrenergic function.

These clarifications could be obtained if a method were available to measure femtomole (10^{-15} mole) concentrations of norepinephrine (NE) and dopamine (DA) in small samples of tissue. Accordingly, we have directed our attention to the analytical technique of combined gas chromatography (GC) and mass spectrometry (MS) (*2*). Identification of an unknown substance by MS usually requires at least 10^{-6} mole of a compound to record its fragmentation pattern and establish its identity. When the fragmentation pattern of the compound is known, it is then possible to identify as little as 3×10^{-15} mole of the substance by the technique of mass fragmentography