ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

Fusion of clonal neuroblastoma cells with rat glioma cells yielded clonal hybrid cell lines which synthesize, store and excrete acetylcholine; properties which are not expressed by the parental cell lines. Cells from one hybrid line were found to form synapses with cultured striated muscle cells. Synapses between hybrid cells and muscle cells closely resemble the synapses between normal motor neurons and striated muscle before they are fully developed. Under appropriate conditions, hybrid cells establish synaptic connections with virtually every muscle cell tested; thus, synaptic connections are formed in abundance. Marked differences were observed in the efficiency of transmission across different synapses. Axonal activities which were found to be regulated include choline acetyltransferase, acetylcholinesterase, Na^+ action potential ionophore specific activities, and the rate of choline transport into cells.

Eight species of receptors have been found thus far with the hybrid cell line which forms synapses. Receptor mediated shifts in cAMP levels, cGMP levels and membrane potentials have been identified and characterized. Thus, the foundation has been laid for studies on the effects of receptor-mediated reactions on synaptic transmission. In addition, more than 100 cell lines which synthesize acetylcholine have been obtained and are being studied to determine whether some cell lines are defective with respect to synapse formation. Much remains to be done but it seems clear that the experimental approach and model systems which have been established afford extraordinary opportunities to explore synapse properties and correlate biochemical events with developmental and electrophysiological phenomena.

Synaptogenesis by normal neurons also was studied. Neurons dissociated from chick embryo retina and maintained in vitro were found to reaggregate and form in vitro approximately 1×10^9 synapses per mg of protein. Three types of synapses and several subtypes were identified which closely resemble those of the intact retina. Studies with this system are described in other sections of this report.

A histochemical technique for detecting and localizing nicotinic acetylcholine receptors was devised previously which depends upon the formation of a complex between peroxidase coupled to an antibody for α-bungarotoxin and the nicotinic acetylcholine receptor. Using this method, clusters of nicotinic acetylcholine-receptors on cultured muscle cells were shown to contain at least 7 times the concentration of receptors found in other membrane regions. Receptor clusters are not characteristically associated with folds in the plasma membrane.

Insert (1) ~~The hybrid cells which form synapses possess abundant morphine receptors.~~ Morphine and other narcotics ~~were shown to be~~ potent inhibitors of adenylate cyclase in cells which possess opiate receptors but not in cells which lack these receptors. Exposure of cells with opiate receptors to morphine for 12 to 48 hours results in ~~an~~ increase in adenylate cyclase activity ~~which compensates for the inhibition of enzyme activity by morphine.~~ Cells have normal

cAMP levels and appear tolerant to morphine because the increase in adenylate cyclase activity is approximately equal to the inhibition of enzyme activity by morphine. However, the cells then are dependent upon morphine to maintain normal cAMP levels. Withdrawal of morphine, or displacement of the narcotic from the opiate receptor by the antagonist, naloxone, reverses the inhibition and results in the synthesis of abnormally high levels of cAMP. Thus, dual regulation of adenylate cyclase by narcotics accounts for the phenomena of narcotic dependence and tolerance. The recently discovered endogenous opiate peptides, Met-enkephalin and Leu-enkephalin, also were shown to be potent inhibitors of adenylate cyclase. These results show that the endogenous opiate peptides and narcotics act as pleiotropic regulators of other species of receptors which are coupled to the activation of adenylate cyclase. By this mechanism opiates alter the perception of neurons to incoming messages.

A cell line with muscarinic inhibitory acetylcholine receptors, and another line with muscarinic excitatory receptors were found. The number of receptors, the receptor affinities for cholinergic ligands and other receptor properties were determined by measuring the binding of [^{3}H]-quinuclidinyl benzilate to receptors. The apparent dissociation constant of the ligand·receptor complex is 6×10^{-11} M. Activation of muscarinic acetylcholine receptors results in a transient increase in cGMP, and a profound, long-lived inhibition of adenylate cyclase activity. Exposure of cells to acetylcholine or carbachol for 24 hours markedly decreases the number of acetylcholine receptors and increases adenylate cyclase activity 50 to 500%. Removal of carbachol results in the return of adenylate cyclase activity and acetylcholine receptor levels to normal values after approximately 3 and 24 hours, respectively. Similarly, exposure of cells with α-receptors to norepinephrine inhibits adenylate cyclase activity and elicits a delayed, compensatory increase in adenylate cyclase activity. These results show that prolonged activation of α-receptors, muscarinic acetylcholine receptors, or opiate receptors results in cell tolerance to and dependence upon norepinephrine, acetylcholine, or opiates, respectively. Withdrawal of the receptor activator elevates intracellular cAMP levels and shifts cells to a supersensitive state with respect to other species of receptors which activate adenylate cyclase.

Desensitization of muscarinic receptors decreases the affinity of the receptor for agonists by a process which exhibits negative cooperativity; whereas interactions between the receptor and antagonists are not cooperative. Both muscarinic excitatory and inhibitory acetylcholine receptors were solubilized and the properties of membrane-bound and soluble receptors were compared.

Chick embryo retina was found to be a rich source of both muscarinic and nicotinic acetylcholine receptors. Both muscarinic and nicotinic acetylcholine receptors are synthesized before synapses appear in the retina; however, during development, nicotinic acetylcholine receptors become associated predominantly with neurites in the synaptic layers of the retina. The properties of muscarinic acetylcholine receptors were determined at different developmental ages and were compared with the properties of muscarinic inhibitory and excitatory receptors of neuroblastoma cells.

Evidence for a new type of PGE_1 receptor coupled to cGMP accumulation was obtained. Cell lines with PGE_1 receptors coupled only to cAMP were found as well as cell lines with 2 species of PGE_1 receptors, one coupled to cAMP accumulation, the other to cGMP accumulation. The 2 species of PGE_1 receptors also desensitize at different rates. These results show that the coupling of PGE_1 to increases in cAMP and cGMP levels are clonally inherited properties which can be expressed independently.

Two pathways for γ-aminobutyric acid synthesis were found in chick embryo retina. The first pathway depends upon the conversion of putrescine to ornithine, catalyzed by ornithine decarboxylase and the subsequent conversion of ornithine to γ-aminobutyric acid. The second route of synthesis is dependent upon the conversion of glutamic acid to γ-aminobutyric acid, catalyzed by glutamic acid decarboxylase. Elevation of cAMP levels in neuroblastoma cells was shown to induce ornithine decarboxylase activity. In the developing embryo, neurotransmitters which affect cAMP levels may regulate ornithine decarboxylase activity and thereby control the rate of GABA synthesis from ornithine.

Previous results led to the conclusions that (1) veratridine, batrachotoxin, and aconitine activate the action potential Na^+ ionophore by interaction with a single class of sites; (2) scorpion venom activates the ionophore by interaction with a different class of sites; (3) two species of toxin bound to separate sites are allosterically coupled and interact in a cooperative manner; and (4) tetrodotoxin and saxitoxin act at a 3rd site which is involved in ion transport.

A toxin which activates the action potential Na^+ ionophore has been purified from scorpion venom. The toxin binds to a single class of sites and acts cooperatively with each of the three alkaloids. Depolarization of cells causes a 30-fold increase in the apparent dissociation constant. The results suggest that the scorpion toxin binds to a voltage sensitive component of the Na^+ ionophore that acts cooperatively in regulating ion transport activity. Binding studies with an ^{125}I-labelled derivative of scorpion toxin showed that the concentration of toxin binding sites is approximately 3 to 6 fmole per mg protein.

Clonal skeletal muscle myoblasts have substantial action potential Na^+ ionophore activity. A small increase in activity accompanies cell fusion. The activity in both myoblasts and myotubes is relatively insensitive to inhibition by saxitoxin and tetrodotoxin and thus resembles denervated rat striated muscle which has been shown to be relatively insensitive to these toxins. Chronic electrical stimulation of muscle cells <u>in vitro</u> does not increase tetrodotoxin sensitivity.

At least 3 ionophores are involved in the action potential in adult heart: a rapidly activated axon-like Na^+ ionophore responsible for the rising phase of the action potential, a slower Ca^{++}/Na^+ ionophore responsible for the plateau phase, and a K^+ ionophore responsible for the repolarization phase. Studies with specific inhibitors of the fast Na^+ ionophore (tetrodotoxin) and the slow Ca^{++}/Na^+ ionophore (D-600) show that the role of these two types of ionophore in beating changes during development of the embryonic chick heart.

In early embryonic hearts, the fast Na^+ ionophore is present but is not required for beating. During development *in ovo* or in monolayer or aggregate culture *in vitro*, changes in the requirement for activity of the fast Na^+ ionophore in beating are accompanied by changes in the sensitivity of the slow Na^+/Ca^{++} ionophore to D-600. When the slow Na^+/Ca^{++} ionophore is able to maintain beating without participation of the fast Na^+ ionophore, its sensitivity to D-600 is high whereas when the fast Na^+ ionophore is required for beating, the slow Na^+/Ca^{++} ionophore is relatively insensitive to D-600. Transitions between these two states can be induced in 2 hours *in vitro* by inhibition of beating. Thus, the activity of these ionophores is regulated during development by a process dependent on the rhythmic activity of the cells.

The developmental changes in action potential ionophores of embryonic hearts occur between days 4 and 7 *in ovo*. During this time, vagal innervation of the heart takes place. Consequently we have studied the muscarinic acetylcholine receptors in embryonic hearts using both physiologic and ligand binding methods to determine whether changes in their properties are temporally correlated with changes in the action potential ionophores. In early embryonic hearts, muscarinic agents are ineffective in inhibiting beating. Between days 5 and 7, sensitivity to inhibition by muscarinic agents increases to the adult level. QNB binds specifically to muscarinic acetylcholine receptors in embryonic and adult heart as assessed by competition studies with muscarinic and nicotinic agents. Receptors, as detected by QNB binding, are present in unresponsive early embryonic hearts and the number per mg heart protein does not increase dramatically during development. Receptor desensitization is accompanied by a small change (3 fold increase) in the K_D for muscarinic agonists as measured by competition with ^3H QNB. This change in K_D occurs only in hearts that are responsive to muscarinic agents and thus may be associated with the physiologic action of the receptor. Competition curves for agonists suggest the involvement of negative cooperativity in activation of the receptor. These results suggest that muscarinic acetylcholine receptors, like action potential ionophores, are present in early embryonic hearts in modified "precursor" or "inactive" forms and undergo activation during development.

Studies on the mechanism of catabolite repression in *E. coli* show that glucose inhibits adenylate cyclase activity reversibly in cells treated with toluene, and that phosphate is required for high adenylate cyclase activity and for glucose dependent inhibition of enzyme activity. Other sugars also inhibit adenylate cyclase provided that transport systems for the sugars are induced. Mutant strains of *E. coli* defective in phosphoenolpyruvate:sugar phosphotransferase system components were examined. Cyclic AMP levels were normal in an HPr mutant, but were markedly depressed in a leaky Enzyme I mutant. Adenylate cyclase activity was low in the Enzyme I mutant whereas the HPr mutant had normal enzyme activity. The Enzyme I mutant under starvation conditions exhibits high adenylate cyclase activity and the adenylate cyclase of this mutant is unusually sensitive to variations in carbon source. The addition of phosphoenolpyruvate leads to a substantial increase in adenylate cyclase activity in permeabilized cell preparations of the Enzyme I mutant. These results suggest that Enzyme I is involved in the regulation of adenylate cyclase activity. Studies are in progress to test the hypothesis that Enzyme I interacts with adenylate cyclase and that the PEP-dependent phosphorylation of Enzyme I is responsible for activation of adenylate cyclase.

Levels of cGMP were compared with cAMP levels in E. coli grown under different conditions. The results show that cGMP and cAMP concentrations are inversely coupled in E. coli and that the regulation of cGMP levels can be uncoupled from that of cAMP.

Since specific species of tRNA are involved in amino acid mediated repression and end-product inhibition, the effect of amino acid deprivation upon tRNA of relaxed and stringent strains of E. coli was studied. In relaxed control E. coli, leucine starvation results in the formation of new isoacceptor species of leucine, histidine, arginine, valine, and alanine-specific tRNA and quantitative changes in the concentration of some other isoacceptors. Experiments with stringent strains or the use of uracil starvation or rifampicin addition provided evidence for the de novo synthesis of new species of leucine-tRNA. The new species of leucine-tRNA is not formed by aggregation of tRNA or nuclease catalyzed hydrolysis, and it is not grossly deficient with respect to methylation. Since there is some evidence from the recent work of others that tRNA formed under conditions of amino acid starvation is deficient in the minor bases 5,6-dihydrouridine and 4-thiouridine, we think that the biochemical explanation for the accumulation of new tRNA species under conditions of amino-acid starvation may be that the enzymes responsible for these tRNA modifications are unstable and require continued protein synthesis to maintain their levels of activity.

Further information was obtained on the mechanism of arginyl-tRNA synthetase which does not catalyze an amino acid dependent ATP-PPi exchange in the absence of added tRNA. A new purification procedure was devised which yields homogeneous enzyme in approximately 10% yield. Pulse labelling experiments indicate that no enzyme bound arginyl-adenylate is formed in the absence of added tRNA. Equilibrium experiments show that no arginyl-adenylate accumulates either in the presence or absence of $tRNA^{arg}$. These data further validate our earlier suggestion that the mechanism of this reaction is probably concerted.

A series of additional studies carried out with the purified preparation of arginyl-tRNA synthetase from E. coli indicate that metals may have two functional roles in the catalytic mechanism. Complete metal activation is observed when $MgCl_2$, $MnCl_2$, $CoCl_2$, or $FeCl_2$ is present at a concentration (5 mM) in excess of the total ATP concentration (2 mM). When $CaCl_2$ is substituted for $MgCl_2$, activity is not observed unless a small amount (0.1 mM) of $MgCl_2$, $MnCl_2$, $CoCl_2$, $FeCl_2$ or $ZnCl_2$ is added. On the basis of these experiments, we visualize a model in which the enzyme possesses a site for free metal which, when filled, lowers the K_m for the three substances (arginine, $tRNA^{arg}$, and metal·ATP) and increases the V_{max} of the reaction.