ence does not lie in different rates of absorption or elimination of phenobarbital. Several other NAD-linked enzymes such as alcohol, lactic, malic, and α-glycerophosphate dehydrogenases are not materially affected.

The increase in capacity for aldehyde oxidation in whole liver after treatment with phenobarbital is of the order of three- to fivefold in animals whose supernatant enzyme activity increases by tenfold. This is because the mitochondria contain from 50 to 75 percent of the total capacity for aldehyde oxidation in the liver when propionaldehyde (*10*) or acetaldehyde (*11*) is used as substrate (*1*). Redmond and Cohen (*7*) indicate a twofold increase in the capacity for aldehyde oxidation in whole homogenates of mouse liver after treatment with phenobarbital. I have found that after treatment with phenobarbital there is a twofold increase in enzyme activity in mouse liver supernatant which is not dependent on genotype. This is a quantitatively but not qualitatively different response from that obtained by Redmond and Cohen (*7*) and may be due to the strain of mice used or to the difficulty in measurement of aldehyde dehydrogenase activity in homogenates of whole liver.

It seems unlikely that this reaction to phenobarbital has any relation to cross tolerance of barbiturate and ethanol as has been suggested (*7*). The response is present only in some animals; it is not brought about by other barbiturates; and, it does not extend to the brain aldehyde dehydrogenase (*9*).

The fact that a tenfold increase in aldehyde dehydrogenase in liver supernatant can be brought about by treatment of selected animals with phenobarbital, indicates that this is a system in which may be studied the function of this enzyme in alcohol, aldehyde, and amine metabolisms and the relationship of the enzyme to ethanol intoxication and addiction. Animals that do not respond to treatment with phenobarbital with an increased activity of aldehyde dehydrogenase make ideal controls for these experiments. The mechanism of the increase in aldehyde dehydrogenase activity is unknown, but the genetic basis for the effect provides a possible means for its discovery. Phenobarbital induces microsomal enzymes (*12*) and a mitochondrial enzyme, δ-amino levulinic acid synthetase (*13*). My studies indicate that administration of phenobarbital is associated with increased activity of a soluble aldehyde dehydrogenase found in the liver supernatant and that this responsiveness is inherited as a dominant characteristic in accordance with classical Mendelian genetics.

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[^{3}H]Adenosine Triphosphate: Release during Stimulation of Enteric Nerves

Abstract. *The isolated taenia coli of the guinea pig takes up tritiated adenosine, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate, in preference to tritiated inosine and adenine. After uptake, [^{3}H]adenosine is converted and retained primarily as [^{3}H]adenosine triphosphate. Tritium is released from taenia coli treated with [^{3}H]adenosine upon activation of the nonadrenergic inhibitory nerves. These results are consistent with the previous evidence that adenosine triphosphate may be the transmitter from the nerves.*

Although adrenergic inhibitory nerves in the gut are well known, the existence of nonadrenergic inhibitory nerves supplying the mammalian gut was not established until recent years (*1*). The cell bodies of these nerves are localized in Auerbach's plexus, and their axons supply both longitudinal and circular muscle coats; they are controlled by preganglionic vagal fibers in the stomach, but in the colon they are without extrinsic nerve connections (*2*). In ultrastructural studies, the nerves appear to be represented by profiles containing a predominance of large unique granular vesicles (1000 to 2000 Å), which distinguish them from adrenergic nerves (*3*).

Evidence has been presented recently that adenosine triphosphate (ATP) or some related nucleotide is the transmitter substance released by nonadrenergic inhibitory nerves in the gut (*4*). The results of experiments were described which, in broad outline, satisfied the criteria summarized by Eccles (*5*) for the establishment of a neurotransmitter substance: ATP and the enzymes necessary for its formation are present in nonadrenergic inhibitory nerves; ATP or its breakdown products (or both) are released on nerve stimulation; enzymes which inactivate ATP are present in gut; some drugs produce parallel effects on responses to both

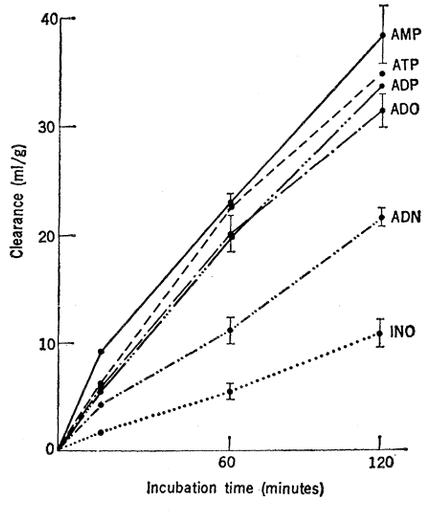

Fig. 1. Uptake of purine derivatives by guinea pig taenia coli. The tissue was incubated with the tritiated derivatives (10^{-7} mole/liter), digested, and assayed for tritium. Each point represents the mean value of six to eight preparations; the vertical bars represent 2 S.E.M. After incubation for both 60 and 120 minutes, the uptake of adenine (ADN) was significantly smaller than that of adenosine (ADO), AMP, ADP, or ATP ($P < .02$) and larger than that of inosine (INO, $P < .01$) as determined by the *t*-test.

ATP and nerve stimulation; and ATP mimics the nerve-mediated response.

We have used isotopes to test this hypothesis, namely, by measuring the uptake of tritium-labeled nucleosides and nucleotides, their conversion and storage in the tissue, and their release during stimulation of the enteric nerves in the taenia coli of the guinea pig. The taenia coli was selected for this study because radioactivity released during the inhibitory responses to both adrenergic and nonadrenergic nerves can be monitored and compared in the same preparation. [2,8-^{3}H]Adenosine and [^{3}H]inosine (generally labeled) were obtained from the New England Nuclear Corp. (Boston, Mass.), and [2-^{3}H]adenosine 5′-monophosphate ([^{3}H]AMP), [2-^{3}H]adenosine 5′-diphosphate ([^{3}H]ADP), [2-^{3}H]adenosine 5′-triphosphate ([^{3}H]ATP), and [8-^{3}H]-adenine were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). Strips of taenia coli together with the underlying nerves of Auerbach's plexus were isolated from guinea pigs (200 to 300 g) of either sex and maintained at 38°C in Krebs bicarbonate solution equilibrated with 95 percent oxygen and 5 percent carbon dioxide.

In uptake studies, 1-cm lengths of taenia coli were incubated with the labeled purine derivatives for different time periods and then washed with three changes of Krebs solution at 10-minute intervals. Tissues were then blotted, weighed, and digested with Soluene (Packard Corp.), and the radioactivity was counted by liquid scintillation spectrometry. The uptake was expressed as milliliters of incubation fluid cleared per gram (wet weight) of tissue. Strips of taenia took up tritiated adenosine, AMP, ADP, and ATP to a considerable extent, concentrating the radioactivity contained in the bath solution in a volume as much as the equivalent of 20 times the tissue volume in 60 minutes. On the other hand, tritiated inosine and adenine, the possible metabolites of ATP, were concentrated to a much smaller degree (Fig. 1).

In the studies of biotransformation, therefore, [^{3}H]adenosine was used exclusively because such a precursor would more likely follow the uptake, distribution, enzymatic conversion, and storage of endogenous ATP. Some portions of the taenia were incubated with [^{3}H]adenosine (0.14 μmole/liter) for 1 hour, washed, homogenized with ten volumes of 0.4N perchloric acid, and then centrifuged at 20,000g for 20 minutes. The supernatant was subjected to cellulose thin-layer chromatography, with a mixture of *tert*-amylalcohol, formic acid, and water (3 : 2 : 1) used as the solvent system. Authentic adenosine, AMP, ADP, ATP, adenine, inosine, and adenosine 3′,5′-phosphate (cyclic AMP) dissolved in the extracts and applied on the same plate were detected by ultraviolet lamp; the R_F values were 0.60, 0.41, 0.21, 0.14, 0.65, 0.51, and 0.47, respectively. The cellulose on the chromatogram was scraped off in narrow bands, shaken with a mixture of toluene and methanol (85 : 15) containing scintillators, and counted. From the ^{3}H activity contained in the bands corresponding to the above R_F values, and corrected for the total recovery, it was estimated that approximately 60 percent of [^{3}H]adenosine was converted into and retained as [^{3}H]ATP; a small fraction was accountable as [^{3}H]AMP, [^{3}H]inosine, or cyclic [^{3}H]-AMP, but virtually no [^{3}H]ADP could be detected (Table 1).

Table 1. Tritiated constituents retained in taenia coli after incubation with [^{3}H]adenosine. The data are expressed as means and standard errors ($n = 3$).

Tritiated constituent	^{3}H recovery (% total)
Adenosine	6.5 ± 2.6
AMP	16.5 ± 7.8
ADP	1.7 ± 0.3
ATP	59.3 ± 10.9
Inosine or cyclic AMP	12.5 ± 2.4
Adenine	1.0 ± 0.5

In release studies, 3-cm lengths of taenia coli were first incubated with [^{3}H]adenosine (0.14 μmole/liter) for 1 hour, connected to a Statham isometric strain gauge, superfused with Krebs solution at a rate of 3 ml/min, and subjected to transmural stimulation of intramural nerves by means of two parallel platinum wire electrodes under the conditions described earlier (*6*). The Krebs solution contained atropine sulfate (10^{-7} g/ml) and guanethidine sulfate (10^{-6} g/ml). The superfused fluid was collected every 1 or 2 minutes, and its ^{3}H activity was counted. When transmural stimulation with biphasic pulses 0.05 or 0.1 msec long at 15 volts or above was applied at a frequency of 30 pulses per second for 30 to 300 seconds, a marked transient reduction of the muscle tone was registered which was accompanied by a marked increase in overflow of ^{3}H into the superfused fluid. Nicotine (5×10^{-6} g/ml), which in the presence of guanethidine activates nonadrenergic inhibitory nerves but not the adrenergic nerves (*7*), also elicited the relaxation and a release of tritium (Fig. 2). A more pronounced and sustained relaxation was obtained with transmural stimulation at 5 rather than 30 pulses per second and in the presence of *l*-noradrenaline bitartrate (3×10^{-8} g/ml). This was, however, accompanied by lit-

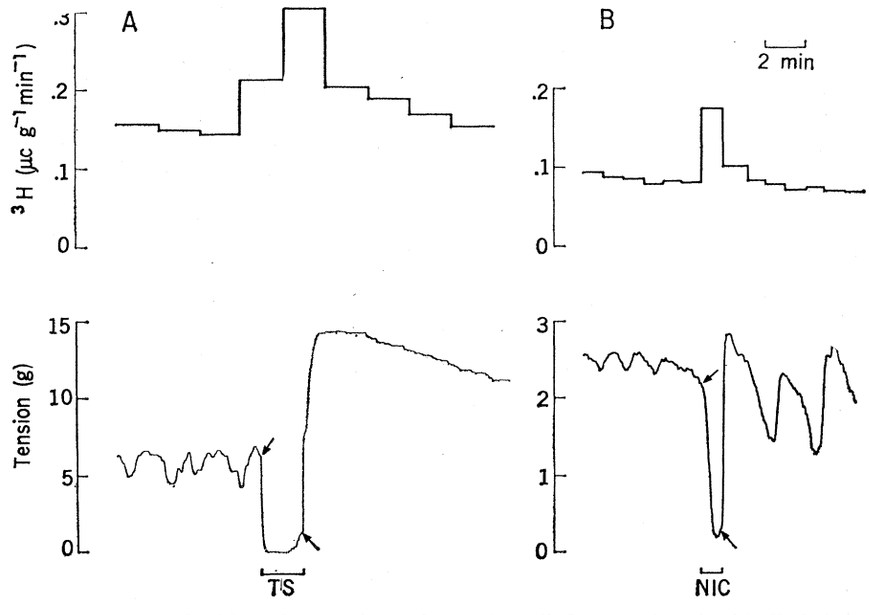

Fig. 2. Release of tritium from guinea pig taenia coli first incubated with [^{3}H]adenosine. The upper chart shows tritium activity in superfused fluid; the lower shows muscle tension. In preparation A, transmural stimulation (*TS*, 0.1-msec pulses, 30 per second, 15 volts) was applied for 2 minutes; in preparation B, nicotine dihydrochloride (*NIC*, 5×10^{-6} g/ml) was applied for 1 minute.

tle or no increase in the ^3H overflow. Thus, although [^3H]adenosine may be taken up by smooth muscle cells as well as by neurons, the increase in overflow after stimulation at 30 pulses per second or application of nicotine cannot be attributed to a release of ^3H from smooth muscle cells as a consequence of change in the muscle tone, but rather to a release of ^3H from neurons. Muscle relaxation and increased overflow of ^3H were also elicited in three taenia coli preparations in which electrical stimulation (0.1 msec, 20 volts, 30 per second for 120 seconds) was applied by two closely positioned platinum rings, through which taenia coli was pulled. Both responses were abolished within 30 minutes of application of tetrodotoxin (2×10^{-7} g/ml). These results are consistent with the evidence that ATP, ADP, and AMP are released during stimulation of Auerbach's plexus, from turkey gizzard, dissected free of smooth muscle (*4*, *8*).

In further experiments, a preparation consisting of perivascular nerve and taenia was used in which the sympathetic supply to the taenia could be selectively stimulated (*7*). In the absence of guanethidine, stimulation of the perivascular sympathetic nerve (2 msec, 15 to 60 volts, 30 per second for 60 to 120 seconds) elicited a reduction in the muscle tone and increased overflow of ^3H in those preparations which were first incubated with [^3H]adenosine. Introduction of guanethidine sulfate (10^{-6} g/ml) abolished both responses to 15- or 30-volt stimulation. With 60 volts, the mechanical response was also abolished, and the ^3H overflow was reduced by guanethidine. From the exclusively adrenergically innervated rabbit aorta previously incubated with [^3H]-adenosine, guanethidine blocked the ^3H overflow following nerve stimulation (*9*). It is not known whether nucleotides are released from cholinergic nerves.

Our results indicate that ATP can be synthesized from adenosine, stored as ATP (but not ADP) in the taenia coli, and released upon activation of both nonadrenergic and adrenergic inhibitory nerves. Although it appears that this property is not unique to the nonadrenergic inhibitory system, it is consistent with the previous evidence that ATP may be the transmitter at this neuroeffector junction (*4*). It remains to be determined what quantitative and other features of ATP release are unique to the nonadrenergic inhibitory neuroeffector system.

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Acetylcholine Binding to Torpedo Electroplax: Relationship to Acetylcholine Receptors

Abstract. *Binding of [^3H]acetylcholine to a particulate fraction of* Torpedo *electroplax was measured by equilibrium dialysis. Two high-affinity sites present on phospholipoproteins bound acetylcholine reversibly, and binding was blocked by nicotinic drugs. Characteristics of this binding suggest that these phospholipoproteins may be acetylcholine receptors.*

Physiologically, one recognizes the acetylcholine receptor (AChR) by the changes in membrane potential or conductance that suitable cholinergic agents cause in the membrane of which AChR is a part. Attempts to isolate AChR require some biochemical index, of which the most favorable appears to be the binding of cholinergic agents having appropriate specificity, affinity, amount, and reversibility. Several previous attempts, in which the binding of *d*-tubocurarine or gallamine triethiodide was used as an index (*1*), were unsuccessful because the high concentrations of ligand or the low ionic strengths of buffers used led to isolation of nonspecific macromolecules. It became apparent that cholinergic ligands also bind to a variety of enzymes, hormones, and mucopolysaccharides (*2*); and the characteristics of their binding are important in distinguishing AChR.

The direct way to identify AChR in vitro is to examine binding of the natural transmitter acetylcholine (ACh). However, acetylcholinesterase (AChE), which hydrolyzes ACh very rapidly, is always present in high concentrations in tissues rich in AChR. Consequently, we started by studying the binding of five cholinergic ligands which are not hydrolyzed by AChE in particulate preparations of electroplaxes from *Torpedo* and *Electrophorus* (*3*), as well as a supernatant fraction of housefly brain centrifuged at 100,000*g* (*4*). Multiple sites for binding cholinergic ligands were revealed in the electroplaxes, and the ones suggested to be on AChR were phospholipoproteins of nicotinic nature (*5*, *6*).

Recently, we also found that several organophosphates, when added at a concentration of 10^{-4} mole/liter, irreversibly inhibited all the AChE present in *Torpedo* electroplax (as judged by the inability to detect any hydrolysis of ACh after dialysis); but the organophosphates did not interfere with the binding of muscarone, nicotine, or acetylcholine (*7*). The degree of inhibition of AChE and blockade of binding varied among the organophosphates and cholinergic tissues tested. Only at higher concentrations did organophosphates reversibly block binding to macromolecules suggested to be AChR. These results were in good agreement with those reported for the physiological effect of organophosphates on AChR of the monocellular preparation of the electric eel (*Electrophorus electricus*) electroplax (*8*).

Being able to phosphorylate the esteratic site of AChE without affecting AChR activity enabled us to proceed and study binding of [^3H]ACh (specific activity 50 mc/mmole; New England Nuclear) to the particulate preparation of *Torpedo* electroplax. The label was on the acetate, so that binding would be observed only if all AChE was inhibited and ACh was intact, because we found the acetate anion did not bind. Binding of choline could be mistaken for binding of ACh, as has happened in an earlier study (*9*).

We measured binding by equilibrium dialysis (at 4°C for 16 hours) in 100