Reports

Action of Lipid-Soluble Quaternary Ammonium Ions on Conducting Membranes

Bioelectric currents which propagate the nerve impulse are carried by ions. The membrane theory, proposed at the turn of the century and which, with small modifications, is still the basis of most modern concepts, assumes that a change in ion permeability of the conducting membrane is responsible for the currents. Such a change was experimentally demonstrated by Cole and Curtis (*1*). Fundamental for the understanding of the process is the question: What is the mechanism by which this permeability change is produced? How does the potential source of electromotive force, the ionic concentration gradients between inside and outside, become suddenly effective? Two opposing views confront each other: (i) Conduction is a purely physical process (*2*), and (ii) chemical reactions change the membrane permeability and control the ion movements (*3*).

Recently, A. V. Hill and his associates have shown, with very fast recording instruments and *Maja* nerve at 0°C, that the previously recorded small initial heat production consists of two distinct phases: a rather large positive heat, roughly coinciding with the electrical activity, followed by a negative heat taking place within 100 to 300 msec after the active phase (*4*). The data lend strong support to the assumption that chemical reactions underlie the activity. This is also supported by the high Q_{10} of the action potential. With the isolated single electroplax of *Electrophorus electricus,* Schoffeniels (*5*) found a Q_{10} of 3.6; the activation energy is 21,000 cal/mole. The results of these physical recordings thus favor the assumption of chemical reactions.

The necessity of chemical processes was maintained by Nachmansohn and his colleagues. They proposed 15 years ago that the action of acetylcholine is essential for the permeability change in conduction. This view is based on the various physiological and biochemical features of the acetylcholine system present in all conducting tissue throughout the animal kingdom. The system has the prerequisites required to generate bioelectric currents: It has the necessary speed, it is inseparable from conduction, it precedes in the sequence of energy transformations all other reactions, and its association with electrical events has been demonstrated in a variety of ways. Nachmansohn's view was nevertheless opposed essentially because of the failure of acetylcholine to affect conduction when applied externally. This lack of effect was explained by the failure of acetylcholine to reach the active sites since it could be experimentally demonstrated that acetylcholine does not penetrate to the inside of the cell (*6*).

A new development started with the preparation of lipid-soluble quaternary ammonium ions, analogs of acetylcholine. The first such compound was pyridine aldoxime dodecyliodide (PAD), a lipid-soluble analog of pyridine aldoxime methyliodide (PAM) (*7*). This latter structure was designed by Wilson on the basis of molecular complementariness and proved to be a powerful antidote against nerve gas (*8*). This and several other lipid-soluble quaternary ammonium ions block in low concentrations conduction of crab and lobster axons. They depolarize the electroplax of *Electrophorus electricus.* In the first records obtained with these cells the polarity was even reversed (overshoot) (*9*), but this could not be reproduced. When they are applied to muscle, the lipid-soluble quaternary ammonium ions produce contraction which can be repeated many times even when the myoneural junction is completely blocked by curare (*10*). Thus, the biological action must be attributed to a direct action of the acetylcholine analogs on the conducting membrane.

Recently, Staempfli tested one of these compounds, noracetylcholine 12 (in which one methyl on the N of acetylcholine is replaced by a dodecyl group) on Ranvier nodes of frog nerve (personal communication). In $10^{-3}M$ concentration, the compound produced a complete and irreversible depolarization of the membrane. In $10^{-4}M$ concentration, a reversible depolarization was obtained. In concentrations of $3 \times 10^{-5}M$, the compound increased the amplitude and duration of the action potential. The effects on the membrane take place within seconds. This is a high speed, only slightly less rapid than that observed with K^+. Similar and even slightly more potent effects on the conducting membrane of the desheathed frog tibialis nerve were obtained by Dettbarn with PAD (*11*). For the interpretation of the effects, the question is crucial whether or not they are produced by a specific action upon the acetylcholine receptor postulated to be the physiological cell constituent whose reaction with acetylcholine is essential for the permeability change; if it were possible to demonstrate directly that this is really the basis for the depolarizing action of the lipid-soluble quaternary ammonium ions, that the modified molecule has the intrinsic ability to react with the receptor and to affect the change normally effected by acetylcholine, this would be new and conclusive evidence.

Evidence in this direction has now been obtained (*12*). If PAD depolarizes the membrane by reacting with the acetylcholine receptor—that is, if it is a receptor activator—a receptor inhibitor should by competitive action protect the active surface of the protein against the quaternary ammonium ion. This is indeed the case. Eserine, as is well known, has a high affinity for the acetylcholine system. It is a potent cholinesterase inhibitor; it is also a receptor inhibitor. When eserine is added to the desheathed frog tibialis nerve in $7 \times 10^{-3}M$ concentration, addition of a markedly depolarizing concentration of PAD has no effect. Lower concentrations of eserine strongly counteract the effect of PAD but do not abolish it. Higher concentrations of PAD may overcome the protective action of eserine. The antagonism between the two compounds is thus typical for competitive action. A description of the details is in preparation (*11*).

The fact that these lipid-soluble analogs of acetylcholine have a fast and reversible depolarizing action on nerve in concentrations of a few micrograms per milliliter and produce muscular contraction outside the myoneural junction as postulated by theory, and the evidence

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Instructions for preparing reports. Begin the report with an abstract of from 45 to 55 words. The abstract should *not* repeat phrases employed in the title. It should work with the title to give the reader a summary of the results presented in the report proper. (Since this requirement has only recently gone into effect, not all reports that are now being published as yet observe it.)

Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* 125, 16 (1957)].

that they act specifically on the acetylcholine receptor, are new supports for the proposed role of acetylcholine in conduction, especially when considered in connection with the huge amount of physical and chemical data accumulated in the last two decades in favor of this concept.

WOLF D. DETTBARN
IRWIN B. WILSON
DAVID NACHMANSOHN
Department of Neurology, College of Physicians and Surgeons, Columbia University, New York

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12. This work was supported in part by the Division of Research Grants and Fellowships of the National Institutes of Health (grants No. B-573 C10 and B-400 C4) and in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army (contract No. DA-49-007-MD-740).

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An Immunological Investigation of Human Pituitary Growth Hormone

Abstract. Growth hormone isolated from human pituitaries has been demonstrated to be a good antigen in the rabbit. With the rabbit antiserum to human somatotropin, it is possible to detect as little as 0.1 μg of the hormone by precipitin test. The antiserum was also capable of neutralizing the growth-promoting activity of human somatotropin.

It is the purpose of this report to present evidence for the antigenicity of human pituitary growth hormone (somatotropin) in rabbits and guinea pigs. Although Cruickshank and Currie have recently produced antisera to pituitary somatotropin from humans (*1*), they were unable to demonstrate by means of precipitin tests the presence of any antibodies that were hormone-specific. Absorption of the somatotropin antiserum with their human ACTH or TSH preparations resulted in the loss of antibodies to somatotropin itself. Heijkensköld and Gemzell (*2*) did not detect any precipitating antibodies to human somatotropin in the sera of rabbits, guinea pigs, and rats that had been injected with the hormone.

The production of a potent antiserum to highly purified bovine pituitary growth hormone has recently been reported (*3*). This antiserum, freed of certain nonspecific antibodies by absorption, was found to be capable of detecting as little as 0.5 to 1 μg of bovine somatotropin by the precipitin ring test, and it gave no cross reaction with comparable and considerably higher doses of all the other known anterior pituitary hormones. Furthermore, it was observed that relatively small amounts of the antiserum could completely neutralize the biological activity of the bovine somatotropin (*3*).

In the present investigation, highly purified human pituitary growth hormone prepared according to a method previously outlined (*4*), was employed for the sensitization of guinea pigs and the immunization of rabbits. Guinea pigs were readily sensitized to the human somatotropin preparation. A dose of 10 μg of the hormone suspended in 0.4 ml of the adjuvant Bayol-Arlacel (*5*) was injected subcutaneously; the same dose was repeated 3 days later. Three weeks after the initial sensitizing dose, the animals were challenged intracardially with varying doses (0.025 to 0.100 mg) of the hormone in 0.5 ml of saline solution. Eight out of 11 of these animals showed typical signs of severe anaphylactic shock and succumbed within 3 to 5 minutes after the injection, whereas no signs of anaphylaxis following similar doses of somatotropin were demonstrable in six control animals that had undergone no prior sensitization.

The antiserum was prepared in the following matter. Young albino female rabbits, weighing approximately 2.6 to 2.8 kg each, were injected with a total of 4 mg of somatotropin suspended in Freund's adjuvant according to the procedure of Cohn (*5*); of this dose, 2 mg was administered subcutaneously and 2 mg intraperitoneally. Two weeks later the rabbits were bled from the marginal ear vein and were injected the following day with 4 mg of somatotropin in the same manner as before. After another interval of 2 weeks, the animals were each injected with 2 mg of the hormone, 1 mg subcutaneously and 1 mg intraperitoneally, in the alum precipitate form (*6*), bringing the total dose of somatotropin per rabbit to 10.0 mg. The rabbits were bled by cardiac puncture 10 days later, and the serum was frozen and stored until use.

Precipitin ring tests (*6*) were performed with the rabbit antiserum to determine the smallest amount of human somatotropin that could be detected. Tubes were set up containing a serial dilution of antigen ranging from 0.5 down to 0.00005 mg in 0.5 ml of saline. One-tenth milliliter of human somatotropin antiserum from which certain nonspecific antibodies had been removed by absorption was carefully layered under the antigen phase. Tests were read after incubation for 1 hour at 30°C. The minimal amount of somatotropin giving a definite positive precipitin test was 0.1 μg of the hormone.

The antiserum was also tested to determine whether or not it was capable of neutralizing the biological activity of the human somatotropin, as determined by bioassay according to the standard 4-day tibia test in hypophysectomized rats (*7*). The experimental animals, all hypophysectomized, were apportioned into three groups. The first group, consisting of six animals, served as controls and received normal rabbit serum only. The five animals in group 2 were injected with normal rabbit serum plus somatotropin, and the six animals in group 3 received antiserum and somatotropin. The daily dose of hormone was 0.010 mg in 0.5 ml of saline solution, injected subcutaneously, and the daily dose of serum was 0.25 ml, administered intraperitoneally. The injections of serum were begun 4 hours prior to the first injection of hormone, so that over the 4-day injection period, five injections of serum were administered. The animals were sacrificed approximately 24 hours after the final injection of hormone. The results showed that the total dose of 0.040 mg of somatotropin had increased the width of the tibial epiphyseal cartilage plate to 237 ± 4 μ (mean ± standard error), as compared with 160 ± 1 μ for the controls that had received normal rabbit serum only. The simultaneous administration of antiserum with the somatotropin had resulted in a complete neutralization of the hormonal effect (164 ± 3 μ). It has previously been demonstrated (*3*) that the injection of bovine somatotropin antiserum alone does not have any significant influence upon the width of the tibial epiphyseal cartilage plate in the hypophysectomized rat (157 ± 4 μ, as compared with 161 ± 3 μ for the uninjected hypophysectomized controls). The fact that the biological activity of somatotropin was neutralized completely when the hormone and relatively small amounts of the antiserum were injected into two entirely distinct sites by two different routes in the same animal suggests the specificity of the antigen-antibody reaction in question. The findings from the precipitin tests, supported by the results of the antihormone tests, have further suggested that an immunological method of assay for the presence of