electrode was reconstructed and shown by the thin continuous line.

In all six experiments on *Nitella megacarpa* and five experiments on different species of *Nitella* (probably *flexilis*), it was found that the peak of the action potential roughly coincides with the peak of the efflux of tagged potassium. The slight discrepancy between the peaks of the two curves can be attributed partly to the difference in the time at which different parts of the cell reach the peak of activity. The retention of the radioactive potassium in some part of the path of running fluid is another factor that is responsible for the discrepancy in the peaks and also for the efflux after the end of the action potential. In a number of more recent experiments, it was possible to obtain a closer parallelism.

In this connection it is to be pointed out that Osterhout (*3*) had predicted an outward movement of K^+ during activity in *Nitella*. The time course of the efflux observed can be interpreted as a reflection of the increased membrane conductance during activity (*4*). Results from using cesium-134 and rubidium-86 instead of potassium-42 were qualitatively the same.

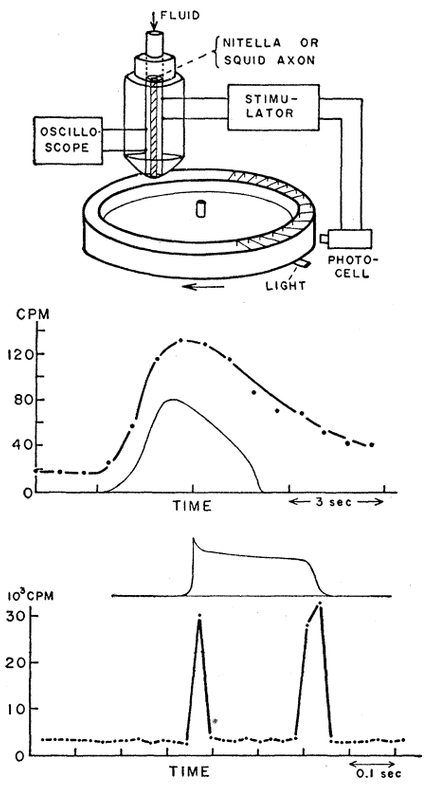

Fig. 1. (Top) Principle of the method of fractionating tracer effluxes during action potential (not to scale). (Middle) Efflux of K^{42} from a cell of *Nitella* (dots) during the action potential (thin continuous curve). (Bottom) Efflux of K^{42} from a squid giant axon treated with tetraethylammonium chloride (dots) during the action potential (thin continuous curve).

Using chloride-36 instead of tagged cations, we tried to demonstrate an increased efflux of tagged univalent anions during activity. We could bring the radioactivity of the cell up to 1000 count/min or slightly more. We could not influence the loss of this radioactivity from the cell by stimulation. We found also that complete substitution of the chloride ion in the medium with glutamate did not bring about any appreciable change in the amplitude of the action potential.

We encountered serious difficulties when attempts were made to apply the same technique to the squid giant axon which requires much higher time resolution in order to correlate the effluxes of tagged ions with the action potential. Such difficulties were partly overcome by prolonging the action potential by injecting tetraethylammonium chloride uniformly over the entire length of the axon. By this procedure, combined with a lowering of the temperature to about 7°C, it was possible to increase the duration of the action potential to 0.5 to 1.0 sec or more. We tried to reduce the thickness of the stagnant layer of the fluid by inducing turbulance in the running fluid around the axon. In several favorable cases, we could attain a time resolution up to the order of 20 msec. In many instances, however, the resolution was poorer than about 100 msec, and, as a consequence, we could not detect an increase in the efflux associated with the action potential. We attribute this variability in the time resolution to the variability in the thickness of the connective tissue layer on the surface of the axon.

The bottom diagram in Fig. 1 shows the result of one of six successful experiments in which the efflux of potassium-42 or sodium-24, or both, was investigated in axons treated with tetraethylammonium chloride. In this example, an isotonic potassium chloride solution containing potassium-42 was injected in a portion of an axon treated uniformly with tetraethylammonium chloride. It is shown in this diagram that there is one phase of increased efflux at the onset and another phase at the end of the prolonged action potential. There was no appreciable increase in the efflux during the plateau. A similar result, with double peaks in the efflux, was obtained with sodium-24. The ratio of the flux at the onset to that at the end varied considerably from preparation to preparation. At this preliminary stage of the experiments, it is not possible to make a definite statement as to this ratio. The results obtained on the relative impermeability of *Nitella* to chlorine-36 are contradictory to the experiments of Gaffey and Mullins on *Chara* (*5–7*).

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6. A report of our interpretation of the experiments presented here and their implications for prevailing concepts of excitation is in preparation. Recently it has come to our attention that C. Terzuolo is in the process of studying the efflux of isotopes from *Nitella* autoradiographically.
7. We are grateful to Mrs. W. J. V. Osterhout for her interest and help.

23 February 1961

Osteogenic Induction across Millipore Filters in vivo

Abstract. Immunization of host mice to homograft tissues prior to subcutaneous implantation of homograft bone within diffusion chambers did not prevent new bone formation on the host side of the filter, thereby indicating its origin from host cells in response to a diffusible osteogenic inductor from within the chamber rather than from undetected escaping homograft cells.

In a previous study (*1*) designed to provide information about the origin of newly formed bone around bone autografts, it was found that subcutaneous implantations of 2- to 3-day-old isologous mouse calvaria within Millipore diffusion chambers (*2*) for periods ranging from 2 to 8 wk resulted in the formation of new bone and cartilage exclusively *within* the chambers. In more recent experiments, bone formation on the host side of the filter (which

has a pore size of 0.45 μ) was obtained as well, if thinner (100 μ) Microweb filters were utilized or if homologous bone was implanted within chambers made up with "standard" thickness (150 μ) Millipore Microweb filters. However, the escape of isologous or homologous bone-forming cells from within the chamber could conceivably have accounted for these results, particularly since it was found that similar bone homografts implanted subcutaneously without the protection of a diffusion chamber showed excellent new bone formation after 2-wk implantation before any host reaction could develop. The following experiment, designed to distinguish by immunological means the source of new bone on the host side of the filter by bringing about early destruction of "escaping" homograft bone cells (should they escape at all), was performed.

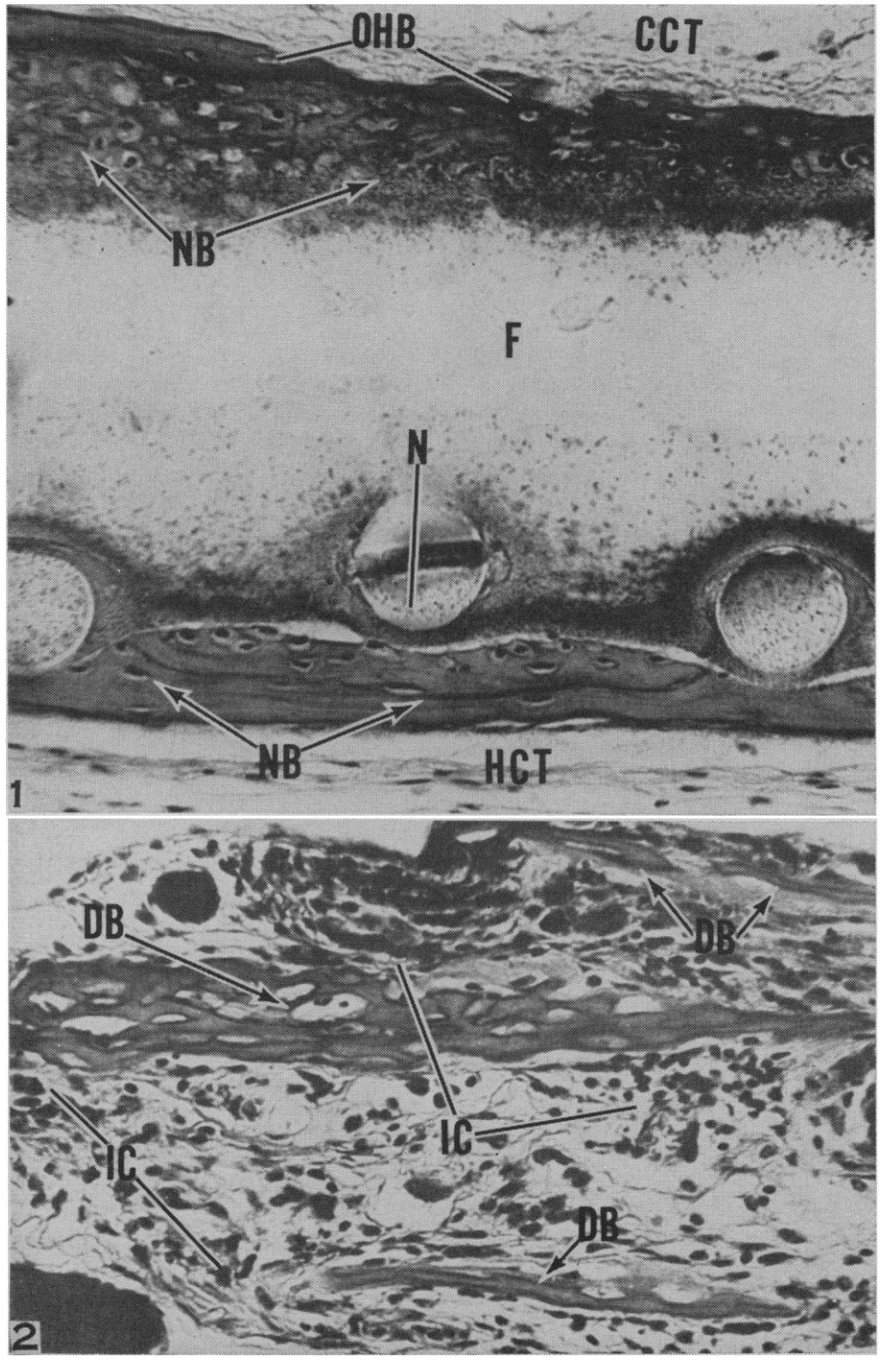

Fig. 1. Histological section (hematoxylin and eosin) of 2-mo chamber containing homograft bone in immunized host (× 185) shows new, vital bone with "resting lines" on host side of filter opposite less organized new bone on inner aspect of filter. Note dark staining "acellular zone" and basophilic droplets within body of filter. *OHB*, original homograft bone; *NB*, new bone; *F*, filter; *N*, cross section of nylon reinforcement; *HCT*, host connective tissue; *CCT*, chamber connective tissue. Fig. 2. Histological section (hematoxylin and eosin) of 2-mo free implant of homograft bone in immunized host (× 185) shows dead, fragmented bone surrounded by inflammatory cells. *DB*, dead bone; *IC*, inflammatory cells.

Young male Swiss albino mice (Webster strain) were immunized to tissues of C57 bl/6 mice by a modification of the technique of Algire *et al.* (*3*); three separate subcutaneous implantations, at 2-wk intervals, of spleen fragments from adults of the latter strain were used. After this 6-wk immunization period, a diffusion chamber containing half of a 1- to 2-day-old C57 bl/6 calvarium was implanted subcutaneously into each host, while the opposite half of the calvarium was implanted at some distance from the chamber as a "free implant" control. A duplicate, nonimmunized group was set up as well. Animals from both groups were killed at intervals of 2, 3, and 4 mo. Twenty-six intact chambers were recovered from immunized and nonimmunized animals. It was found that of 14 chambers recovered from *immunized* mice, slight to considerable patches of new, vital bone were noted on the host side of the filter in 12 animals. Characteristically, this new bone appeared opposite an area of new bone formation on the inner aspect of the filter (Fig. 1). On the other hand, the *free implant bone* in the immunized animals was uniformly found to be dead, surrounded by an inflammatory reaction, and it showed little, if any, attempt at new bone formation (Fig. 2). In the *nonimmunized* group, 9 of 12 intact chambers showed similar patches of new, vital bone on the host side of the filter. Although the free implant control bone showed in these nonimmunized animals evidence of extensive new bone proliferation at an earlier stage after implantation, the tissue appeared to be dead or dying and it was surrounded by inflammatory cells at the time that the animals were killed.

In view of the inhibition of new bone formation and the enhanced destruction of freely implanted bone homografts by the preimplantation immunization procedure, it is assumed that any homograft bone cells escaping from diffusion chambers in immunized animals would have suffered a similar

fate. It is therefore concluded that the new bone on the host side of the filter must have come from the host. With regard to the possibility that the Millipore filters per se were responsible for the bone induction, this seems unlikely in view of my inability in other experiments to demonstrate bone formation around freely implanted Millipore filter material as well as in view of the failure of other investigators (*4*) to note new bone formation on the host side of Millipore filters. Rather, the experimental results indicate that the new, vital bone found in immunized mice on the host side of diffusion chambers containing homograft bone is derived from host tissue in response to a *diffusible osteogenic inductor* coming from the new bone laid down on the inner aspect of the filter, thereby representing the in vivo extension of the in vitro findings of Grobstein (*5*) and Lash *et al.* (*6*) with respect to the passage of inductor substances through Millipore filters (*7*).

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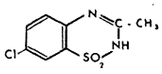
7. Further experiments are now in progress to determine the specificity and chemical nature of the diffusible osteogenic inductor reported here. This study was supported by the U.S. Army, Office of the Surgeon General, under contract No. MD-2018. I gratefully acknowledge the technical assistance of Miss G. Cirulis and G. Pettengill.

20 February 1961

New Class of Antihypertensive Agents

Abstract. Effective antihypertensive agents of the benzothiadiazine series, devoid of diuretic activity, are described. There follows a method of synthesis and a description of the pharmacological activity of one of these substances.

We have synthesized 7-chloro-3-methyl-1,2,4-benzothiadiazine-1,1-dioxide (I), the first compound in the 1,2,4-benzothiadiazine-1,1-dioxide series in which a separation of the antihypertensive and diuretic activities has been achieved. A characteristic of the diuretic agent chlorothiazide (*1*) (II) and other related benzothiadiazine diuretics, including the 3,4-dihydro derivatives, is their property of moderately reducing the blood pressure of hypertensive subjects.



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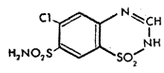

II

Although the precise mechanism of this action is not known, it has generally been assumed to be related to the diuretic and natriuretic properties of the compounds (*2*).

Compound I was synthesized as follows: 2,4-dichloronitrobenzene was converted into 2-benzylthio-4-chloronitrobenzene, with a melting point of 132° to 134°C (found: N, 5.03) by reaction with benzylmercaptan in the presence of potassium hydroxide in ethanol solution. Treatment of the benzylthio compound with chlorine in aqueous acetic acid, followed by reaction of the resulting sulfonyl chloride with ammonia, afforded 5-chloro-2-nitrobenzenesulfonamide, with a melting point of 159° to 160°C (found: N, 11.71; Cl, 14.53). Reduction of the latter with iron filings and ammonium chloride solution yielded 2-amino-5-chloro-benzenesulfonamide, with a melting point of 152° to 153°C, λ_{max} (MeOH), 253 mμ (ϵ, 12600), 321 mμ (ϵ, 3100); λ_{max} (Nujol), 6.14 μ (found: N, 13.27; Cl, 17.22) which, upon heating with ethyl orthoacetate at 100° to 110°C, furnished 7-chloro-3-methyl-1,2,4-benzothiadiazine-1,1-dioxide (I), with a melting point of 330° to 331°C, λ_{max} (MeOH), 268 mμ (ϵ, 11300), λ_{max} (Nujol), 6.22 μ (found: N, 12.40; Cl, 15.41).

When compound I was administered orally at a dose of 5 mg/kg per day (in two divided doses) to renal hypertensive dogs, a gradual (in 2 to 6 days) fall in blood pressure was observed, which was maintained for the duration of the experiment (12 days) without evidence of diuresis. Upon withdrawal of the drug, the blood pressure returned to approximately pretreatment levels in 3 to 6 days. Essentially similar antihypertensive effects were obtained by using metacorticoid hypertensive rats. These results have also been confirmed clinically, the extent of the pressure reduction in many cases exceeding that observed with the benzothiadiazine diuretics.

Chemically, compound I differs importantly from the diuretic 1,2,4-benzothiadiazine-1,1-dioxides in being devoid of the benzenoid sulfamyl group. Further experiments have indicated that other compounds of this type and other classes of compounds which differ from known diuretic sulfonamides in that the sulfamyl group is replaced by hydrogen, alkyl, halogen, trifluoromethyl, or the like, demonstrate a similar antihypertensive activity separate from diuretic action. It thus appears, from our studies, that removal of the sulfamyl group from substances having diuretic properties usually results in compounds without diuretic effect but exhibiting antihypertensive activity. We are synthesizing and biologically evaluating an extensive series of these compounds.

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5 May 1961

Stereotypy and Intermittent Reinforcement

Abstract. Three pigeons were trained to peck at a horizontally oriented rubber srtip 10 in. long. The spatial distribution of responding along this strip is found to be nonrandom when every peck is reinforced with food. The degree of nonrandomness increases markedly when the pecking is intermittently reinforced.

Antonitis demonstrated that hungry rats will form strong preferences for particular locations when they are given equal opportunities to be fed at a number of different locations (*1*). The experimental situation he used consisted of a box that had a long, narrow, horizontal slot in one wall, and the rat was given a single pellet of food after it put its snout into any part of the slot. Each rat developed a marked preference for some location along the slot. Later, Antonitis discontinued de-