formation by molecular rearrangement of a given sulfanilide. We are interested in determining the structural configurations limiting the practical application of this double molecular rearrangement.

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ISOLATION OF A CRYSTALLINE PROTEIN POSSESSING THE PROPERTIES OF TOBACCO-MOSAIC VIRUS

A CRYSTALLINE material, which has the properties of tobacco-mosaic virus, has been isolated from the juice of Turkish tobacco plants infected with this virus. The crystalline material contains 20 per cent. nitrogen and 1 per cent. ash, and a solution containing 1 milligram per cubic centimeter gives a positive test with Millon's biuret, xanthoproteic, glyoxylic acid and Folin's tyrosine reagents. The Molisch and Fehlings tests are negative, even with concentrated solutions. The material is precipitated by 0.4 saturated ammonium sulfate, by saturated magnesium sulfate, or by safranine, ethyl alcohol, acetone, trichloracetic acid, tannic acid, phosphotungstic acid and lead acetate. The crystalline protein is practically insoluble in water and is soluble in dilute acid, alkali or salt solutions. Solutions containing from 0.1 per cent. to 2 per cent. of the protein are opalescent. They are fairly clear between pH 6 and 11 and between pH 1 and 4, and take on a dense whitish appearance between pH 4 and 6.

The infectivity, chemical composition and optical rotation of the crystalline protein were unchanged after 10 successive crystallizations. In a fractional crystallization experiment the activity of the first small portion of crystals to come out of solution was the same as the activity of the mother liquor. When solutions are made more alkaline than about pH 11.8 the opalescence disappears and they become clear. Such solutions are devoid of activity and it was shown by solubility tests that the protein had been denatured. The material is also denatured and its activity lost when solutions are made more acid than about pH 1. It is completely coagulated and the activity lost on heating to 94° C. Preliminary experiments, in which the amorphous form of the protein was partially digested with pepsin, or partially coagulated by heat, indicate that the loss in activity is about proportional to the loss of native protein. The molecular weight of the protein, as determined by two preliminary experiments on osmotic pressure and diffusion, is of the order of a few millions. That the molecule is quite large is also indicated by the fact that the protein is held back by collodion filters through which proteins such as egg albumin readily pass. Collodion filters which fail to allow the protein to pass also fail to allow the active agent to pass. The material readily passes a Berkefeld "W" filter.

The crystals are over 100 times more active than the suspension made by grinding up diseased Turkish tobacco leaves, and about 1,000 times more active than the twice-frozen juice from diseased plants. One cubic centimeter of a 1 to 1,000,000,000 dilution of the crystals has usually proved infectious. The disease produced by this, as well as more concentrated solutions, has proved to be typical tobacco mosaic. Activity measurements were made by comparing the number of lesions produced on one half of the leaves of plants of Early Golden Cluster bean, *Nicotiana glutinosa* L., or *N. langsdorffii* Schrank after inoculation with dilutions of a solution of the crystals, with the number of lesions produced on the other halves of the same leaves after inoculation with dilutions of a virus preparation used for comparison.

The sera of animals injected with tobacco-mosaic virus give a precipitate when mixed with a solution of the crystals diluted as high as 1 part in 100,000. The sera of animals injected with juice from healthy tobacco plants give no precipitate when mixed with a solution of the crystals. Injection of solutions of the crystals into animals causes the production of a precipitin that is active for solutions of the crystals and juice of plants containing tobacco-mosaic virus but that is inactive for juice of normal plants.

The material herein described is quite different from the active crystalline material mentioned by Vinson and Petre[1] and by Barton-Wright and McBain,[2] which consisted, as Caldwell[3] has demonstrated, largely of inorganic matter having no connection with the activity. These preparations were less active than ordinary juice from diseased plants, and the activity they possessed diminished on further crystallizations.

The crystalline protein described in this paper was prepared from the juice of Turkish tobacco plants infected with tobacco-mosaic virus. The juice was brought to 0.4 saturation with ammonium sulfate and the precipitated globulin fraction thus obtained was removed by filtration. The dark brown globulin portion was repeatedly fractionated with ammonium sulfate and then most of the remaining color was removed by precipitation with a small amount of lead subacetate at pH 8.7. An inactive protein fraction was removed from the light yellow colored filtrate by adjusting to pH 4.5 and adding 2 per cent. by weight of standard celite. The celite was removed, suspended in

[1] C. G. Vinson and A. W. Petre, *Contrib. Boyce Thompson Inst.*, 3: 131, 1931.

[2] E. Barton-Wright and A. McBain, *Nature*, 132: 1003, 1933.

[3] J. Caldwell, *Nature*, 133: 177, 1934.

water at pH 8, and the suspension filtered. The active protein was found in the colorless filtrate. This procedure was repeated twice in order to remove completely the inactive protein. Crystallization was accomplished by adding slowly, with stirring, a solution containing 1 cubic centimeter of glacial acetic acid in 20 cubic centimeters of 0.5 saturated ammonium sulfate to a solution of the protein containing sufficient ammonium sulfate to cause a faint turbidity. Small needles about 0.03 millimeters long appeared immediately and crystallization was completed in an hour. Crystallization may also be caused by the addition of a little saturated ammonium or magnesium sulfate to a solution of the protein in 0.001 N acid. Several attempts to obtain crystals by dialyzing solutions of the protein gave only amorphous material. To date a little more than 10 grams of the active crystalline protein have been obtained.

Although it is difficult, if not impossible, to obtain conclusive positive proof of the purity of a protein, there is strong evidence that the crystalline protein herein described is either pure or is a solid solution of proteins. As yet no evidence for the existence of a mixture of active and inactive material in the crystals has been obtained. Tobacco-mosaic virus is regarded as an autocatalytic protein which, for the present, may be assumed to require the presence of living cells for multiplication.

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ACTION POTENTIALS DURING HIGH AND LOW FREQUENCY STIMULATION OF MEDULLATED NERVE

IT has been shown by Hill and his collaborators (Feng and Hill,[1] Bugnard,[2] Hill,[3] Bugnard and Hill[4]) that when the frequency of a stimulus applied to frog medullated nerve is increased above about 500 shocks per second at room temperature there is a falling off in the total response as measured either by the heat produced or the accompanying electric potential. At a frequency of 2,500 one-way or 5,000 two-way shocks per second the response is only about 10 per cent. of that at the optimal frequency. Recently Cattell and Gerard[5] made the observation that a high frequency stimulus, itself producing a very small response, does not prevent the nerve from responding to a stimulus of lower frequency, applied above, at or below the electrodes giving the high frequency stimulation. These results indicated that the decreased effectiveness of high-frequency excitation involves a local change occurring at the stimulating electrodes. In the light of these observations Bugnard and Hill[6] have made a further analysis of the problem, interpreting the results on the basis of refractory period, summation and post-excitatory changes in irritability. All the experiments mentioned above were carried out with a technique which measured only the total response over a period of time and have the drawback that they do not permit observations of the individual action potentials in relation to the various combinations of stimulation frequencies, a deficiency which has had to be supplied by inference. We have therefore made a series of similar experiments, recording the details of the potential picture on the cathode-ray oscillograph. Individual responses were then directly observed under different experimental conditions with the following results.

1. The effect of increasing shock frequency. As the stimulation rate is progressively increased successive supermaximal shocks fall within the refractory period of the preceding responses and the individual action potentials therefore become smaller. Then there follows the well-known alternation in magnitude, and finally, with frequencies above 1,000 per second, only small irregular responses occur. This last state is the potential picture of the phenomenon originally described as "inhibition" by Bugnard. If the intensity of the stimulus is increased greater responses can be obtained.

2. The ability of the rapidly stimulated or "inhibited" nerve to conduct superimposed impulses. If, against a background of high frequency stimulation, extra stimuli of the same or lower intensity are applied through different electrodes, there are produced responses to these extra stimuli which are transmitted through the region of high frequency stimulation, as was observed by Cattell and Gerard. These responses can be reduced in magnitude by increasing sufficiently the intensity of the background stimulation; an effect which is presumably due to increased background activity, as described in the preceding paragraph.

3. The ability of the nerve to respond to superimposed stimuli. Not only can the "inhibited" nerve be stimulated at electrodes other than those carrying the high frequency background, but extra stimuli applied through the same electrodes are also capable of producing a response. (Indeed, in this case a curious phenomenon has been regularly observed: following the response to the extra stimulus there is an increased

[1] T. P. Feng and A. V. Hill, *Proc. Roy. Soc. B*, 113: 366, 1933.

[2] L. Bugnard, *Jour. Physiol.*, 80: 441, 1934.

[3] A. V. Hill, Suppl. SCIENCE, Vol. 79, 9, 1934.

[4] L. Bugnard and A. V. Hill, *Jour. Physiol.*, 83: 383; 394, 1935.

[5] McK. Cattell and R. W. Gerard, *Jour. Physiol.*, 83: 407, 1935.

[6] L. Bugnard and A. V. Hill, *Jour. Physiol.*, 83: 416, 1935.