3595

Time of Onset of Vagal Function in the Heart of Mammals.

HOWARD B. KELLOGG. (Introduced by A. C. Ivy.)

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Electrical stimulation of the peripheral end of the right vagus in ten pig fetuses which were about three-fourths term did not cause inhibition of the heart. Stimulation of the peripheral ends of the right and left vagus nerves of rabbits and dogs ranging from one hour to five days after birth caused no cardiac inhibition in 20 per cent of 32 animals examined; very weak inhibition was found in 36 per cent, and strong inhibition in 44 per cent. One rabbit and one dog showed a strong cardiac inhibition on stimulation of the sino-auricular node, even though stimulation of the vagi caused no such effect. It was very noticeable that in a large number of cases where inhibition was obtained it resulted from a vagal heart block, in which the atrial beat was increased in rate and frequently in amplitude. Stimulation of the central end of the vagus nerves inhibited respiration in all of the rabbits and dogs.

Apparently the afferent respiratory fibers of the vagus nerves are functional at birth, but in a large percentage of animals the cardio-inhibitor fibers either react weakly or are non-functional. There is some indication that the post ganglionic mechanism of the vagus in the heart may be functional before the preganglionic fibers are capable of transmitting impulses.

3596

Precipitation of Proteolytic Enzymes of Bacillus Proteus by Azine and Azonium Bases.

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That there is a definite chemical union between a proteolytic enzyme and an azine or azonum base was first suggested by Robertson,[1] who observed that if a drop of a saturated solution of safranine was added to a solution of trypsin a flocculent precipitate was formed which gradually settled to the bottom of the tube.

Holzberg[2] tested the proteolytic activity of this safranine-trypsin precipitate and found that it hydrolized casein, thus proving that the enzyme was precipitated by the safranine. Marston[3] made a study of the chemical nature of this compound. He found that the proteolytic enzyme trypsin was completely precipitated from its solution by safranine, and also by several other compounds which were water soluble and contained the azine nucleus, such as neutral violet, neutral red and the more complex indulines.

Marston states that the type of union between the protease and the azine base in a direct combination of the enzyme with the azine nitrogen, the acid nature or property of the enzyme being responsible for this combination. As evidence of this union he says that with the reduced leuko compound of safranine where the nitrogen bonds are all satisfied with hydrogen, no precipitation of the proteolytic enzyme takes place. The results of the work quoted above suggested the possibility of purifying, and studying the chemical nature and action of bacterial proteases.

With this in view a series of experiments were begun. *Bacillus proteus* was selected as the organism to use as it excretes a very active proteolytic enzyme. A culture of this organism was inoculated into sugar-free nutrient broth, and incubated for five days. At the end of this period the broth culture was filtered through Berkefeld filters to remove the organisms. The clear filtrate consisting of the broth, plus the soluble products of the bacteria, was first tested for the presence of the enzyme. The enzyme was then precipitated by adding to the filtrate an equal volume of a 5 per cent aqueous solution of safranine. This was filtered through a Buchner filter, washed several times with absolute alcohol, and dried. The protease is thus quantitatively removed from the broth, as is shown by the fact that the filtrate is devoid of all proteolytic powers. This precipitate consists of the protease combined with the safranine and also a small fraction of the proteins which are also precipitated by the azine bases. Amino acids, pepteds, or peptones do not react with the azines. They, therefore, must be related to the more highly organized proteins. A further study of this protein is being made for the reason that having the same reaction with the azine bases, it is probable that it may have other chemical and physical properties in common with the proteases.

The precipitate from the broth culture of *B. proteus* is small, being only a few milligrams per 100 cc. of medium. It is bluish in color and insoluble in any solvents thus far used. Marston dissolved the safranine precipitate of trypsin and pepsin in a M/15 solution of

sodium phosphate pH 7.5, but this solution had no effect on the bacterial protease safranine compound.

The precipitate is, however, soluble in the presence of proteins and it is probable that the compound is also dissociated. The precipitate is very active proteolytically; minute quantities of it will rapidly liquefy relatively large amounts of gelatin. The enzyme in combination with the azine base seems to be quite stable, retaining its proteolytic powers for at least one month, the longest interval at which it was tested.

Other azine bases will be used as precipitants, and of the many such bases it would seem at this time that three of them would be especially interesting to try. These three are paradiazine, phenazine and diketopiperazine. Paradiazine is one of the simplest of the azines, and if it acts as a precipitant of the protease, it is probable that the resulting compound would be more soluble, and more easily dissociated than the compounds formed by the more complex azine bases. This would be a distinct advantage in the attempt to purify the enzyme.

Phenazine has a structure similar to that of neutral red in that it has the azine nucleus with two nitrogens in the paraposition and two benzine rings attached but differs from it, and all other azine bases thus far used, in that it has no amino groups attached. Therefore if phenazine precipitates the protease it will be evidence in favor of the theory that the azine nitrogen is the point of attachment. If it does not precipitate the protease this would indicate that the nitrogen was not, and that possibly the amino group was the point of union.

Abderholden[4] suggested that proteins were not straight chains, but rather were composed of ring compounds being anhydrides of amino acids, or that proteins were essentially polydiketopiperazines in structure. Marston[3] also enunciated this theory and by it explained the chemistry of proteolytic enzyme action. In the light of this theory it will be well worth while to study the reactions of diketopiperazine and proteases.

This is a preliminary report.

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[1] Robertson, *J. Biol. Chem.*, 1907, ii, 343.

[2] Holzberg, *J. Biol. Chem.*, 1913, xiv, 335.

[3] Marston, *Biochem. J.*, 1923, xvii, 851.

[4] Abderholden, *Z. physiol. Chem.*, 1923, cxxviii, 119.