A cogent question at the present time in regard to the theory of chemical mediation of nerve impulses is whether the choline esterase, available in the neighborhood of a nerve ending, is sufficient to destroy the acetyl choline liberated by a nerve impulse within the refractory period (1). The superior cervical ganglion of the cat is a particularly desirable tissue for enzyme studies in this connection because of the wealth of reliable data available pertaining to this organ. Recently von Brücke (2) measured the choline esterase activity of this tissue by a biological method and demonstrated a decrease in activity parallel with a degeneration of the preganglionic fibres. Other evidence of a more or less indirect nature has also been presented to indicate a relative concentration of the enzyme in the region of the nerve endings by Marnay, Nachmansohn, and coworkers (3, 4) in their studies on muscle tissue.

In the present investigation chemical measurements of the choline esterase activity of superior cervical ganglia were made under conditions carefully controlled to yield a quantitative estimation of the enzyme, and the results obtained were applied to the calculation of theoretical maximum and minimum velocities of acetyl choline hydrolysis. For these calculations it was necessary to know the value of the dissociation constant for the reaction being studied; hence a separate determination of the affinity between enzyme and substrate was performed. In the light of the calculations made, the possibility that the refractory period of the ganglion is determined primarily by the esterase action was considered. A preliminary note concerning these studies has appeared recently (5).

* Fellow of The Rockefeller Foundation.
EXPERIMENTAL

Normal cats were killed by a blow on the head and the superior cervical ganglia were dissected out soon after. The ganglia, freed of all adhering tissue, were placed immediately in vessels fitted with stoppers and kept at -12° until ready for enzyme study. The tissue was used within 24 hours after the animal was killed. A cylinder of tissue was removed from the frozen ganglion by means of a borer having an internal diameter of 1.71 mm., and placed on the head of a rotary freezing microtome. Sections 25μ thick were cut, and determinations of the enzyme activity in single sections were carried out by the micro procedure already described (6). The sections were each placed in a tube with 9.2 c.mm. 30 per cent glycerol. After 1 to 2 hours, 16 c.mm. of 0.1 M veronal buffer, having a pH of 8.0, and containing 0.5 per cent of freshly dissolved acetyl choline chloride, were added. Digestion was allowed to proceed for 1 hour at 38°, after which the reaction was halted by adding 50 c.mm. of the usual eserine-brom-thymol blue solution, and titration was performed in the manner previously described (6). The volume of each section was 0.0574 c.mm., and, since the specific gravity was found to be 1.051, the weight of a section was 0.0603 mg.

The specific gravity was determined by suspending small pieces of the tissue in mixtures of bromo benzene and kerosene having different specific gravities. The specific gravity of the mixture in which the pieces remained suspended was taken as the specific gravity of the tissue.

No significant difference in activity could be observed between right and left ganglia of the same animal. The lowest average value for the enzymatic hydrolysis observed in a ganglion was equivalent to liberation of 3.03, and the highest 3.46 c.mm. N/20 acid in 1 hour under the conditions given. Under these conditions the degree of splitting was a linear function of time throughout the digestion period, and the substrate concentration was sufficiently high to ensure maximum velocity of hydrolysis. In a typical experiment seventeen analyses with 4 controls were conducted on one ganglion (Table I). The fairly constant enzyme activity observed throughout the ganglion would be expected from the homogeneous structure of the tissue.

A requirement of the micro method is that the digestion be carried out at a pH of 8.0. To obtain the corresponding activity at pH 7.4 reference may be made to a previous study on cat brain (6) in which the activity at pH 7.4 was found to be 0.74 times that at pH 8.0. Since 1 c.mm. N/20 acid = 9γ acetyl choline chloride, the quantity of this substance that can be hydrolyzed per milligram of tissue per second at pH 7.4 and 38° is then:
To obtain the value of the Michaelis or dissociation constant of the reaction between the enzyme and substrate, the ultra micro gasometric method of LINDELL-EMSON was employed since this method not only requires very little ganglion material but enables the course of the hydrolysis to be followed continuously. A ganglion weighing 15 mg. was ground with sand and Ringer's

\[
\frac{3.30 \text{ c.mm.} \times 9 \gamma \text{ c.mm.} \times 0.74}{3600 \text{ sec.} \times 0.0603 \text{ mg.}} = 0.10 \frac{\gamma}{\text{sec. mg.}}
\]

**FIG. 1.** Initial velocities of hydrolysis of acetyl choline chloride for various substrate concentrations.

Concentration of substrate (per cent) added to enzyme solutions is indicated on the lines drawn. Control experiment of barometric changes represented by points (x).

ström-Lang and Glick (7) was employed since this method not only requires very little ganglion material but allows the course of the hydrolysis to be followed continuously.
### CHOLINE ESTERASE

#### TABLE I

*Choline Esterase Activities of Microtome Sections of Ganglion*

<table>
<thead>
<tr>
<th>Microtome section No.</th>
<th>Acid liberated in 1 hr. at pH 8.0 and 38°C (c.mm. n/20)</th>
<th>Acid liberated in controls (c.mm. n/20)</th>
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<tr>
<td>1</td>
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</tr>
<tr>
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</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>3.63</strong></td>
<td><strong>0.33</strong></td>
</tr>
</tbody>
</table>

**Diff. = 3.30**

#### TABLE II

*Choline Esterase Activities As a Function of Substrate Concentration*

| Concentration of acetyl choline chloride added to enzyme solution, per cent. | 0.30 | 0.20 | 0.10 | 0.05 | 0.02 |
| Final concentration of acetyl choline, molar                          | 0.0213 | 0.00852 | 0.00426 | 0.00213 | 0.000852 |
| Pressure change per hr., cm. water                                     | 3.30 | 3.10 | 2.85 | 2.40 | 2.00 |
| CO₂ evolved per hr., corrected, c.mm. × 10⁻²                        | 33.23 | 29.78 | 28.76 | 22.73 | 15.87 |
| Relative initial velocities of hydrolysis, c.mm. × 10⁻⁴ CO₂ evolved × a factor | 99.69 | 89.34 | 86.28 | 68.19 | 47.61 |
solution, the mixture was filtered through paper, and the residue washed on the filter with Ringer's solution until a volume of 4 ml. of filtrate was obtained. 0.3 c.mm. of this enzyme solution was pipetted into 1.0 c.mm. of Ringer's solution containing the substrate (acetyl choline chloride). The pipettings were made directly into the Cartesian divers used as reaction vessels.

The 5 per cent CO₂, 95 per cent N₂ gas mixture was passed over the substrate solution both before and after addition of the enzyme. The paraffin oil seal was placed in the neck of each diver and the measurements were conducted at 25°C as described (7). Non-enzymatic hydrolysis of the substrate was found to be negligible under the conditions employed. Fig. 1 shows the course of the reactions with various substrate concentrations. Calculations of the CO₂ evolved by the reactions proceeding at the initial velocity for 1 hour were made in the usual manner (7), and the results (Table II) were expressed as cubic millimeters of gas at standard conditions corrected for solubility in the aqueous phase. The points in Fig. 2 were obtained from the data in Table II, and the extent of the agreement between them and the theoretical pS activity curve for a dissociation constant of 0.001 may be seen. This constant has the same value as that obtained for human serum acting upon acetyl choline chloride (8).

**Maximum Choline Esterase Activity**

The enzyme activity of the ganglion was shown to be equivalent to the splitting of 0.107 acetyl choline chloride per second per milligram of tissue as measured under optimal conditions of substrate

---

**Fig. 2.** Activity-pS relations of ganglionic choline esterase acting upon acetyl choline chloride.

The curve represents the theoretical relations for a dissociation constant of 0.001, the points were derived experimentally.
concentration at a pH of 7.4 and 38°. This figure may be applied to the data of Brown and Feldberg (9) (who found in a typical experiment 22.5 \( \gamma/\text{gm.} \) the concentration of the ester in an unstimulated superior cervical ganglion of the cat): \( \frac{22.5 \ \gamma/\text{gm.}}{100. \ \gamma/\text{gm. sec.}} = 0.225 \ \text{sec.} \) would be the time required for the enzyme to hydrolyze that amount of acetyl choline normally coexistent with it in this ganglion, provided that the enzyme and substrate were in complete combination; that is, the maximum velocity of hydrolysis were maintained. Actually the velocity falls when the substrate concentration becomes less than a certain value; hence 0.225 second represents a limiting least time which might be merely approached in actuality.

Brown and Feldberg (9) also have shown that the greatest output of acetyl choline from the ganglion perfused with eserinized Locke's solution occurs in the first 5 minutes of preganglionic stimulation at 17 per second. In this period 0.1\( \gamma \) was liberated from a ganglion weighing 12.9 mg. Hence \( \frac{0.1 \gamma}{12.9 \ \text{mg.} \times 0.10 \ \frac{\gamma}{\text{mg. sec.}}} = 0.078 \ \text{sec.} \) would be the limiting least time required for the enzyme in a given weight of tissue to destroy the acetyl choline formed during the first 5 minutes of stimulation of this tissue. Furthermore \( \frac{0.015 \sigma}{300 \times 17 \ \text{stimulations}} = 0.015 \sigma \) would then be the limiting least time for splitting the acetyl choline liberated by one nerve impulse. Compared to the refractory period of the ganglion which Brown (10) has found to be of the order of 2\( \sigma \), it is apparent that the enzyme need operate only with an average rate of about 0.75 per cent of its theoretical maximum velocity in order to destroy the acetyl choline liberated by a nerve impulse within the refractory period. After the first 5 minutes, the quantity of acetyl choline liberated per impulse falls until finally only about a fifth of the initial amount is set free (9); under these conditions the enzyme could hydrolyze the acetyl choline within the refractory period at about 0.15 per cent of its maximum velocity.

**Minimum Choline Esterase Activity**

From the foregoing it would appear that the enzyme present is sufficient to destroy the acetyl choline liberated by a nerve impulse.
within the refractory period. However, it must be borne in mind that for the conditions of minimum velocity of hydrolysis, as in the case of an even distribution of enzyme and substrate throughout the tissue, the reaction velocity would be very far indeed from the maximum one, because of the low substrate concentration and affinity for the enzyme. Since \( \frac{dS}{dt} = V_{\text{max}} \frac{S}{K_s + S} \) where \( S \) represents the substrate concentration, \( t \) the time, \( V_{\text{max}} \) the maximum velocity of hydrolysis, and \( K_s \) the Michaelis constant for the affinity between enzyme and substrate, it follows that the time for splitting 99 per cent of the substrate is given by:

\[
 t = \frac{1}{V_{\text{max}}} \int_{s_1}^{s_2} \frac{K_s + S}{S} \, dS
\]

where \( s_2 \) is the original substrate concentration and \( s_1 \) is 1 per cent of \( s_2 \). From this equation we have

\[
 t = \frac{1}{V_{\text{max}}} \left( K_s \ln \frac{s_2}{s_1} + s_2 - s_1 \right) = \frac{4.6K_s + 0.99s_1}{V_{\text{max}}}.
\]

The value of \( s_1 \) or the concentration of acetyl choline developed by one nerve impulse becomes:

\[
 \frac{0.1\gamma}{12.9 \text{ mg.} \times 5100 \text{ stimulations}} = 1.5 \times 10^{-4} \frac{\gamma}{\text{mg.}}
\]

or

\[
 1.5 \times 10^{-4} = 8.3 \times 10^{-4} \text{ molar}
\]

Since it was shown earlier in this paper that the \( K_s \) for the case in question is 0.001, as determined by the procedure described, it follows that

\[
 t = \frac{(4.6 \times 0.001) + \left(0.99 \times 8.3 \times 10^{-4} - \frac{9 \text{ mols}}{\text{liter}}\right)}{0.1 \text{ mols}} = 8.3 \text{ sec.}
\]

In order that this time be reduced to the refractory period of 2\( \sigma \), it would be necessary for the enzyme and substrate to be concentrated within a small portion of the total ganglionic volume, such as at the nerve endings. Evidence for a localization of this type has already been mentioned (1-4).

The calculations given serve to show the great divergence between the times for splitting under minimum and maximum velocities, and
hence the requirement of a state of localization of enzyme and substrate within the ganglion cell if the nerve-liberated acetyl choline is to be destroyed within the brief span of the refractory period.

SUMMARY

The maximum choline esterase activity of the superior cervical ganglion of the cat was measured and found to be, on the average, equivalent to the splitting of 0.10μ of acetyl choline chloride per second per milligram of fresh tissue at a pH of 7.4 and 38°. The least possible time required for destruction of the ester liberated by one nerve impulse was calculated to be 0.015μ.

The dissociation constant of the reaction between the enzyme and acetyl choline chloride was determined, and a value of 0.001 was obtained.

From the value of the dissociation constant, the time for hydrolysis at the minimum rate was calculated to be about 8 seconds.

It was shown that a localization of enzyme and substrate within the ganglion cell would have to exist in order that enzymatic destruction of acetyl choline liberated by nerve impulses occur within the span of the refractory period.

The author wishes to express his appreciation of the interest with which Prof. S. P. L. Sørensen has followed this work, his gratitude to Dr. K. Linderstrøm-Lang for his invaluable suggestions and discussion, and his thanks to Dr. W. L. Doyle and K. Mogensen for providing the ganglia used. It is also a pleasure to acknowledge the stimulating suggestions afforded by Dr. G. L. Brown of the National Institute of Medical Research, London.

BIBLIOGRAPHY