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A METHOD OF DETERMINATION OF CHOLINESTERASE ACTIVITY AND ITS DISTRIBUTION IN INVERTEBRATES

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A METHOD OF DETERMINATION OF CHOLINESTERASE ACTIVITY AND ITS DISTRIBUTION IN INVERTEBRATES

INTRODUCTION

Cholinesterase is the name suggested by Stedman (1) in 1932 for the enzyme or enzymes which hydrolyze choline esters. In view of the importance attached to acetylcholine, which is believed by many investigators to be the humoral agent by which the parasympathetic nerves transmit their impulses to their respective end organs, the fundamental importance of this enzyme in limiting the action of a nerve to its end organ and constantly protecting the body against possible acetylcholine poisoning becomes obvious.

In spite of numerous researches on the action, specificity, distribution and physiological function of the enzyme, there are many observations which have not been explained.

The author began the study of cholinesterase as a result of the observation that when the isolated heart of the marine clam, Venus Mercenaria, was bathed in sea water containing a small quantity of acetylcholine (from .001 to .000001 mg. per milliliter) the heart would momentarily stop beating. When eserine was added (.01 mg. per ml.), which is ordinarily assumed to be sufficient to inhibit cholinesterase activity completely, no increase in sensitivity to acetylcholine was noted. Dr. David Glick very kindly invited me to visit his laboratory and together we studied the distribution of cholinesterase in several marine forms. The results of the study, the first of its kind on species of marine invertebrates common to the New England coast around Woods Hole, Massachusetts, will be presented as the second portion of this thesis.

From the summer of 1938 to the present time, a large number of determinations of cholinesterase activity have been made. Several methods have been tried. Because of the difficulties and limitations attached to all of the methods which have been described to date, the seemingly simple method published by Rinkel and Pijoan (2) was tried. Their method used the Van Slyke manometric apparatus and in their summary they stated that the method was, "simple and accurate".

Preliminary experiments were performed using their exact procedure on the cholinesterase activity of rabbit, dog, horse, and human sera. In the light of previous experience in measuring cholinesterase activity it was soon recognized that determinations made as they suggested were no real measure of the true enzyme activity, since the authors apparently failed to recognize all the chemical reactions upon which their determination depended. Full consideration of these facts will be taken up later.

It is the purpose of the first portion of this thesis to present a method where by the Van Slyke manometric apparatus can be used to obtain as accurate measurements of cholinesterase activity as the design of the apparatus permits.

The results obtained by this new procedure will be compared with other accepted methods and advantages and limitations of each will be discussed.

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A New Method for Determining Cholinesterase Activity.

A. Chemistry of Cholinesterase Determinations.

The enzymatic decomposition of acetylcholine proceeds as follows:

$$\text{Acetylcholine} \xrightarrow[\text{esterase}]{\text{choline-}} \text{choline} + \text{acetic acid} \quad (1)$$

Since acetylcholine is ten thousand times as active physiologically as choline itself, the early methods measured the activity of the remaining acetylcholine on some sensitive organ or eserinized animal. These methods are subject to considerable error and have, for the most part, been abandoned for more precise and accurate chemical methods. No discussion of them will be undertaken here.

The eight chemical methods of enzyme assay (1, 2, 3, 4, 5, 6, 7, 8,) except the colorimetric method of Abdon and Uvnas, (9) depend on the fact that acetic acid is one of the products of the hydrolysis (See equation 1 above). They differ greatly with regard to details of time, temperature, pH, substrate used and concentration of enzyme and substrate employed.

The simplest procedures are the direct titration methods of Stedman, (10) et al., Glick, (6) Roepke (11) and Alles and Hawes (12). The best procedure of this type seems to be that of Alles and Hawes, in which the acetic acid liberated is continuously titrated with standard alkali to a constant pH by the assistance of a glass electrode.

The other type is the manometric methods of Ammon, (3) Stedman and Stedman, (4) Linderstr~~o~~m-Lang and Glick, (5) and Rinkel and Pijoan (2). All of these depend on the reaction of the acetic acid produced, with sodium bicarbonate present in the reaction mixture. The CO_2 so produced, is measured manometrically.

$$CH_3COOH + NaHCO_3 \rightarrow CH_3COONa + CO_2 + H_2O: \quad (2)$$

Since these procedures (see page 56 for details) utilize a gas mixture consisting of 95% Nitrogen and 5% carbon dioxide to keep the reaction anaerobic and saturated with carbon dioxide, the CO_2 resulting from equation 2 will diffuse to the vapor phase and exert pressure on a manometer. The method has general application to esterases since it can be applied validly whenever the acid released by the hydrolysis of the ester used as the substrate, is sufficiently ionized to react with the bicarbonate in solution and release CO_2. Obviously, enough, bicarbonate ion must be present to react with the acetic (or other acid) as fast as it is liberated, otherwise the second reaction becomes the limiting factor.

In general, the following criteria can be applied to test the adequacy of the various chemical methods:

1. The enzyme activity e.i., the acetylcholine split or CO_2 produced, should give a straight line function with time.

2. Total hydrolysis figures should be corrected for the coincident non-enzymatic or alkaline hydrolysis of the ester.

3. The pH and temperature of the enzyme solution should be kept as nearly constant as possible.

Examination of the method and results of Rinkel and Pijoan (2) show that their method not only did not fulfill the three above mentioned requirements but also included certain other points of criticism among which the following are most apparent.

4. No experimental data are presented to prove that the enzyme is not partially inhibited by the mercury with which presumably it comes into direct contact.

5. The constants for the Van Slyke apparatus (19) which they used does not apply in their procedure.

6. Since the only source of bicarbonate for equation 2 in their method, is the 1 ml. portion of blood which they use, and this in many pathologic cases may be far below the normal, the limiting factor in their procedure may be the bicarbonate content of the blood.

A low result might be due to low bicarbonate rather than too low cholinesterase.

In order better to appreciate these difficulties, the author has taken the liberty of quoting their original method in order that a clearer discussion of the six difficulties can be presented.

METHOD AND PROCEDURE OF RINKEL AND PIJOAN (2)

"REAGENTS. Acetylcholine chloride C.P.; CO_2-free distilled water; 1 N KOH.

PROCEDURE. The Van Slyke extraction apparatus having been cleaned several times with distilled water, with the reaction chamber filled with mercury, 1 cc. of serum is admitted into the cup from a pipette. The serum is allowed to run into the reaction chamber neck and the stop-cock sealed with mercury. Lower the bulb until the surface of the mercury ~~of the mercury~~ and serum is first below the reaction chamber. Close stop-cock e (see Van Slyke and Neill for directions of gas extraction) and shake for 3 minutes. Open stop-cock e and allow the mercury to rise into the chamber. Allow the serum to rise in deliver cup. Place 1cc. of CO_2-free distilled water containing 50 mgm. of acetylcholine into delivery cup and allow it to run into reaction chamber with the serum, taking care not to allow any air to enter into reaction chamber. Seal with mercury. Shake for 10 seconds and take Po reading in manometer scale with the level of serum at the 2 mark. Lower to half way in reaction chamber. Do not shake, and take readings with serum level at the 2 mark every five minutes for 20 minutes, (P, P_1, P_2, P_3, P_4). It is well to keep the water temperature in the mantle surrounding the reaction chamber at 29°-30°C. After readings have been taken for 20 minutes the mercury level is brought to the half way mark once more and 1 cc. of 1 N KOH are admitted into the reaction chamber. This will absorb the CO_2 evolved. A reading with the serum level at the 2 mark will agree within 2 to 3 per cent of the initial Po reading."

A study of their procedure shows that an undetermined portion of the bicarborate content of the serum is removed by their initial gas extraction. This tends to make the reaction mixture more alkaline. Also they do not shake the enzyme mixture nor do they lower to the fifty cc. mark. According to Van Slyke and Sendroy (15) the factors which they use do not apply unless the mercury is lowered to the 50cc. mark and the solution shaken 200 times or more per minute.

The author performed 25 initial determinations on rabbit, dog, horse and human precisely as these authors (2) state. A typical curve is shown in Fig. I. For comparison, a curve showing a determination of cholinesterase activity by the direct titration procedure (1), and also a curve typical of the Warburg manometric procedure of Ammon (3) are shown.

It can be seen that whereas the second and third methods give straight lines, the first gives a curve whose slope changes rapidly. The rate changes during each 5 minute inteval~~s~~. Expressed as mm. of mercury, the rate varied as follows:

0-5 minutes	51 mm.	15-20 minutes	20 mm.
5-10 minutes	82 mm.	20-25 minutes	10mm.
10-15 minutes	44 mm.	25-30 minutes	1.5 mm.

Rinkel and Pijoan used the reading at 20 minutes as the rate but comparison of several curves determined on different samples

showed that this procedure for establishing rates might lead to errors as great as 20%.

Realizing that to make the method usable would necessitate the introduction of serious changes in the procedure, the author began an orderly study of the entire method, including the fundamental Henderson-Hasselbalch equation upon which the determination is based.

B. Experimental Verification of a Method for Calculating the pH.

Before any satisfactory answers to the other problems could be obtained, a method for determining the pH during the course of the reaction had to be devised. In the previous discussion of the chemistry of the method it was shown that when cholinesterase acted on acetylcholine salts, acetic acid was formed. This in turn reacted with the sodium bicarbonate in the solution to form sodium acetate and carbonic acid. In this case the sodium bicarbonate-carbonic acid equilibrium is acting as a buffer system. This system has been intensively studied because of its importance in acid-base balance in physiological solutions.

In order to explain the action of certain buffers which tend to maintain the pH of physiological solutions close to the neutral point, Henderson (14, 15) used the well known mass action equation

$$K[Ha] = [H^+] \times [a^-] \qquad (3)$$

in which $[Ha]$ is the concentration of the undissociated weak acid, $[H^+]$ the concentration of hydrogen ions and $[a^-]$ the concentration of the anion. K is the constant of proportion or dissociation. He showed that Ostwald's dilution law

$$K[Ha] = [H^+]^2 \qquad (4)$$

which holds for pure solutions of weak acids, can never be applied in biological solutions since they always contain alkali salts of the acid. Therefore, for the most part, the $[a^-]$ does not come from the dissociation of the weak acid according to equation 3 but rather from the alkali salts. These salts are partially ionized, and we express their ionization by the equation

$$[a^-] = \gamma [Ba] \tag{5}$$

where $[Ba]$ represents the total molar concentration of the salt and γ, representing the fraction ionized, has a value between 0.6 and 0.9. By substituting $\gamma[Ba]$ in place of $[a^-]$ in equation 3, Henderson obtained equation 6 to which his name is attached.

$$K[Ha] = [H^+] \times \gamma [Ba] \tag{6}$$

Now if we rearrange this equation and let $K/\gamma = K'$ we get the usual form of the equation

$$[H^+] = K' \frac{[Ha]}{[Ba]} \tag{7}$$

K' is sometimes called the "apparent dissociation constant". If we invert equation 7 we get

$$\frac{1}{[H^+]} = \frac{1}{K'} \frac{[Ba]}{[Ha]} \tag{8}$$

Taking the logarithm of each side and using the connotation of Sörensen, - log H^+ = pH, etc., Hasselbalch (16) obtained the following equation

$$pH = pK' + \log \frac{[Ba]}{[Ha]} \qquad (9)$$

which we know as the Henderson-Hasselbalch equation. In the example which we are considering this becomes

$$pH = pK' + \log \frac{BHCO_3}{H_2CO_3} \qquad (10)$$

In this case, to be exact, one should use the symbol pK_1' since Buytenduyk (17) has shown that the value of K in the equation

$$K[H_2CO_3] = [CO_2] \times [H_2O] \qquad (11)$$

is so high that only a fraction of a percent of the free carbonic acid in water solution is in the form of H_2CO_3; over 99 per cent remains as anhydrous CO_2.

In order to avoid confusion the author has adopted the following abbreviations and symbols.

Abbreviations and Symbols

pK_1'	as the apparent first dissociation constant of carbonic acid;
H_2CO_3	to represent all the uncombined carbonic acid ($H_2CO_3 + CO_2$) in solution;
pCO_2	to represent the tension of CO_2 above a solution in millimeters of mercury;
$[CO_2]$	to represent the total CO_2 content of the liquid and gaseous phases after the solution has been acidified, the content expressed in millimoles per liter;
$p[CO_2]$	the total CO_2 pressure above the solution after acidifying expressed in millimeters of mercury.

In order to be able to calculate the pH in the enzyme mixture by means of the Henderson-Hasselbalch equation, the following quantities must be determined.

1. The amount of bicarbonate ion present in the solution.
2. The amount of carbonic acid ($CO_2 + H_2CO_3$) in the solution.
3. The proper value of the constant pK_1'.

To determine the bicarbonate content of a solution containing both bicarbonate ion and carbonic acid, the following procedure was devised.

The total bicarbonate and carbonic acid content of the liquid is determined by the method of Van Slyke et al. Van Slyke and Stadie (18) developed the equation

$$V_0^{o},760 = P \times \frac{ia}{760\ (1+0.00384\ t)} \times \left(1 + \frac{S\alpha'}{A-S}\right) \quad (12)$$

in which V is the required volume of gas at standard conditions; P is the pressure of the gas in millimeters of mercury; i is the reabsorption coefficient; a is the fixed gas volume at which the pressure is read; (1 + 0.00384 t) is tho correction for the effect of temperature expanding mercury more than glass; S is the volume of solution in the apparatus; α' is the ratio in which carbon dioxide distributes itself between equal volumes of aqueous and gaseous phases when extraction is complete; and A is the total volume of the gas chamber.

Van Slyke and Neill (19) then worked out a table of factors for various values of a, i, and S at various temperatures by which one can calculate the $[CO_2]$, as defined on page 17, if the pressure of CO_2 above the acidified solution is known.

Since

$$[CO_2] = BHCO_3(\text{liq.}) + H_2CO_3\ (\text{liq.}) + CO_2\ (\text{gas}). \quad (13)$$

then

$$BHCO_3 = [CO_2] - [H_2CO_3 + CO_2]. \quad (14)$$

By means of equation 12, the sum of the carbonic acid content of the liquid and the CO_2 in the vapor phase can be calculated if the tension of CO_2, pCO_2, above the solution is known. This can be determined easily in the Van Slyke manometric apparatus.

To get the amount of carbonic acid in solution we utilize a variation of equation 12. In equation 12, the last expression

$$\left(1 + \frac{S\alpha'}{A-S}\right) \qquad (15)$$

is a correction factor for the amount of carbonic acid in the solution. If we represent this expression 15, by the symbol U, then U - 1 is that part of the carbon dioxide which is dissolved in the solution and which we shall call H_2CO_3. Then U-1/U is the ratio of the amount of CO_2 in solution to the total CO_2. Since α' is a function of the temperature(19)

$$\alpha' = \alpha \times Kt \qquad (16)$$

it follows that U-1/U will vary with the temperature. The numerical value of the fraction depends on the value of the temperature, and the quantities S and A-S. When $\alpha = 0.84$, S = 3,0 ml., and A-S = 47 ml., U-1/U is equal to 0.0545 at 20° C., and 0.0425 at 30° C. This factor will be referred to as factor T. (See Table I.)

Having computed factors for obtaining the bicarbonate content and the carbonic acid concentration of the reaction mixture, the only value required to calculate the pH is the pK_1'. Van Slyke, Sendroy, Hastings and Neill (20) calculated that with the solubility coefficient, α, equal to 0.051, the value of pK_1' should be 6.10. Some of the calculations of a more probable value will be presented later but until such data were available the author accepted the value, 6.10.

To simplify the calculation of the pH, the author has used the following symbols to designate the different factors.

pCO_2 x factor R = CO_2 in gaseous and liquid phases in mM./liter.

$p[CO_2]$ x factor S = $[CO_2]$ in the solution after acidifying in mM./liter.

CO_2 in mM./liter x factor T = H_2CO_3 in mM./liter.

The values for these factors for various temperatures appear in Table I. An explanation of the detailed application of them in the calculation of the pH will be reserved for the section on the procedure. The six-step calculation is as follows:

FORMULA A

1) $pCO_2 \times R = CO_2$ in mM./liter.

2) $p[CO_2] \times S = [CO_2]$ in mM./liter.

3) $[CO_2] - CO_2 = BHCO_3$ in mM./liter

4) $CO_2 \times T = H_2CO_3$ in mM./liter.

5) $pK_1' = 6.10 + 0.005(37-t)$

6) $pH = pK_{1_t}' + \log \frac{BHCO_3 \text{ mM:/liter.}}{H_2CO_3 \text{ mM./liter.}}$

TABLE I

FACTORS FOR CALCULATING pH IN CHOLINESTERASE ASSAYS
BY FORMULA A

	S=3 ml., a=2 ml., i=1.017			S=6.5 ml., a=2 ml., i=1.017		
Temp.	R	S	T	R	S	T
20	0.0399	0.0394	0.0545	0.0198	0.0195	0.119
21	0.0397	0.0392	0.0532	0.0197	0.0194	0.116
22	0.0394	0.0390	0.0518	0.0195	0.0192	0.113
23	0.0392	0.0388	0.0501	0.0194	0.0191	0.110
24	0.0390	0.0386	0.0491	0.0193	0.0190	0.108
25	0.0388	0.0384	0.0477	0.0192	0.0189	0.105
26	0.0387	0.0382	0.0466	0.0190	0.0188	0.103
27	0.0385	0.0380	0.0456	0.0189	0.0186	0.101
28	0.0383	0.0379	0.0444	0.0188	0.0185	0.0981
29	0.0381	0.0377	0.0436	0.0187	0.0184	0.0962
30	0.0379	0.0375	0.0425	0.0186	0.0183	0.0942

During the course of a determination the ratio of the bicarbonate content to the carbonic acid content in the solution is constantly changing. The acetic acid, liberated by the esterase, makes the reaction mixture more and more acid. This is of course a disadvantage as the enzyme has its optimum activity at a pH around 8.4.

It occurred to the author early in this study that it would be much simpler and more accurate if the pH could be kept constant by means of a suitable buffer as in the other two methods, namely, titration and the Warburg manometric procedures. Such a buffer would have to be the salt of an acid with an ionization constant lower than the first apparent ionization constant of carbonic acid. Otherwise the stoichiometric relationship between the quantity of acetic acid liberated and the increase in CO_2 pressure above the liquid would be altered. If the ionization constant were higher bicarbonate would react with the buffer. An illustration of this is the well known reaction

$$NaH_2PO_4 + NaHCO_3 \longrightarrow Na_2HPO_4 + H_2CO_3 \qquad (17)$$

Obviously, any CO_2 so produced would not depend on the activity of cholinesterase.

If the buffer chosen was the salt of an acid weaker than carbonic acid, such as sodium phenolate, then H_2CO_3 already formed would react with it.

$$H_2CO_3 + NaC_6H_5O \longrightarrow NaHCO_3 + C_6H_5OH \qquad (18)$$

There may be some solution to this apparent dilemma but so far none of the attempts to maintain the pH constant have been successful. The problem in the case of the Van Slyke manometric apparatus is entirely different than in the other two procedures already referred to.

The calculated pH was compared with the pH as determined by a glass electrode. The glass electrode (Leeds and Northrop) was standardized before and after each determination against a 0.05 M solution of specially purified potassium acid phthalate which has been shown to have a pH of 4.000.

The determination of the pH was made alternately by the two methods on a standard mixture containing:

7 ml. sodium bicarbonate solution (containing about 8 mg. $NaCO_3$.)
8 ml. human serum
33 ml. Ringer solution
48 ml. Total volume

In each of the experiments in the group No. 221- No. 230, 3 ml. of this standard mixture was used. In those in which the pH was to be calculated, the liquid was introduced into the Van Slyke manometric chamber, and shaken under vacuum (using regular Van Slyke gas extraction procedure) until a constant pressure reading was obtained. This reading is the pCO_2 to be substituted into formula A. Then 0.5 ml. of 1 N lactic acid was introduced and $p[CO_2]$ was determined in the usual technique for manometric blood gas analysis. Finally the CO_2 was absorbed by introducing 0.2ml. of 5 N NaoH. From these three readings the pH can be calculated and was calculated by means of formula A. The readings were corrected by blank determinations on the reagents. These will not be described since they were carried out according to the directions given in Peters and Van Slyke. (21)

In those analyses in which the pH was determined by glass electrode, the liquid (3 ml.) was extracted as before and when constant pCO_2 readings were obtained, the liquid was allowed to rise slowly from the 50 ml. mark until about half of the extraction chamber was filled with mercury. Then the stop-cock at the top of the extraction chamber was carefully opened until the contents of the chamber came into equilibrium with the atmosphere. The liquid was then transferred by means of an Ostwald-Folin pipette directly into a special chamber so designed that a pH determination could be made on 3.0 ml. of fluid without appreciably exposing it to air. This procedure made it possible to remove the liquid from the chamber and yet retain the same CO_2 tension above the solution that was present during the gas extraction.

The results of these paired determinations are shown in the chart below.

TABLE II

Determination Number	pH as calculated from CO_2 content	Determination Number	pH by glass electrode
221	8.17	222	8.38
224	8.13	223	8.27
226	8.29	225	8.35
228	8.31	227	8.36
230	8.30	229	8.33

Determination No. 222 was probably in error since the pH reading fluctuated very much during the determination. The first reading taken was below 8.30. This is more in agreement with the next determination, No. 223, made only 30 minutes later on another portion of same solution, which gave a pH reading of 8.27.

Because of the technical difficulties of handling 3 ml. of fluid and the need of a special chamber which perhaps introduced some error, a similar series of experiments were performed using a volume of 6.5 ml. instead of 3 ml. used before. This permitted us to use the regular glass electrode chamber. The fluid was transferred more quickly to the glass electrode and with even less contact with air. However, to protect the glass electrode, no oil or mercury was used to maintain anaerobic conditions. No appreciable change was noted when subsequent determinations of the pH were made on the same solution. Since the tension of CO_2 above the solution is so much lower than in blood, the precautions against loss of CO_2 are not necessary (22, 23).

For this series, the following mixture was made and 6.5 ml. of it was used for each experiment.

60 ml.	bicarbonate Ringer (Glick) (5)
20 ml.	human serum (J.J.)
180 ml.	Ringer solution (without bicarbonate)
260 ml.	Total volume

Using the same technique as described before, pairs of determinations were performed, and the pH, as determined by calculation from CO_2 content, was compared with the pH as determined by glass electrode. The results of these experiments are given in Table III on the following page.

TABLE III

Determination Number	pH as calculated from CO_2 content	Determination Number	pH by glass electrode
234	8.27	233	8.30
236	8.27	235	8.34
238	8.27	237	8.36
241	7.68	242	7.70
243	7.69	244	7.79
246	7.74	247	7.84
250	7.82	251	7.92
252	7.83	253	7.93
255	7.81	245	7.90
259	7.86	260	7.96
262	7.89	261	7.98

In Fig. 2 the pH values, as determined by glass electrode, are plotted against the pH as calculated from CO_2 determinations. It can be seen that all the determinations lie on the right side of a line connecting equal pH values, when $pK_1' = 6.10$ is used.

This may perhaps mean that the pK_1' used, namely, 6.10, which was determined for blood at 38° does not apply exactly.

According to Cullen, Keeler and Robinson (J.B.C. Vol. 66, 301 (1925) the pK_1', of carbonic acid decreases as the temperature rises and the decrease is equal to about 0.005 unit per degree centigrade. From the graph Fig. 2, it can be seen that most of the points fall very close to the line which connects points of equal pH value if pK_1' is assumed to be equal to 6.16 for 25°C. instead of 6.10 which was the value used in the calculations.

On the basis of the close correspondence of calculated pH values with those determined by the glass electrode if one assumes pK_1' equal to 6.16, all subsequent calculations were made using this value for the apparent first dissociation constant for carbonic acid.

An excellent review of the work of various authors concerning the use of the Henderson-Hasselbalch Equation, the theory of the dissociation of carbonic acid and determination and variation of the constants used is included in Peters and Van Slyke Vol.I, pages 874-917. The entire Chapter XVIII, "Carbonic Acid and Acid - base Balance", is recommended by the author as a most concise exposition of the whole problem.

The author discovered that there was a logarithmic relationship between the carbon dioxide tension, before referred to as pCO_2, the total bicarbonate and CO_2 content, for which we have used the symbol $[CO_2]$, and the pH. This discovery, which might have been deduced from the work of Peters (24, 25) is very useful since it eliminates the need of calculating the pH. Peters had pointed out that the equation

$$\left[CO_2\right] = 0.1316\alpha(10pH - pK_1' + 1)\,p \qquad (19)$$

can be reduced to the following simple equation $\left[CO_2\right]$ = constant x p if the pH is kept constant. Taking the log of both sides it becomes

$$\log\left[CO_2\right] = \log \text{Constant} + \log p. \qquad (20)$$

This is a rectilinear equation of the form $y = a + x$ in which the slope of the line is 45°.

The author discovered this fact quite accidentally while trying to get a graphic method which would permit one to read off the pH directly when the pCO_2 and p $\left[CO_2\right]$ are known.

Such a graph is shown in Fig. No. 3, To construct such a chart relating CO_2 tension, $\left[CO_2\right]$ content and pH, it is necessary to lay off on x and y axes of logarithmic paper values of the $\left[CO_2\right]$ content in millimeters of mercury and CO_2 tension respectively. Then substituting known values into the formula A page 18, the following table (See Table IV) was calculated. This chart fig.3, constructed from Table IV applies exactly when the total volume is 6.5 ml. and the temperature is 25° C.

TABLE IV

pH Values Corresponding to Various pCO_2 and $p[CO_2]$ Readings.

Volume of solution = 6.5 ml.
Temperature = 25.0° C.
pK_1' at 25° C. = 6.16

pCO_2	$p[CO_2]$		
	100 mm. Hg.	200 mm. Hg.	300 mm. Hg.
	pH	pH	pH
10 mm. Hg.	8.10	8.42	8.61
20 mm. Hg.	7.75	8.10	8.29
30 mm. Hg.	7.52	7.90	8.10
40 mm. Hg.	7.33	7.75	7.96
50 mm. Hg.	7.15	7.62	7.84
60 mm. Hg.	6.98	7.52	7.75
70 mm. Hg.	6.79	7.42	7.66
80 mm. Hg.	6.57	7.33	7.59
90 mm. Hg.	6.25	7.24	7.52
100 mm. Hg.		7.15	7.45

In order to determine what effect temperature has on the pH when pCO_2 and $p[CO_2]$ are kept constant, the pH at various temperatures, 20° - 30°,C., was calculated when pCO_2 = 10 mm. Hg. and $p[CO_2]$ = 100 mm. The effect of temperature on pH and pK_1' ~~are~~ is shown in Fig. No. 4. As can be seen from the chart the pK_1' decreases with increasing temperature, while the pH as calculated increases. The following corrections to the reading apply when the graph fig. 3, relating CO_2 tension, $[CO_2]$ content and pH is used at some temperature besides 25°.

TABLE V

Temp.	Correction Factor	Temp.	Correction Factor
20°	-0.026	26°	+0.005
21°	-0.022	27°	+0.009
22°	-0.016	28°	+0.016
23°	-0.009	29°	+0.019
24°	-0.005	30°	+0.023
25°	0.000		

Thus at 30°, the pH read from the chart would be 0.023 pH units too low, while at 20°, the pH as read from chart would be 0.026 too high. By means of these corrections the log-log graph can be used for any pCO_2 from 4-400 mm. and $p[CO_2]$ from

10-1,000 mm. at any temperature from 20°-30° C., although for practical purposes only pCO_2 pressures from 4-200 mm., and $p[CO_2]$ pressures from 80-500 mm., are used because of the limitations of the height of the mercury manometer from which the pressures are read.

C. Non-enzymatic Hydrolysis of Acetylcholine Salts

It has long been recognized that in accurate determinations of the amount of acid liberated when acetylcholine salts are hydrolyzed by cholinesterase, a correction factor should be applied for the amount of acetylcholine which is decomposed by the alkalinity of the enzyme solution. It is surprising that Rinkel and Pijoan (2) reported no such corrections. But perhaps this may be explained by their procedure in which the pH could not be determined. Obviously, if the pH of the enzyme reaction is not known, no accurate correction for the non-enzymatic hydrolysis would be possible.

Since there was now available a formula by which the pH of the solution could be determined, the problem of applying accurate corrections for alkaline hydrolysis of the ester was greatly simplified.

It is assumed that anyone using the method would wish to make one or more blank determinations to be certain about the correction factors at the temperature, pH and substrate concentration being used. For this reason the author has not attempted to determine the amount of the alkaline hydrolysis at all possible pH values and all possible temperatures.

When one controls the temperature in the Van Slyke manually, 25° C. is a very convenient temperature to use. Since this temperature was the one most used in this study, values for the non-enzymatic hydrolysis at this temperature were determined at various hydrogen-ion concentrations. The chart below gives the corrections which should be applied at various pH values when the total volume of solution is 6.5 ml. containing 50 mg. acetylcholine iodide.

TABLE VI

pH	Correction Factor	pH	Correction Factor
7.0	.67 cmm./30 min.	7.8	5.6 cmm./30 min.
7.2	.80 cmm./30 min.	8.0	8.8 cmm./30 min.
7.4	1.33 cmm./30 min.	8.2	12.8 cmm./30 min.
7.6	2.67 cmm./30 min.	8.4	18.0 cmm./30 min.

The effect of temperature on the non-enzymatic hydrolysis was also studied. The same concentration of substrate was used, and the pH was about 8.30. The results are shown in Fig. No. 5.

In general, the results agree with those of Glick (6, 8) and Alles and Hawes (12). It is noteworthy to point out that using the same concentration of substrate and the same temperature, Glick reports figures for alkaline hydrolysis which differ among themselves as much as 20%. For this reason it is customary to include a determination of the alkaline hydrolysis each time a series of manometric determinations are made on the Warburg apparatus. Solutions of acetylcholine which have been stored in the ice box not infrequently give somewhat different values for the non-enzymatic hydrolysis than solutions freshly made. The reason for this has not been explained. Alles and Hawes also mention this phenomenon in regard to one of the compounds they used.

D. The Effect of Mercury on Cholinesterase.

Having found a method for determining the pH, which made it possible to apply accurate corrections for the non-enzymatic hydrolysis, a further important detail before outlining the general procedure was a study of the effect of mercury on cholinesterase activity.

Early in this study a series of determinations of the effect of mercury on cholinesterase activity in normal horse serum was made. At that time it was found that metallic mercury did not produce any appreciable inhibition of cholinesterase activity of normal horse serum. Later, however, when the method was tried at Woods Hole, results on the esterase content of Limulus ganglia led the author to question the validity of the method.

Consequently, a more thorough study was made of the effect of mercury on the cholinesterase activity of various tissue extracts.

The general procedure was identical with that of Glick (24). The fresh organ was minced, a small sample ground finely with sand, the extract diluted with sufficient Ringer-bicarbonate (5) solution to give good readings on the manometer. Then a small portion of the whole extract was pipetted into the side arm of the Warburg vessel. An appropriate amount of acetylcholine iodide or chloride dissolved in bicarbonate-Ringer solution was introduced into the main vessel. In half of the chambers, a small amount of metallic mercury was introduced. After the usual procedure of attaching the flasks, saturating with the gas mixture consisting of 95% N_2 and 5% CO_2, and equilibrating in the constant temperature bath, the taps were closed and readings were taken at convenient intervals depending on the activity of the extract. The pH of the fluid was about 7.3.

From the results in table VII, it can be seen that in serum and all the crude extracts the effect of mercury on the enzyme was almost negligible. For a time the author considered that metallic mercury did not appreciably effect cholinesterase activity. Recently certain specially purified enzyme preparations have been measured on the Van Slyke manometric apparatus and from preliminary experiments it appears that purification takes away from the enzyme something which previously protected it against poisoning by mercury. A typical curve of the activity of a purified enzyme preparation from human serum is given in Fig. No. 6. The curve shows an abrupt leveling which is quite different from that of the normal serum. The results of these experiments are similar to those of Sumner (25) who showed that purified urease could not be used in the manometric determination of urea.

No explanation of this will be attempted here. The author is studying the effect of adding the various constituents of normal serum in an attempt to find out something about the nature of the substance, or substances responsible for the difference.

TABLE VII

Effect of Mercury on Cholinesterase Activity

Enzyme Source	Amount used	Concentration of Extract	Activity cmm.CO_2/30 min. Without Mercury	With Mercury	0.5 ml.10% Extract
Horse serum	0.5 ml.	10% sol.	150.0	148.0	148.0
Horse serum	0.5 ml.	5% sol.	63.0	62.7	125.5
Horse serum	0.5 ml.	2.5% sol.	45.0	38.0	152.0
Human placenta	0.5 ml.	10% ext.	3.0	2.83	2.83
Beef heart	0.5 ml.	20% ext.	1.50	1.50	1.50
Calf brain	0.5 ml.	20% ext.	29.0	29.0	29.0
Beef liver	0.5 ml.	33% ext.	1.48	1.50	0.45
Beef kidney	0.5 ml.	33% ext.	16.9	15.7	4.71
Calf spinal cord	0.5 ml.	20% ext.	34.6	33.6	16.8
Sheep muscle	0.5 ml.	20% ext.	1.75	1.70	0.85
Rabbit brain	0.1 ml.	32% ext.	94.7	93.4	145.6
Rabbit submaxillary gland	0.2 ml.	8.3% ext.	8.65	8.4	25.2

E. New Method and Recommended Procedure

In the discussion that has preceded, certain fundamental problems have been discussed. The solutions to these problems were a means by which it was hoped the best procedure for determining cholinesterase activity by the Van Slyke manometric apparatus could be determined. In the light of these findings the procedure finally adopted is as follows:

Procedure

Reagents:

1. Acetylcholine iodide (or chloride) C.P.
2. Ringer's solution containing 0.2 g. sodium bicarbonate per liter.
3. 1 N lactic acid.
4. 5 N NaOH.
5. Boiled distilled water.

Into the well-cleaned extraction chamber of the Van Slyke manometric apparatus, the sample to be assayed is delivered by means of a rubber tipped pipette. According to the size of the sample, enough gas-free distilled water is added to make a total volume of 3.5 ml. Then 2 ml. bicarbonate-Ringer solution and 1 ml. of acetylcholine solution (containing either 32.5 mgm. acetylcholine chloride or 50 mgm. acetylcholine iodide) are delivered successively into the chamber, making a total volume of 6.5 ml. The upper stop-cock is sealed with mercury and the chamber evacuated until the mercury meniscus is lowered to the 50 ml. mark, and shaken for one minute. In the case of extracts containing considerable protein some foaming may be encountered. It has been found by experience that rapidly raising and lowering the level of the mercury in the extraction chamber will quickly break the bubbles and permit an accurate reading of the gas pressure. The mercury is then admitted to the chamber (see Peters and Van Slyke Vol. II, page 277, for specific directions for CO_2 analyses) and the gas volume brought to 2.0 ml. A reading is taken on the manometer, and the mercury meniscus quickly lowered to the 50 ml. mark and the contents shaken for 3 minutes. Subsequent pressure readings are taken at each 3 minute interval, care being taken to keep the temperature in the water jacket constant.

Readings are made at 3 minute intervals for a sufficient number of intervals to determine the rate in mm. of mercury per 3 minute period. Usually 6 or 7 readings are sufficient, but more may be necessary when the enzyme activity is small.

When the rate has been established, the liquid is left at the 2 ml. mark and 0.5 ml. of 1 N lactic acid is delivered into the chamber through the upper stop-cock. Then the stop-cock is again sealed with mercury and the mercury meniscus again lowered to the 50 ml. mark. The contents of the chamber are shaken for

3 minutes. Then mercury is admitted into the chamber and a reading made on the manometer, when the gas volume has been brought to 2.0 ml. as before. When two successive readings agree within 1 mm., the fluid is left at the 2 ml. mark and 0.2 ml. of 5 N NaOH introduced into the reaction chamber. The stop-cock is again sealed with mercury. The fluid contents are allowed to rise into the neck of the chamber in order to thoroughly mix with the alkali. The mercury meniscus is lowered to the 50 ml. mark and the reaction chamber shaken for 3 minutes. The gas volume is again reduced to 2.0 ml. by admitting mercury and a reading taken.

The addition of the lactic acid and NaOH have nothing to do with determining the activity of the preparation being assayed but permit the calculation of the pH to be made.

In order to correct the pressure readings for the effect of change in volume and dissolved gases, a blank determination on the reagents is made. Introduce 6.5 ml. acidified Ringer solution into the chamber as before, and repeat the exact procedure as in the esterase determination. After constant pressure readings are obtained, 0.5 ml. of 1 N lactic acid is introduced and after shaking, a pressure reading is made with the gas volume at 2.0 ml. mark. Then 0.2 ml. of 5 N NaOH is introduced and the pressure reading made as before.

The first reading in the blank is called P_1', the reading after introducing the lactic acid is called P_2' and the reading after adding the NaOH is called P_3'.

When calculating the pH, the reading of the CO_2 tension at the point in the assay where the pH is desired is called P_1, the reading on the manometer after the lactic acid has been added is called P_2, and the final reading after adding NaOH is called P_3.

To apply the corrections, the values are substituted in the following formulae:

<u>FORMULA B</u>

$$pCO_2 = P_1 - \left[P_3 + (P_1' - P_3')\right]$$

$$p\left[CO_2\right] = \left[P_2 - (P_2' - P_1')\right] - \left[P_3 + (P_1' - P_3')\right]$$

In order to illustrate the method, a typical assay (No. 267) is reproduced in full.

First exactly 0.5 ml. of diluted horse serum (1 ml. horse serum + 4 ml. Ringer solution) was pipetted into the cup. This was washed into the chamber with 2.5 ml. of CO_2 -free distilled water, added in small portions. Next 2.0 ml. of bicarbonate-Ringer solution are introduced and finally 1.5 ml. of acetylcholine chloride solution containing 32.5 mg. The stop-cock was sealed and the contents mixed at 1:16.

ANALYSIS NO. 267

Time	Temperature	Reading	Difference
1:19	25.0°	159.8	
			9.2
1:22	25.0°	169.0	
			8.0
1:25	25.0°	177.0	
			8.0
1:28	25.0°	185.0	
			8.0
1:31	25.0°	193.0	
			8.0
1:34	25.0°	201.0	
			8.0
1:37	25.0°	209.0	
			7.5
1:40	25.0°	216.5	
			9.0
1:43	25.0°	225.5	
			6.5
1:46	25.0°	232.0	
			8.0
1:49	25.0°	240.0	

.5 ml. 1 N degassed lactic acid is added

1:59	25.0°	510.5

.2 ml. 5 N NaOH is added

2:13	25.0°	144.7
2:16	25.0°	145.0

Then, at the beginning of the analysis, P_1 = 159.8 mm., while at the end of the assay, P_1 = 240.0 mm.; P_2 = 510. 5 mm.; while P_3 = 145.0 mm.

A blank determination on the reagents gave P'_1 = 96.7 mm.; P'_2 = 99.8 mm.; and P'_3 = 98.9 mm.

$$(P'_3 - P'_1) = 2.2 \text{ mm.}$$

$$(P'_2 - P'_1) = 3.1 \text{ mm.}$$

To calculate the initial pH value then

$$pCO_2 = 159.8 - [145.0 + 2.2]$$

$$pCO_2 = 12.6 \text{ mm.}$$

$$p[CO_2] = [510.5 - 3.1] - [145.0 + 2.2]$$

$$p[CO_2] = 360.2 \text{ mm.}$$

The factors from Table I for formula A on page 18, have the following values at 25° C.

Factor R = 0.0188

Factor S = 0.0192

Factor T = 0.105

Substituting the above values and these factors in formula A

$$CO_2 = 12.6 \times 0.0189 = 0.2376 \text{ mM. } CO_2 \text{ /liter.}$$

$$[CO_2] = 360.2 \times 0.0192 = 6.899 \text{ mM. } [CO_2] \text{ /liter.}$$

6.899 - 0.238 = 6.661 mM. bicarbonate /liter.

Substituting these values in the Henderson-Hasselbalch equation we get,

$$pH = 6.16 + \log \frac{6.661}{0.025}$$

$$pH = 6.16 + 2.43$$

$$pH = 8.59$$

By the same series of calculations the pH at the end of the assay when P_1 = 240.0 mm., is 7.62.

Since these calculations are rather lengthy, the graphic method using the log-log chart, Fig. 3, though a little less accurate, gives results sufficiently close for ordinary purposes.

To calculate the rate, one may use the factors in Table VIII which permits one to express his answer in mM./liter, volumes per cent, or in cubic millimeter of CO_2 produced in unit time. Pijoan and Rinkel (2) expressed their results in volumes per cent. In order to compare the results of this method with the results of the other two methods (1, 3) which have been referred to, it is convienient to express the rate in cmm. CO_2 produced in some unit time, such as 30 minutes.

If, in determination No. 267, we take the average increment per 3 minute interval as 8 mm., then the rate would be

8 x 10 x 0.1245 = 9.96 mM./liter/30 min.

8 x 10 x 0.2972 = 25.776 volumes per cent /30 min.

8 x 10 x 2.729* = 218.33 cmm./30 min.

*The factor for converting mm. of Hg. into cmm. is calculated by equation 12.

TABLE VIII

Factors for Converting Pressure Readings in mm. Hg. into mM./liter, Volumes Per Cent, or Cubic mm. CO_2

	When a = 2, i = 1.017 and S = 3 ml.			When a = 2, i = 1.017 and S = 6.5 ml.		
Temp. C.	mM. per liter	Volumes per cent	cubic mm.	mM. per liter	Volumes per cent	cubic mm.
20	0.1364	0.2628	2.628	0.1464	0.2820	2.820
21	0.1358	0.2616	2.616	0.1454	0.2802	2.802
22	0.1351	0.2603	2.603	0.1445	0.2784	2.784
23	0.1344	0.2589	2.589	0.1434	0.2763	2.763
24	0.1338	0.2577	2.577	0.1425	0.2746	2.746
25	0.1331	0.2565	2.565	0.1416	0.2729	2.729
26	0.1325	0.2552	2.552	0.1404	0.2706	2.706
27	0.1319	0.2541	2.541	0.1399	0.2696	2.696
28	0.1312	0.2529	2.529	0.1391	0.2779	2.679
29	0.1307	0.2518	2.518	0.1383	0.2665	2.665
30	0.1301	0.2507	2.507	0.1375	0.2650	2.650

The average correction factor for the non-enzymatic hydrolysis of acetylcholine at that temperature and substrate concentration is 8.8 cmm./30 min., therefore the corrected rate would be 209.5 cmm./30 min. for 0.10 ml. of normal horse serum.

From the results of the last analysis, No. 267, (See Fig. 7) it can be seen that the primary objectives of this thesis have been accomplished. A method has been devised for assaying serum and crude enzyme preparations to determine their cholinesterase activity using the Van Slyke manometric apparatus. The method fulfills the requirements outlined on page 9.

If the analyses are made according to the precautions given when the Van Slyke manometric apparatus is used for blood gas analyses, duplicate determinations will agree within ± 1%. Alles and Hawes state that the titrimetric method using the glass electrode has an accuracy of about ± 2%. The accuracy usually claimed for the manometric method of Ammon is about ± 5%.

F. SUMMARY

1. The method of Rinkel and Pijoan has been critically examined and a new procedure has been devised which avoids the inherent limitations of their original method.

2. A formula and set of factors are given by which one can calculate the pH from determinations of the total CO_2 and H_2CO_3 content of the reaction fluid. A graphic method for determining the pH is also described. The calculated values agree closely with the values obtained with a standardized glass electrode if the value of the apparent first dissociation constant of carbonic acid at 25° C. is assumed to be 6.16. This value agrees with the accepted value for this temperature.

3. Measurements of the non-enzymatic hydrolysis at different pH values, and at various temperatures have been made and corrections for the non-enzymatic hydrolysis are given.

4. The effect of metallic mercury was almost negligible on crude cholinesterase preparations and did not inhibit the cholinesterase activity of normal sera. Mercury did appear to poison the enzyme in purified preparations.

5. The new method and procedure are described in detail. It is more accurate than previously described methods, the accuracy being ± 1%.

II. THE CHOLINESTERASE ACTIVITY OF EXTRACTS OF SOME INVERTEBRATE TISSUES

A. Introduction.

In the course of experiments on the effect of cholinergic drugs on invertebrate tissues, it was found that in the case of the heart of Venus mercenaria, which is extremely sensitive to choline esters, no potentiation of the inhibition normally produced by the addition of various choline esters occurred when the heart was eserinized in the usual manner. This observation suggested that perhaps the clam heart contained little or no cholinesterase activity.

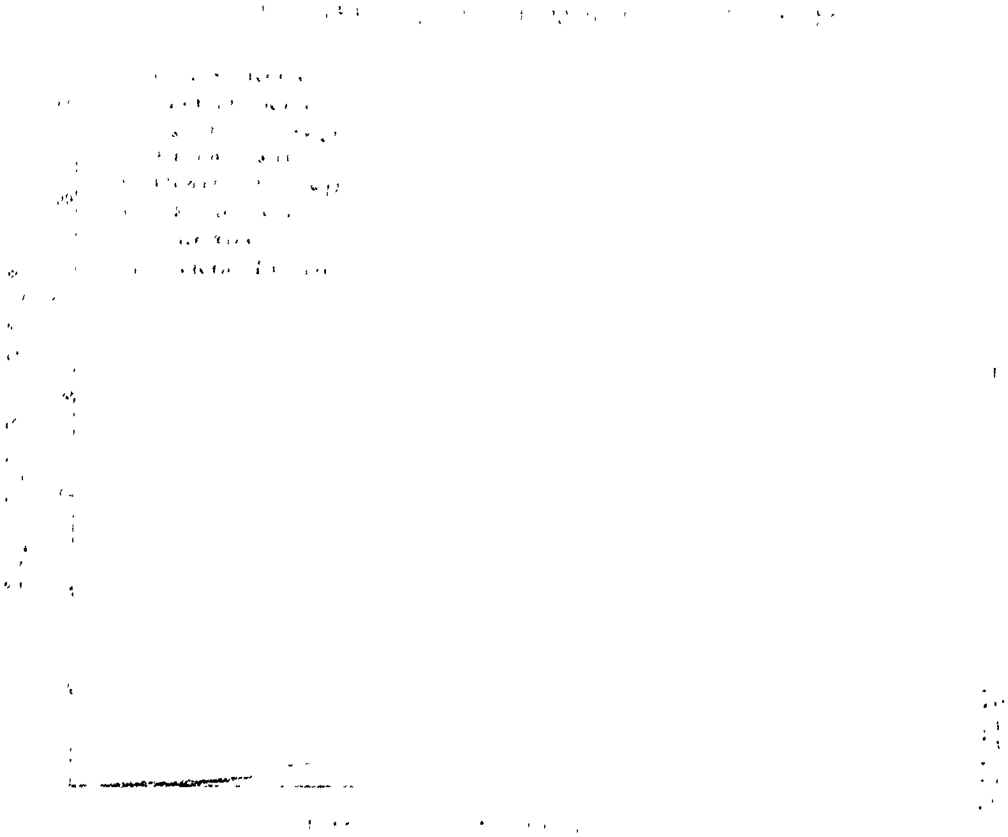
A study of the literature revealed the fact that to date determinations of the cholinesterase activity in invertebrates had been entirely confined to European species. It was the purpose of these experiments to provide some data concerning the distribution of cholinesterase in the hearts of some of the invertebrates common to the region around Woods Hole, Massachusetts, as a basis for further studies on their physiology. A more complete study of Limulus polyphemus was made since this animal has been the object of important studios in invertebrate neurophysiology.

B. Experimental Part.

METHOD. The method used was essentially that of Ammon (3) and Glick (24). The tissue to be used was removed, washed once with bicarbonate-Ringer, dried quickly on filter paper and weighed in a closed tared vessel. The tissue was then quickly ground with sand until a smooth suspension was obtained when a calculated amount of bicarbonate-Ringer (5) as shown in the Tables 9 and 10, was added. In some experiments the suspension was centrifuged and the activity of the supernatant fluid and the residue were determined separately. Precautions were taken to keep the materials as cool as possible.

The substrate used in each experiment consisted of 15 mg. of acetylcholine chloride dissolved in 1.5 ml. of bicarbonate-Ringer. The substrate was placed in the main vessel, and 0.5 ml. of the enzyme suspension was placed in the side arm. The vessels are then attached to the manometers and saturated with a moist gas mixture containing 95 parts nitrogen and 5 parts carbon dioxide. The final pH after saturation is 7.2. The contents of the flasks were mixed and placed in the constant temperature bath. After equilibration, the taps are closed and readings of the pressure made at convenient intervals, usually each 5 minutes for 30 minutes or longer.

A summary of the results is given in Tables 9 and 10, and in Fig. 8 and 9.



DISCUSSION The concentration of cholinesterase activity seems to parallel, in general, the amount of nervous tissue present. This is well illustrated by the fact that considerably higher activities were noted in the 5 - 6 segments of the Limulus heart as compared with the 1 - 2 segments. The 1 - 2 segments are known to have fewer nerves and ganglia than the 5 - 6 segments. The activity of extracts of the Venus heart agrees with its histology and explains certain interesting observations on its pharmacology. No explanation can be given for the very high activity found in the Modiolus demissus heart. Further experiments on its physiology are planned.

The results agree well with the findings of other investigators who have measured the cholinesterase activity of a large number of European marine and fresh water invertebrates.

SUMMARY

1. Assays of 12 different tissue extracts from a total of six species of marine invertebrates shows that all extracts studied have cholinesterase activity. The highest activity was found in nervous tissue; the lowest, in the muscle extracts.

2. The activity of the various extracts seemed to parallel the amount of nervous tissue found there.

3. The observation that the heart of the clam, Venus mercenaria, is not sensitized to acetylcholine by eserine can be explained perhaps on the basis of its low cholinesterase content.

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