STUDIES ON ENZYMATIC HISTOCHEMISTRY

XXV. A Micro Method for the Determination of Choline Esterase and the Activity-pH Relationship of This Enzyme*

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(Accepted for publication, October 21, 1937)

Within recent years a great deal of interest has been aroused concerning the enzyme hydrolyzing choline esters because of its relation to the metabolism of acetyl choline. Up to the present time there has been no adequate quantitative method for the determination of this enzyme in minute amounts of material, a determination particularly valuable for investigations of the enzyme in microtome sections of fresh frozen tissue for correlation of the activity with the histological structure of the tissue in question. The procedure to be described was based on the principle of the micro method developed for estimation of esterases hydrolyzing esters of simple alcohols (1). The sensitivity of the method is such that splitting can be measured down to the equivalent of the liberation of 0.20 c.mm. N/20 acid, corresponding to 1×10^{-8} mol of ester, or 1.81γ of acetyl choline chloride.

The pH range within which digestions may proceed in the method employed is limited, hence it is necessary to ascertain beforehand whether this range of pH coincides with the region of maximum activity of the enzyme. Previously it had been shown that maximum activity in human serum occurred at a pH of 8.4–8.5 (2). In order to determine whether this optimum holds as well for the enzyme from different sources, and also because of the fact that the data would

* This paper also belongs to the series of investigations by the author and coworkers as "Studies in histochemistry. XIII," the preceding papers of which have appeared chiefly in the *Journal of Biological Chemistry*.

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be of value in other connections, studies of the activity-pH relationships were undertaken for the enzyme from horse serum, gastric mucosa of the pig, and cat brain.

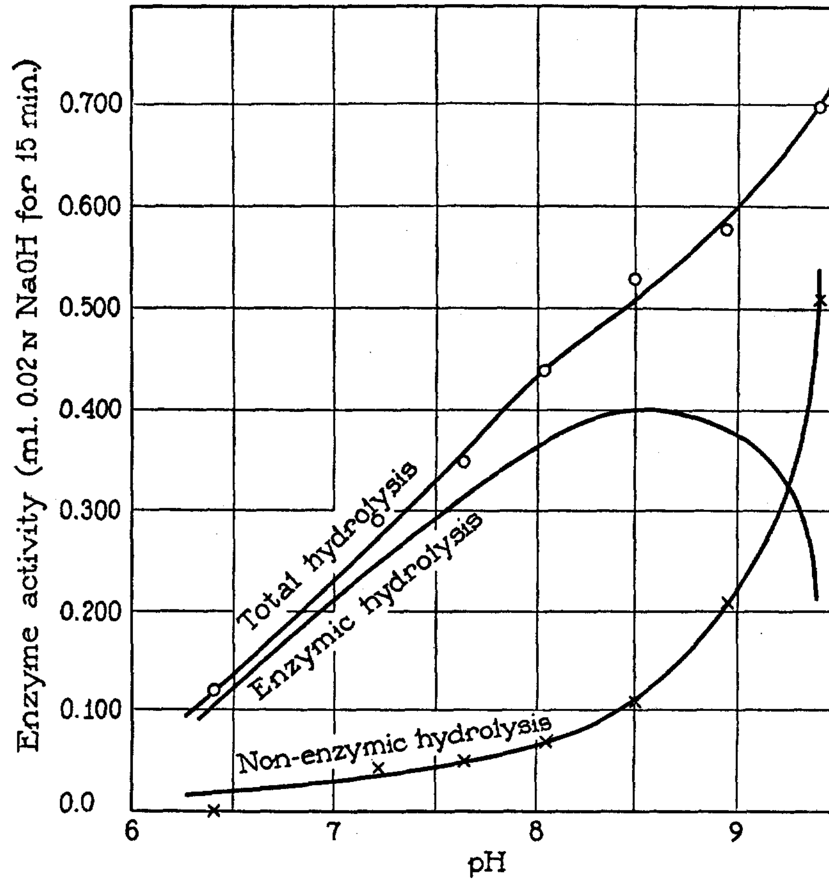

Fig. 1. Activity-pH curve for choline esterase in pig's gastric mucosa

The Activity-pH Relationships

The method used for the purpose of investigating the activity-pH relationships was the continuous electrometric titration procedure employing a glass electrode in the manner previously described (2). The advantages of this method, which eliminates the effects of buffer, indicators, etc., have already been discussed (2). For the study at hand, mechanical stirring was employed, and a burette graduated in 0.01 ml. divisions was used for titration, the quantity of alkali added

being measured on the burette rather than by counting the number of drops delivered. Measurements were made at 25° and the reaction mixture consisted of 20 ml. of 0.2 per cent acetyl choline chloride plus 1 ml. of enzyme preparation or 0.2 ml. serum. In the case of dog stomach mucosa and cat brain the preparation was made by extracting 100 gm. of finely ground tissue with 300 ml. of 30 per cent glycerol at 0° for 10 days, and subsequently filtering through paper. The results are given in Figs. 1–3 and it may be seen that the optimum occurs at a pH of 8.5 in every case.

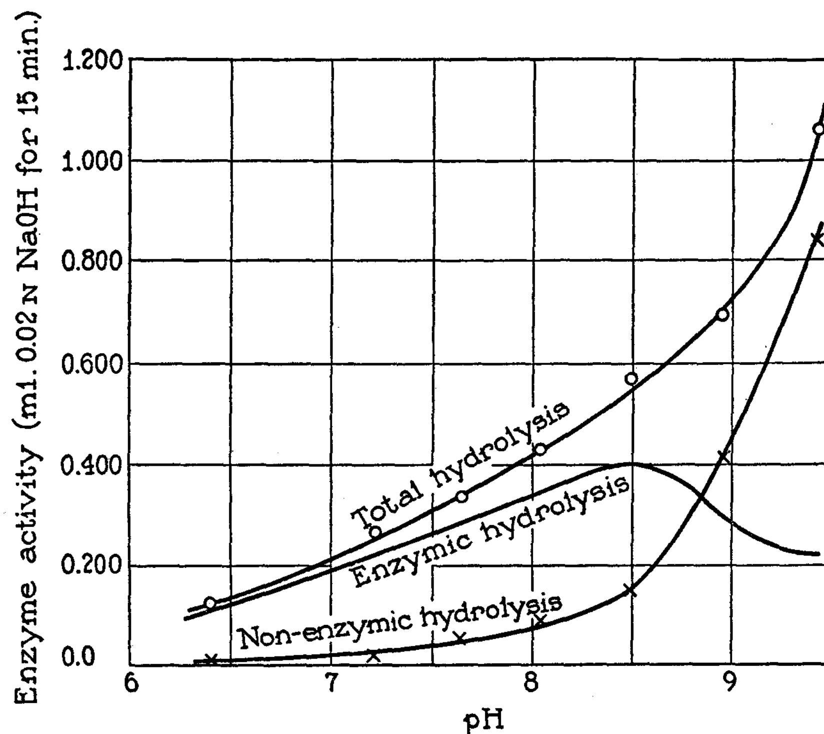

FIG. 2. Activity-pH curve for choline esterase in horse serum

The Micro Method for the Estimation of Choline Esterase

In the micro method for determination of esterases splitting esters of simple alcohols, to which reference has already been made, a glycine-NaOH buffer having a pH of 8.7 was employed, the enzyme activity was halted at the end of the reaction period by a phenol solution, and the titration was finally carried out to an end point of pH 6.5 with brom-thymol blue indicator.

From the curves in Figs. 1–3 it is apparent that at the pH of optimum activity

the non-enzymatic hydrolysis begins to assume relatively large values. In order to minimize this effect without deviating greatly from the conditions for optimum activity, it was decided to employ a pH of 8.0. However, at this pH the glycine buffer approaches the lower limit of its buffering range; hence the veronal buffer of Michaelis (3) was used instead. The veronal buffer was prepared by adding 7.15 ml. of 0.1 M sodium diethylbarbiturate to 2.85 ml. of 0.1 M HCl.

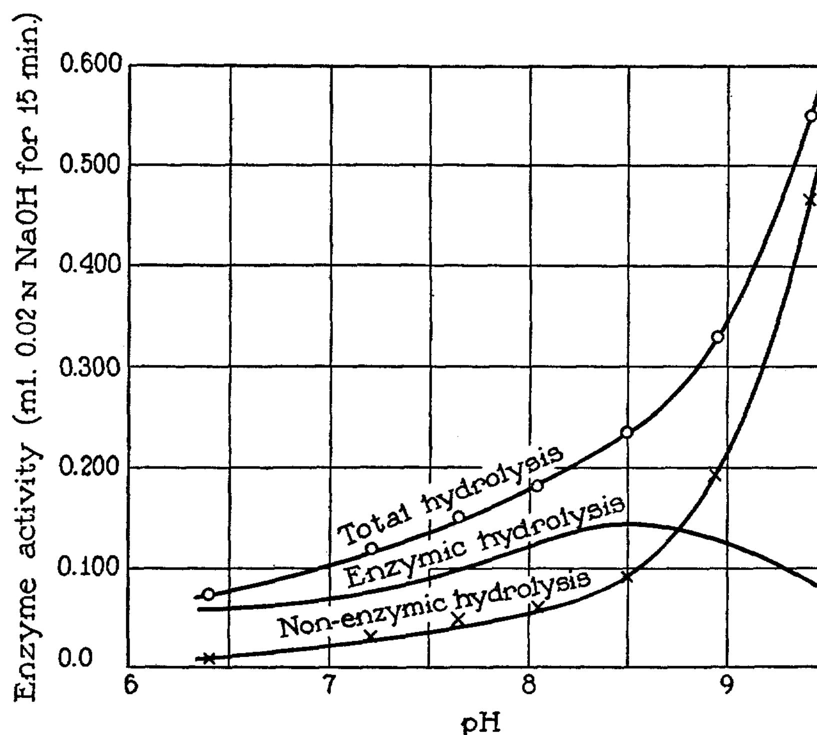

FIG. 3. Activity-pH curve for choline esterase in cat brain

Since the veronal has a lower pK (8.0) than the glycine buffer (9.7) it is necessary to carry the titration to an end point of lower pH in order to obtain a sharp color change. The lowering of the end point pH, however, is limited by the fact that the pK of the acetic acid liberated from the substrate is 4.7, and the pH of the end point must not be allowed to approach this value too closely or the sharpness of the color change will suffer. The pH finally chosen as most suitable for the end point was 6.2. As in the previous esterase

method (1), the standard color-comparison tube was filled with phosphate buffer and indicator, but the buffer was made up to a pH of 6.2 in this case.

Because of its powerful inhibiting action upon choline esterase, eserine was used to stop the enzymatic reaction at the end of the digestion period. A solution of 10 ml. of 0.1 per cent eserine sulfate

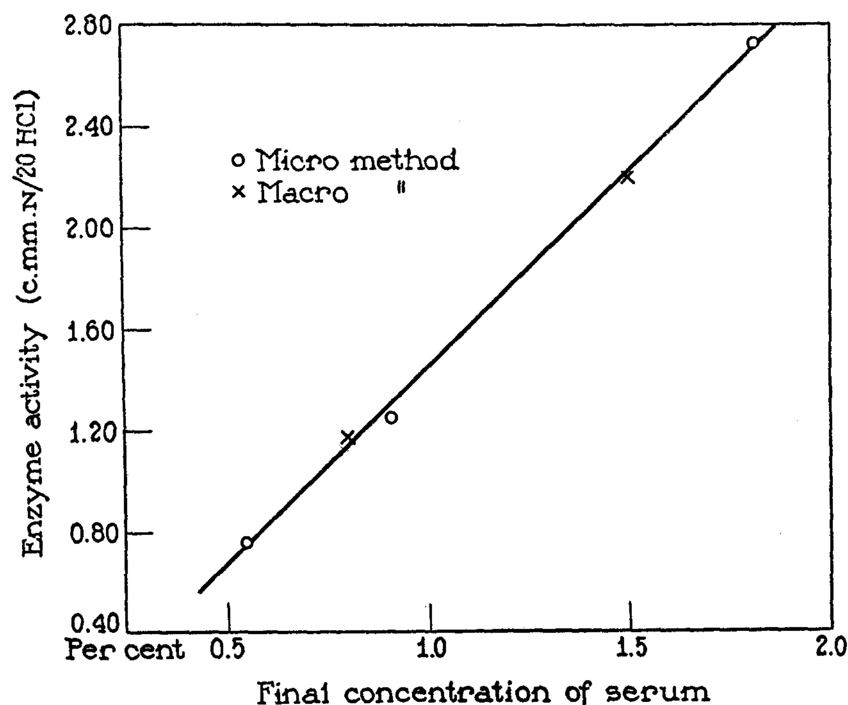

FIG. 4. Comparison of micro and macro methods

added to 1.5 ml. of 0.04 per cent brom-thymol blue was used for the purpose.

The most desirable substrate concentration would be that giving maximum enzymatic hydrolysis with the least accompanying non-enzymatic splitting. In the case of choline esterase there exists for a given concentration of enzyme a concentration of substrate beyond which no significant increase in enzyme hydrolysis is demonstrable (2, 4). Therefore it is not only of no value to employ substrate concentra-

tions greater than this quantity, but it is definitely objectionable since the magnitude of the non-enzymatic hydrolysis becomes greater, thus decreasing the accuracy of the measurement. For the present purposes a final concentration of about 0.4 per cent acetyl choline chloride was found to be entirely suitable.

TABLE I

Measurements of Choline Esterase Activity by the Micro Method

Final serum concentration, *per cent*	0.55	0.91	1.82
N/20 HCl required, *c.mm*	10.66	10.00	8.36
	10.70	9.98	8.36
	10.52	9.86	8.24
	10.58	9.90	8.44
	10.60	10.10	8.22
Average	10.61	9.97	8.32
Controls	11.44	11.30	11.10
	11.28	11.16	11.02
	11.40	11.20	11.00
Average	11.37	11.22	11.04
Difference	0.76	1.25	2.72

TABLE II

Measurements of Choline Esterase Activity by the Macro Method

Final serum concentration, *per cent*	0.80	1.50
N/50 NaOH required, *ml*	2.98	4.96
Non-enzymatic hydrolysis	0.60	0.60
Difference	2.38	4.36
Expressed as c.mm. N/20 HCl for each 25.2 c.mm. of reaction mixture	1.19	2.20

Preliminary experiments with horse serum showed that practically the same activity was obtained in the presence of veronal as in glycine buffer. Furthermore the eserine solution employed was found to completely inhibit the enzymatic action.

The micro method described was finally compared with the macro electrometric procedure used for the activity-pH studies. This comparison should demonstrate

any effect of veronal buffer on the esterase activity. Accordingly a reaction mixture was employed for the micro titrations consisting of 16 c.mm. of 0.1 M veronal buffer at pH 8.0 containing ½ per cent acetyl choline chloride, plus 9.2 c.mm. of horse serum solution. Digestion was allowed to proceed for 2 hours at 25°, after which 50 c.mm. of eserine-indicator solution were added and titration carried out with N/20 HCl. Suitable control experiments were included in which the enzyme and substrate-buffer (in the same tube) were not allowed to mix. Table I gives the results obtained.

The reaction mixture used in the macro experiments contained the same final substrate concentration. After addition of horse serum solution to the substrate the volume was made up to 20 ml. with distilled water. After digestion for 2 hours at 25° and at a pH of 8.0, the quantity of N/50 NaOH consumed bv the acid liberated is given in Table II.

The agreement between the two methods is apparent from Fig. 4. Hence it may be concluded that veronal has no measurable effect upon choline esterase under the conditions given.

SUMMARY

The activity-pH relationship for choline esterase from horse serum, gastric mucosa of the pig, and cat brain was investigated, and an optimum was observed at pH 8.5 in each case.

A micro method for the determination of choline esterase was developed capable of measuring the hydrolysis down to the order of that given by 1×10^{-8} mol of ester.

The author wishes to thank Professor S. P. L. Sørensen for his interest in this work, and to express his appreciation to Dr. K. Linderstrøm-Lang for his many helpful suggestions, and to G. Haugaard for the use of his glass electrode equipment.

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