CCCVI. A MODIFIED COLORIMETRIC ESTIMATION OF CARBONIC ANHYDRASE.

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Introduction.

The work described here is the result of an attempt to devise for class purposes a method of estimating carbonic anhydrase which does not involve the use of special apparatus as in the boat method of Meldrum & Roughton [1933]. It is a modification of the colorimetric method of Brinkman [1933], who mixed solutions of CO_2 and $NaHCO_3$ plus phenol red and measured the time required for the colour change.

In our method the $NaHCO_3$ is replaced by Na_2CO_3 (plus a little $NaHCO_3$). The reaction begins at *p*H about 10·5, in the middle of the $NaHCO_3$-Na_2CO_3 buffer range, and the end-point is taken at *p*H 7·0, using bromothymol blue as an indicator. The reaction therefore begins slowly in the well-buffered part of the titration curve and ends on the steep part of the curve, thus producing a rapid and very sharp end-point. The net reaction is approximately:

$$CO_2 + CO_3'' + H_2O \rightarrow 2HCO_3'.$$

The chief merit of the changes introduced is probably the dramatic suddenness of the end-point, which not only permits great accuracy of timing but also is an asset for class purposes. The method gives good results for moderately purified enzyme preparations, but for other cases (notably for enzyme estimations in tissue extracts) it has certain drawbacks which make it compare unfavourably with the boat method. These are:

(*a*) The detailed mechanism of the reaction alters during its course, owing to the *p*H change.

(*b*) The choice of ionic strength of the solutions used is restricted by several practical considerations.

(*c*) The reaction time can be shown theoretically to be rather sensitive to extraneous buffering in the *p*H region 7–9.

(*d*) Haemoglobin has a curious effect on the calibration curve which is described below.

From the research point of view therefore the applicability of the method is probably limited to routine estimations under fairly constant conditions, or to problems involving purified enzyme preparations. For these however the rapidity (15 estimations per hour) and ease of manipulation will be found advantageous.

Experimental.

Solutions. (*a*) Na_2CO_3. 0·3 *M* solution, containing 0·206 *M* $NaHCO_3$. The latter was added to keep the reaction from becoming too alkaline since carbonic anhydrase is only stable from *p*H 4 to 11. This solution was prepared from a solution saturated with Na_2CO_3 and $NaHCO_3$ at room temperature by diluting to 27·3 parts in 100. The carbonate was estimated by titration with HCl to *p*H 8·3, running in the HCl under the surface with rapid stirring, to avoid loss of

CO_2, and waiting about 30 sec. after each addition. The bicarbonate was estimated by titrating to *p*H 4·0 and subtracting twice the carbonate. The indicators used were cresol red and bromophenol blue respectively.

(*b*) CO_2. A 0·00263*M* solution of $NaHCO_3$ was saturated with CO_2 by bubbling from a cylinder. The $NaHCO_3$ was added to keep the initial reaction from being too acid and destroying the enzyme.

(*c*) Enzyme. A crude preparation, made from pig corpuscles by the $CHCl_3$ method, was used, diluted 1/50. (This preparation was kindly supplied by Mr V. H. Booth.)

(*d*) Indicator. Bromothymol blue.

Apparatus.

Fig. 1 shows the final set-up. Most of the accessories are unnecessary for rough work.

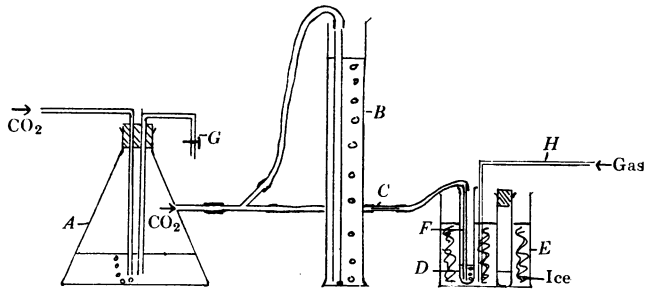

Fig. 1. Diagram of apparatus. *A*, reservoir of CO_2 solution; Büchner flask. *B*, pressure regulator (bubbling from bottom of wide tube). *C*, fine capillary, about 5·5 cm. long. *D*, reaction vessel; pyrex boiling-tube 2·5 cm. diameter. *E*, vessel for colour standard. *F*, CO_2 bubbling tube. *G*, clip for obtaining CO_2 solution from reservoir. *H*, gas bubbler to stir the ice and water surrounding the reaction vessel (air or any gas will do). The flask *A*, tubes *D* and *E*, the reagents and clean reaction tubes are all kept in ice and water.

Procedure.

x ml. enzyme and $11-x$ ml. CO_2 solution are measured with pipettes into the cooled reaction tube. (The CO_2 solution is run from *G* into a test-tube and then measured accurately with a pipette.) Ten drops of indicator and one drop of octyl alcohol (to prevent frothing) are added and CO_2 is bubbled through. After 2 min. 1 ml. of Na_2CO_3 is blown in from a graduated pipette and the time is measured for the colour to change from blue to a yellow-green. CO_2 is bubbled the whole time. The end-point is judged by matching the colour with the standard tube which contains phosphate buffer at *p*H 7·0 and the same indicator. With reaction times below 30 sec. the end-point is so sharp that exact matching is out of the question.

Precautions.

The 2 min. of preliminary bubbling are to ensure saturation with CO_2 and to allow temperature equilibrium to be reached. If the surrounding ice and water are kept well stirred this is quite long enough.

It was found that the reaction time was affected by the rate of bubbling of CO_2 during the reaction. This was due to

(*a*) the effect on the rate of the initial mixing,

(*b*) the fact that CO_2 continues to dissolve during the reaction to replace that which is used up.

The two factors concerned were the rate of flow of CO_2 and the nature of the bubbling tube *F*.

The rate of flow was standardized by introducing the pressure regulator *B* and the capillary *C*. We finally decided to define the conditions as follows:

Bubbler: length 19 cm.; cross-section 4·5–6·5 sq. mm.

Rate of flow: 70–90 ml. in 15 sec.

This gives a reaction time nearly as short as that obtained with much larger rates of flow. The rate of flow is adjusted by (*a*) the length and bore of the capillary *C*, and (*b*) the head of water in the pressure regulator *B*.

It was found that, within these limits, variations in the above conditions caused less than 5% systematic error, since the total error, both random and systematic, was never more than 5%.

Definition of unit. If the reciprocal of the reaction time is plotted against the amount of enzyme the result is a straight line within the useful range (time: 11–40 sec.). Thus the number of enzyme units in a solution giving a reaction time t is $K\,(t_0/t-1)$, where t_0 is the reaction time of the blank (54 sec.) and K is a constant equal to 17·7. The value of K was found empirically by comparing our measurements with data kindly obtained for us by Mr V. H. Booth on the same preparation by means of the boat method.

If the purified enzyme is replaced by haemoglobin or defibrinated blood the calibration curve is shifted along the concentration axis, with a small non-linear region near the origin. Corrections for this can be made, but it is better not to attempt absolute measurements in the presence of haemoglobin.

SUMMARY.

A modified colorimetric estimation of carbonic anhydrase is described, capable of 15 estimations per hour, with an accuracy of about 5% over a two-fold range of enzyme concentration.

The method requires no special apparatus and has, so far, been tested on moderately purified enzyme preparations and on haemoglobin solutions. In other cases further controls would be necessary.

We are grateful to Prof. R. A. Peters for his interest in this work.

REFERENCES.

Brinkman (1933). *J. Physiol.* **80**, 171.
Meldrum & Roughton (1933). *J. Physiol.* **80**, 113.