A Re-investigation of the Question of Activators of Carbonic Anhydrase

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The claim that certain substances are activators of the enzyme carbonic anhydrase was first made by Leiner (1940), who found that human serum could increase the apparent activity of the enzyme as observed by Brinkman's (1933) colorimetric method. This was followed by a second paper (Leiner & Leiner, 1940), in which it was asserted that there are two main groups of activators. The first, which includes cysteine, glutathione, histidine, histamine and certain purines, activates principally the hydration of carbon dioxide, while the second, which includes a thermolabile organic compound present in plasma, activates principally the dehydration of carbonic acid.

Using the Philpot & Philpot (1936) colorimetric method, Maine & Locke (1941) found that a large number of organic substances have slight or marked powers of activating carbonic anhydrase. Histamine was found to activate maximally in a concentration as low as 0.24 mM. In some cases, e.g. catechol and histamine, activation effects were observed to be additive. Maine & Locke believe that compounds which contain >NH groups and which form carbamates can activate the enzyme.

The subject was then taken up by Kiese (1941), who not only pointed out that Leiner's views were thermodynamically unsound, but was unable to detect by careful manometric methods any activation in most of the instances brought forward by Leiner. In a later paper (Kiese, 1942), however, he states that glutathione can activate carbonic anhydrase, but insists that its effect is the same for both hydration and dehydration reactions. Bakker (1942) found that when using both colorimetric and manometric methods, most of the substances claimed by Leiner to be activators had no effect, although boiled tissue extracts, cysteine and glutathione appeared to increase the activity of the enzyme to some extent. He was unable to find any evidence in support of Leiner's ideas on unidirectional activation.

In spite of this adverse criticism, Leiner has published several more papers (1942, 1943, 1944) in which he enlarges upon his earlier views and presents

further evidence in support of them. Some of his observations made by Brinkman's method have been confirmed by Van Goor (1943*a*). Unfortunately, much of this work has been carried out under very imperfectly controlled conditions. Thus, in some of his manometric experiments on the rate of dehydration of carbonic acid, Leiner uses mixtures of phosphate buffer and bicarbonate solutions, but gives the boats (alternatively his modified Warburg flasks) a preliminary evacuation at the water pump. He apparently has failed to take into consideration the effect of reducing the pressure on the composition of bicarbonate solutions. As a further example of inadequate control, it may be noted that in a recent review, Van Goor (1948) states that when using the Brinkman method, the 0.02M-sodium bicarbonate solution should be given a preliminary boil, but he makes no mention of any checks to ascertain the extent of the conversion of bicarbonate to carbonate. Although the proportions of HCO_3^- to CO_3^{--} may be the same in any given set of experiments, it is desirable that quantitative work on enzyme kinetics should be carried out with solutions of known composition. In this particular case, variation in the ratio between the concentrations of the two ions concerned will mean that the initial pH of the system will not be the same in every case. Bakker (1941) has also suggested boiling the sodium bicarbonate solution with the object of adjusting the time taken for the non-enzymically catalysed reaction to occur.

It may also be pointed out that in some instances, investigators have compared activities obtained manometrically at 15° with those obtained colorimetrically at 0°. Since the temperature coefficients for the enzymic and non-enzymic reactions are different (Roughton, 1948), such uncritical methods can introduce very large errors.

Roughton (1943) and Roughton & Booth (1946*b*) have drawn attention to the possibility that many of the so-called activations may be nothing more than the reversible restoration of activity lost by adsorption at interfaces or by the action of traces of inhibiting substances present. This possibility can be eliminated only by controlling the experimental conditions with the greatest care and by using the highest possible enzyme concentrations. Other objections to the methods used by previous workers

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in this field will be considered when discussing our own results, but enough has been said to indicate a very real need for the complete re-investigation of the subject. The experiments to be described below represent a start in this direction.

EXPERIMENTAL

Methods and materials

Dehydration of carbonic acid. This was studied by the rapid manometric method (Meldrum & Roughton, 1933) at 0°. The solutions used were 2.0 ml. of 0.186 M-NaHCO₃ in 0.038 M-NaOH (Hodgson, 1936) and 1.0 ml. of 0.4 M-phosphate buffer, pH 6.8, the final volume being made up to 4.0 ml. by the addition of the enzyme solution together with any substance to be tested for activating properties. All solutions were prepared with glass-distilled water. Under certain conditions when the manometric method is being used, it is necessary to apply corrections for the limiting effect of diffusion, and a procedure for doing this has been worked out by Roughton & Booth (1946a), but this question did not arise during the course of the present investigation, in which the concentration of the enzyme solutions was kept within the range where there was strict proportionality between the amount of carbonic anhydrase added to the boats and the observed catalytic effect.

In the absence of any protecting agents, dilute solutions of purified carbonic anhydrase rapidly lose their activity on standing. This loss is greatly accelerated if the solutions are shaken violently, a point of considerable practical importance for, in the rapid manometric method, the boats are shaken at rates ranging from 350 cyc./min. (Meldrum & Roughton, 1933; Clark, 1949) up to 600 cyc./min. (Kiese, 1942). For this reason Leiner has advocated the use of Warburg manometers on the grounds that the lower shaking rate is less likely to bring about the rapid inactivation of the enzyme. However, if this low rate of shaking is used, the range of enzyme concentrations that can be covered without diffusion constituting a troublesome factor becomes very much reduced. On the other hand, the observations of Scott & Mendive (1941) and Scott & Fisher (1942) on the stabilizing action of peptone on carbonic anhydrase have shown that it is possible to obtain satisfactory results with a rapid manometric method even when using highly purified enzyme preparations. It is suggested, therefore, that the boat method is quite satisfactory and should be retained provisionally as the standard procedure.

A method of using the unimolecular velocity constant for the determination of carbonic anhydrase activity has been described by Mitchell, Pozzani & Fessenden (1945). An extension of the method, which enables stabilizations to be distinguished from activations, has been suggested by Clark (1949). Values of the velocity constant are determined at 20 sec. intervals over a total period of 3 min. Extrapolation back to zero time gives the true, initial value of the constant. If the addition of a particular substance to the enzyme solution brings about an increase in the value of the constant, then it would be regarded as a true activator, provided that it had no appreciable effect on the velocity constant for the non-enzymically catalysed reaction. The sensitivities of different preparations to inactivation are compared by calculating the per cent decrement, D , in the value of the unimolecular velocity constant during the first 100 sec. of

the reaction. The ability of an agent to protect or stabilize the enzyme is arbitrarily defined as $100(1 - d/d')$, where d and d' are the values of D in the presence and absence of the stabilizing agent respectively. The degree of stabilization is denoted by the letter S .

The hydration of carbon dioxide. The manometric method of Roughton & Booth (1946) was used in some of the preliminary experiments. The method, however, suffers from the disadvantage that, owing to the relatively large velocity constant of the non-enzymically catalysed reaction, it is difficult to cover a very wide range of enzyme concentrations. Consequently, even when corrections are made for the limiting effects of diffusion, the method is restricted to reaction velocities not more than ten times the uncatalysed rate. The same objection of course also applies to manometric output methods. The difficulty has been overcome in two ways: (a) by the use of a new colorimetric technique which is free from most of the objections that may be levelled against the older Brinkman or Philpot methods, and (b) by a further development of Faurholt's (1924) carbamino method, in which the use of rapid-flow methods has enabled higher reaction velocities to be determined than were possible in the original method.

(a) *A new colorimetric method.* With this method it has been possible to measure velocity constants of up to 700 times the uncatalysed value (Perrin & Clark, 1949). The method, which may be regarded as a development of one suggested by Roughton & Booth (1946b), involves the rapid mixing at room temperatures of a saturated solution of CO₂ in water with a veronal buffer solution having a pH of approximately 8.6 and a pK of about 8. In the presence of a suitable indicator (phenol red), the pH of the solution is recorded continuously as a function of the time. This was achieved most easily by the stopped-flow technique, using the rapid-reaction apparatus of Chance (1940), together with a pen recorder to note the changes in the photocell current. The apparatus was calibrated with standard pH buffers so that for any run, a pH-time curve was readily constructed. The concentration of carbon dioxide at any time, t , is given by the equation

$$[\text{CO}_2]_t = [\text{CO}_2]_0 - [\text{B}']_0 + \frac{[\text{B}'] + [\text{HB}]}{1 + [\text{H}^+]/K},$$

where $[\text{B}']$ is the buffer ion concentration, $[\text{HB}]$ the buffer acid concentration and K the dissociation constant of the buffer. The derivation of this equation is given in the Appendix. The unimolecular velocity constant for the hydration of CO₂ is obtained by plotting $\ln [\text{CO}_2]$ as a function of time.

(b) *Faurholt's carbamino method.* Solutions of CO₂ and a suitable buffer are mixed rapidly and, after a certain time has elapsed, dimethylamine is added to the system. The amine forms a carbamino compound with any free CO₂ present, while that fraction of the gas which has been hydrated to form carbonic acid can be precipitated as carbonate by the addition of alkaline BaCl₂. In the present investigation, the solutions were mixed in a rapid-flow apparatus similar to that used by Millikan (1936). The CO₂-buffer mixture, after travelling along the flow tube, was expelled into a centrifuge tube containing dimethylamine, together with 10⁻³ M-sulphanilamide if the enzyme concentration is very high. Alkaline BaCl₂ solution was then added immediately from a third syringe. After centrifuging, the CO₂ present as a carbamino compound in the super-

natant fluid was estimated with the Van Slyke apparatus. A preliminary set of control experiments showed that neither carbonic anhydrase (up to a concentration of 25 mg./100 ml.) nor 2% boiled horse plasma interfered with the complete precipitation of the barium carbonate.

As the experiments with the carbamino method were essentially of an exploratory nature, boiled plasma was the only substance tested systematically for activating powers by this method. However, the technique is being developed further, for it has an advantage over the colorimetric methods in that the change in pH during the estimation is less, while there is no indicator present which could interfere by inhibiting the enzyme. On the other hand, the procedure is somewhat tedious since each run can establish only a single point on the graph of $\ln [\text{CO}_2]/\text{time}$. In most cases, three estimations together with the initial value for the concentration of CO_2 are sufficient for the velocity constant to be determined.

Enzyme solutions. Carbonic anhydrase solutions at three stages in purification were used: (a) a solution of lysed red cells, (b) the crude CHCl_3 preparation of Meldrum & Roughton (1933), and (c) highly purified preparations obtained by dialysis and precipitation of the crude CHCl_3 solutions with $(\text{NH}_4)_2\text{SO}_4$, followed by fractional adsorption with Alumina C_γ (Keilin & Mann, 1940). For comparisons of the relative activities of different preparations, the unit adopted was that amount of enzyme which doubles the initial rate, in $\mu\text{l./sec.}$, of liberation of CO_2 from the phosphate buffer-bicarbonate mixture when the latter was shaken in the boats at 350 cyc./min. at 0° . Except when using lysed red cell solutions, 0.05% peptone was added as stabilizer.

RESULTS

Peptone as a protecting agent. It has already been mentioned that small amounts of peptone are able to retard the gradual loss of activity that purified preparations of carbonic anhydrase undergo on dilution. Peptone is also able to restore some of the activity of purified enzyme solutions which have been diluted with distilled water in the absence of any protecting agent. The restoration of activity may be as high as 90%. That peptone acts merely as a stabilizer and not as a true activator was shown manometrically by Clark (1949). The same point can be demonstrated very clearly with the new colorimetric technique. In Fig. 1 is shown the effect of adding peptone on the relation between the concentration of carbonic anhydrase and the observed value of the unimolecular velocity constant. The fact that, beyond a certain enzyme concentration, both curves are virtually parallel shows conclusively that the peptone is not an activator, but merely protects the enzyme from adsorption or from the effects of some inhibiting substance which manifests itself when the enzyme concentration is low.

The type of curve (Fig. 1, curve A), in which there is no direct proportionality between the observed activity and the enzyme concentration until the latter has passed a certain critical value, has also been observed by Roughton & Booth (1946b) in their manometric work on purified carbonic an-

hydrase. The explanation which they offered for this kind of effect is that some definite amount of metal or other enzyme poison is present, in spite of the precautions taken to ensure cleanliness of all glassware, and that an equivalent amount of the enzyme is inactivated by combination with such impurity. As was the experience of Scott and his colleagues, we found it quite essential to use a small amount of peptone in all manometric work with purified carbonic anhydrase. With the two new methods, on the other hand, sufficiently high enzyme concentrations can be used to reduce the errors due to inactivation of the enzyme. Nevertheless, as it is established that peptone itself does not activate, it was included at a final concentration of 0.05% as routine procedure in all experiments in which other substances were being tested for activating properties. There are no obvious grounds for believing that the peptone might prevent the effect of some true activator from manifesting itself.

Fig. 1. Effect of 0.05% peptone on the activity of highly purified carbonic anhydrase, as measured by the new colorimetric method. Final solution was 0.02M in veronate, pH 8.6, 0.017M in CO_2 and 0.001% phenol red. Temperature 18° . Ordinate: first order velocity constant. Abscissa: concentration of enzyme preparation/ml. A, without peptone; B, with peptone.

The activators. So many substances have been claimed by Maine & Locke and by Leiner to be activators that little point would be served by considering them all in detail. Attention, therefore, will be confined to those which have been asserted to be particularly effective.

Leiner & Leiner (1940) and Van Goor (1943a), working with the Brinkman colorimetric method, found boiled plasma of different mammalian species to be a powerful activator. According to Van Goor, the degree of activation of lysed red cell solutions may be as high as 600–800%. One would expect such a high degree of activation to be observable

just as readily with one of the alternative methods for estimating the enzyme activity. On the contrary,

being a powerful activator is, in fact, a mild inhibitor (Figs. 2 and 3). With the manometric method, if peptone is omitted, there is a tendency for the

Fig. 2. Effect of 10% boiled horse plasma on the activity of purified carbonic anhydrase in the presence of 0.05% peptone. Manometric CO_2 output at 0° . Plot of $\log(a-x)$ against time from the equation $k = \frac{2.3}{t} \log \frac{a}{a-x}$. Curve A, boiled horse plasma; curve B, boiled horse plasma and carbonic anhydrase; curve C, carbonic anhydrase.

Fig. 3. Effect of boiled horse plasma on the activity of carbonic anhydrase, using the new colorimetric technique. Curve A, enzyme + 0.05% peptone; curve B, enzyme + 0.05% peptone.

measurements made manometrically, by the new colorimetric method and by the carbamino method showed conclusively that boiled plasma, instead of

Fig. 4. Effect of 0.1 mM-glutathione. Manometric CO_2 output at 0° . Curve A, glutathione only; curve B, enzyme only; curve C, enzyme + glutathione; curve D, enzyme + 0.05% peptone, with or without glutathione.

Fig. 5. Effect of glutathione, using the new colorimetric method. $\circ$ — $\circ$, enzyme + 0.05% peptone; $\bullet$ — $\bullet$, enzyme + 0.05% peptone + 0.1 mM-glutathione.

mild inhibition to be obscured by the weak stabilizing action of boiled plasma. This is especially the case with the more highly purified enzyme preparations.

Glutathione is the only substance that Kiese found to possess activating properties, but we have been unable to confirm this either manometrically or colorimetrically (Figs. 4 and 5). In the manometric experiments there were evidences for small stabilizing and protecting effects, but the estimations made in the presence of peptone definitely excluded all question of glutathione being a powerful activator of carbonic anhydrase. For reasons given below, we feel that all claims concerning activators which are based on either the Philpot or Brinkman techniques should be rejected. This would include the observations on glutathione made by Leiner and by Maine & Locke (1941). Kiese's work, however, was based on the manometric method. Moreover, he was well aware of the common sources of error, namely the limiting effects of diffusion and the tendency for the enzyme to lose its activity owing to the violent shaking. On the other hand, he did not make use of any stabilizer or protecting agent. Consequently, it does seem possible that the apparent activations he observed on adding glutathione were in fact due to the restoration of activity lost by adsorption or by the action of inhibitors. Support for this explanation is provided by the observation that the apparent activity of purified carbonic anhydrase diluted with distilled water can be increased nearly 30 % by the addition of glutathione, but the increased activity is always less than that observed when peptone is used in place of glutathione.

Table 1. *Stabilizing properties of certain substances on purified solutions of carbonic anhydrase when tested manometrically*

(*D* is the amount of inactivation of the enzyme within 100 sec. from the commencement of shaking and when no stabilizer is present. *S* is the degree of stabilization brought about by the substance in question. The method of calculating *D* and *S* is described in the text, p. 496.)

Enzyme preparation (units/mg. dry wt.)	Substance	<i>D</i> (%)	<i>S</i> (%)
1750	—	35	—
	Catechol (0.5 mm)	34	3
	Quinol (0.5 mm)	34	3
	Glycine (5.0 mm)	30	14
	Cysteine (0.01 M)	27	23
	Histamine (4.0 mm)	18	49
	Adrenaline (1 : 5000)	15	57
	Horse serum (1 : 40)	0	100
	Peptone (0.05 %)	0	100
4800	—	48	—
	Histamine (4.0 mm)	35	27
	Peptone (0.05 %)	0	100

Several other substances mentioned by Leiner and by Maine & Locke, such as cysteine, histamine, adrenaline, catechol, quinol and glycine, were

examined manometrically and, in the case of the first three, by the new colorimetric method, but none was found to possess activating powers (Table 1). Cysteine appeared to be a mild inhibitor, which is in agreement with the observations of Kiese & Hastings (1940). In certain cases when the manometric method was tried in the absence of peptone, catechol and quinol were found to bring about small increases in the apparent activity of the enzyme, although they had little or no stabilizing action. Since the effect varied considerably with different enzyme preparations, while the increased activity was always well below the level produced by the addition of peptone alone, it is possible that these two substances act by way of antagonism of traces of some enzyme poison present.

DISCUSSION

In many instances, evaluation of the work on activators of carbonic anhydrase has in the past been rendered difficult by the failure of different workers to agree on both qualitative and quantitative issues. Thus, whereas Leiner, Schmidt & Klawonn (1944) find the plasma activators to manifest themselves in very high dilutions, suggesting that they have some important role in physiological regulations, Van Goor (1943*a*) states that their effect is clear only if the plasma is undiluted and the enzyme concentration low. The latter investigator concludes, therefore, that the activators are of little or no physiological importance. Even within the work of one investigator, there are some discrepancies. While examining the properties of plasma activators, Van Goor (1943*a*) found that unboiled sheep plasma inhibits the carbonic anhydrase activity of lysed red cells by as much as 90 %. On the other hand, in a second paper which deals with thermolabile and thermostable fractions of the enzyme, he finds (Van Goor, 1943*b*) that unboiled sheep plasma activates the purified enzyme solutions. The explanation offered by Van Goor for this last effect, namely that the plasma provides some factor which has been split off from the enzyme during the purification of the latter, is not entirely convincing. An alternative explanation which may be suggested is that the sheep plasma contains an inhibitor of carbonic anhydrase (see also Booth, 1938) which can also protect and stabilize the enzyme when the latter is highly purified.

A significant observation made by Leiner *et al.* (1944) is that the effect of the activators depends on the order in which the enzyme and activator solutions are mixed. If they are mixed together before dilution, the observed 'activation' is greater than if the enzyme is diluted separately and then mixed with the activator. Protection of the enzyme against adsorption at interfaces seems to be the simplest explanation for this effect.

From what has already been said, it is apparent that it is most important to make a clear distinction between true activation of the enzyme and mere stabilization or protection. Although Leiner (1943) appears to be aware of stabilizing effects, he maintains that they cannot account for the activations observed by him with Brinkman's method. His argument is that in a colorimetric method there can be no inactivation of the enzyme through shaking, while the duration of the experiment is so short that there can hardly be any denaturation by dilution. He gives no consideration to the possibility of reversible adsorption at interfaces of a fraction of the enzyme and he was unaware of the inhibitory effect of the carbonate buffer system (see below). In view of Roughton's suggestion that reversible restoration of activity may be the underlying explanation for many apparent activations, it is significant that almost all investigators agree that the effects of activators are greater with purified enzyme solutions than with lysed red cell preparations. It may be suggested that in the latter instance there is always sufficient protein present, in the form of haemoglobin, to protect adequately the enzyme from adsorption or surface denaturation. In this connexion it would seem that Leiner's estimation that one carbonic anhydrase molecule requires many thousands of 'activator' molecules for full activity would fit into a picture of physical protection of the enzyme rather than with true activation. It is almost certain that the activations which Bakker (1942) observed with his simplified manometric technique were also in the class of stabilization and protection of the enzyme.

There is no need to give detailed consideration to Leiner's original idea that certain activators affect the enzyme more when it is catalysing the hydration of carbon dioxide than when it is catalysing the dehydration of carbonic acid, as this was the subject of vigorous debate between Leiner and Kiese during the war years. Acceptance of Leiner's view would, as Kiese frequently pointed out, imply that the enzyme is able to displace the equilibrium constant for the reaction $\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}_2\text{CO}_3$, and this is contrary to our knowledge of thermodynamics and enzyme kinetics. Perhaps, however, it is not without some interest to note that Leiner & Leiner (1940) believe unidirectional activation of carbonic anhydrase to have its counterpart in the behaviour of other enzymes. As an example, they cite the activation of trypsin by enterokinase.

The most surprising of all of Leiner's claims is not that we must accept the existence of two classes of activators, but his assertion (Leiner, 1944; Leiner *et al.* 1944) that the important factor involved in activation is the partial or complete removal of zinc from the enzyme molecule. Keilin & Mann (1940)

found a very close correlation between the zinc content of a purified enzyme preparation and the carbonic anhydrase activity. Leiner, on the other hand, does not find this to be the case. He states (Leiner, 1944) that preparations containing from 0.14 to 0.18 % zinc may be more active than those containing 0.3–0.5 % of the metal, but this observation is not very significant, for Keilin & Mann showed that irreversible inactivation of carbonic anhydrase does not result in the liberation of the zinc. Consequently, the presence of zinc in amounts over and above that to be expected from the observed activity of the preparation probably means simply that, during the various stages in purification, a certain amount of the enzyme has become denatured or otherwise irreversibly inactivated. Leiner, however, maintains that there is no direct relationship between the zinc content and the enzyme activity of purified solutions. In the paper written in collaboration with Schmidt & Klawonn, he states that it is possible to remove up to four-fifths of the zinc from carbonic anhydrase by treating the enzyme with twenty times its weight of cysteine, followed by dialysis. The enzyme is then said to be more active than before the treatment. In support of the claim that removal of zinc is the main process underlying activation, Leiner *et al.* (1944) assert that the activating effects of cysteine, histidine and serum can be abolished by the addition of 2.5×10^{-5} M-zinc sulphate. Unfortunately, complete details of these experiments have not yet been published, so further discussion of them is not possible. It may be mentioned, however, that an attempt to confirm Leiner's observations on the effect of treating purified carbonic anhydrase with cysteine was unsuccessful.

There can be little doubt that the unsatisfactory state of knowledge about the 'activators' is due almost entirely to the uncritical use of inadequate methods. Roughton (1943) has pointed out several objections to the use of the Philpot method for studying this subject, the most important being the fact that in the initial stages of the reaction, the buffer system inhibits the enzyme by as much as 90 %. It is obviously most important to ensure that such a high degree of inhibition remains the same in all cases (Roughton & Booth, 1946*b*). An additional source of error which we have observed in the Philpot technique is the considerable protein error of bromothymol blue in the presence of many biological fluids. Fig. 6 shows the effect of boiled horse plasma on the light absorption of the indicator solutions, the pH of which had been checked with a glass electrode. Phenol red, on the other hand, is not so susceptible to such a large error and has the further advantage that it is only a very weak inhibitor of carbonic anhydrase (Kiese & Hastings, 1940; Wilbur & Anderson, 1948).

Objections to the Brinkman method, although less serious, are still sufficient to render the method unreliable for the study of activators. Inhibition by the carbonate-bicarbonate buffer system is still large, but the most serious objection is the extremely low poise of the buffer system. The reaction time which is observed is determined by the time taken to convert the small amount of carbonate present to bicarbonate. Just how small this quantity is can be judged from the fact that in the original method (Brinkman, 1933), no carbonate is added at all, other than that present as impurity in the 0.02M-bicarbonate solution. In Van Goor's modification,

Fig. 6. The effect of 2% boiled horse plasma on the optical density at 620 m μ . of bromothymol blue solutions over the pH range from 6.0 to 8.0. Curve A, indicator alone (0.001 %); curve B, indicator + 2% boiled plasma.

the carbonate concentration may have been increased by the preliminary boiling of the solution. Kreps & Tchenyakaeva (1942) make the final solution 0.0002M in carbonate ion. The sensitivity of the system to traces of hydrogen or hydroxyl ions does not seem to be fully appreciated, the following example serving to illustrate this error. Using the Brinkman method to study the hydration of carbon dioxide and a manometric method to study the dehydration of carbonic acid, Kreps & Tchenyakaeva found that insect blood contains a substance which inhibits the former reaction but not the latter. In view of the thermodynamical implications of this, the work was repeated by Levenbook & Clark (1950), who found that as far as the Brinkman method was concerned, acceleration or inhibition of the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ could be demonstrated at will,

depending upon the pH chosen for the final comparison. Carefully controlled manometric experiments, on the other hand, showed that insect blood has no effect at all on either the hydration or dehydration reactions.

When examining the effects of various tissue extracts on carbonic anhydrase, inhibition by chloride may be a potential source of error. Roughton & Booth (1946b) found that 0.024M-sodium chloride can inhibit by as much as 30 %, probably due to the formation of a complex with the enzyme. The inhibition appears to increase as the pH changes from 8.5 to 7.5, suggesting that the effect of chloride is most likely to introduce errors when one of the 'changing pH' methods is being used.

Fig. 7. Effect of bromothymol blue (2 mg./100 ml.) on highly purified carbonic anhydrase and in the absence of peptone. Manometric CO_2 output at 0°. Curve A, bromothymol blue alone; curve B, bromothymol blue + enzyme; curve C, enzyme alone.

Of the older methods available for the estimation of carbonic anhydrase activity, there can be no doubt that the rapid manometric method is still the most reliable, but even here errors may be introduced if a mild inhibitor also has stabilizing properties. This applies especially if work is being done with the purer enzyme preparations. The case of bromothymol blue may be taken as an example (Fig. 7). In this particular instance, it is apparent that any estimation of activity which utilizes the overall gas volume changes occurring in a given time may be very much in error. Up to the first 60 sec. of the reaction, the inhibiting effect of the dye is quite clear, but as shaking continues the stabilizing property tends to become more evident, so that an estimation of activity based upon figures obtained after 100 sec. would suggest some degree of activation.

In conclusion, we would again emphasize that when all possible precautions were taken to eliminate sources of error, complete agreement has been obtained between all three methods used in this work for the measurement of the enzyme activity. Since all of the substances tested failed to show any signs of true activating properties on highly purified carbonic anhydrase in the presence of peptone, the claims to the existence of activators of this enzyme must be rejected until more reliable and convincing evidence has been produced. Satisfactory evidence is not likely to be forthcoming by mere repetition of the older work, using the less highly developed methods. In fact, we believe that the results obtained in the present investigation show the need for rapid reaction methods in further work of this kind, enabling the highest possible enzyme concentrations to be used. Not only would this tend to eliminate errors due to surface effects and traces of inhibitors, but it would enable a closer approach to be made to the physiological conditions under which carbonic anhydrase functions in the living organism.

SUMMARY

1. Carbonic anhydrase activity was estimated manometrically and by a new colorimetric method which enabled higher enzyme concentrations to be used than were previously possible. Some observations have also been made on a rapid-flow adaptation of Faurholt's carbamino method for measuring the rate of hydration of carbon dioxide.

2. Provided that all precautions were taken to eliminate errors due to stabilization of the enzyme, no evidence could be obtained to support the claims that certain substances are activators of carbonic anhydrase.

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APPENDIX

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The new colorimetric method for studying the hydration of carbon dioxide involves the rapid mixing of a saturated solution of carbon dioxide with a suitable buffer solution in the presence of an appropriate indicator, followed by a continuous recording

of the rate of the subsequent colour change. A modified Chance rapid-reaction apparatus (Chance, 1940) was used, the two solutions being expelled at the same rate from similar all-glass syringes and mixed in a small mixing chamber before being driven