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CARBONIC ANHYDRASE. ITS PREPARATION AND PROPERTIES.

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INTRODUCTION.

THERE have been two main theories as to the mode of carbon dioxide transport in blood: (*a*) that carbon dioxide is carried from the organs of the body to the lung mainly in the form of bicarbonate, and that when the blood reaches the lung the proteins of the blood acting as weak acids (expressible by the formula HP) convert the bicarbonate into carbonic acid (H_2CO_3) which in turn dehydrates to water and dissolved carbon dioxide. The latter then volatilizes as CO_2 gas into the air spaces in the lung from which it is eliminated by expiration; (*b*) that in addition part, and possibly the more important part physiologically, of the carbon dioxide is carried in direct reversible combination with the blood proteins. Of these theories (*a*) has been called the bicarbonate theory and has had the support of Zuntz (quoted by Haldane, 1922), Parsons [1919, 1920], Poulton [1920], L. J. Henderson [1928], Peters and van Slyke [1931], Stadie [1928, 1931] and their co-workers. The direct combination theory (*b*) has, on the other hand, been sponsored by Bohr [1909], Buckmaster [1917 *a*, *b*], Bayliss [1924], Mellanby and Thomas [1920], and by later workers to whom reference will be made below.

From 1917 to 1921 the problem was worked at intensively by numerous British physiologists, with the result that at the end of this period the bicarbonate theory, in the view of most writers, had established a decided lead over its rival. Thereupon the scene shifted to America, and there followed an exhaustive study of the acid-base equilibria in blood by van Slyke and various colleagues. These workers gave full data as to the titration curves of oxyhæmoglobin and reduced hæmoglobin, which, together with their data upon the CO_2 dissociation curves, seemed to show that under physiological conditions the amount of CO_2

in blood in forms other than CO_2, H_2CO_3 or HCO_3^- is negligible. It will, however, be noted below that both in this work, and in that of Stadie and his collaborators [1928, 1931], there are certain features which can only be explained by the simple bicarbonate theory, with the aid of rather forced assumptions. These apparent anomalies readily fall out if a certain proportion of carbon dioxide exists in some form other than CO_2, H_2CO_3, or HCO_3^-, *e.g.* in direct combination with the blood proteins.

Up till 1925, attention had been given solely to the equilibrium states of the process, but in that year Hartridge and Roughton, quoted by L. J. Henderson [1925], following on their measurements of the velocity of oxygen combination and dissociation from hæmoglobin, pointed out the desirability of studying the kinetics of the carbon dioxide processes in blood. In 1926 this aspect was taken up by Henriques with a startling outcome.

According to the bicarbonate theory the detailed chemical reactions which lead to CO_2 evolution in the lungs are as follows:

$$HP\,(\text{protein acid}) + NaHCO_3 \rightarrow NaP\,(\text{protein salt}) + H_2CO_3, \qquad \ldots\ldots(i)$$

followed by

$$H_2CO_3 \rightarrow CO_2 + H_2O. \qquad \ldots\ldots(ii)$$

Of these reactions (i), being purely ionic, is very rapid, but (ii) is known from the work of Thiel *et al.* [1913 *a*, *b*], Faurholt [1924], and the recent paper of Brinkman, Margaria and Roughton [1933] to be rather slow. On the bicarbonate theory reaction (ii) must therefore limit the rate of CO_2 escape in the expired air. Using the velocity constants of previous authors, Henriques (and others independently) calculated that the rate at which CO_2 could be formed by reaction (ii) under physiological conditions would be far less than that actually observed in the expired air. Hence he concluded that either one or both of the following conditions must obtain: (*a*) there must be a catalyst for the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ in the blood; (*b*) the physiological transport of carbon dioxide in the blood must take place by some mechanism other than the bicarbonate one.

The most important of Henriques' [1928] experiments were those in which he compared the rate at which CO_2 is evolved when hæmoglobin solutions, on the one hand, and serum solutions, on the other hand, were violently shaken *in vacuo*. He noted for the first time that CO_2 came off much more quickly from the hæmoglobin solutions especially in the early stages of the shaking, whereas the rate of escape from serum was of the same order as that calculated from Faurholt's velocity constants for

the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$. He concluded that his results could not be explained by a catalyst mechanism, but required instead the existence of a very rapid reversible reaction between CO_2 and hæmoglobin direct, analogous to the reaction between oxygen and hæmoglobin. To this hypothetic complex, which he suggested might be of a carbamate type, he gave the name carbhæmoglobin. Hawkins and van Slyke [1930], however, in experiments of a similar type found distinct evidence of a catalyst, and this was clinched by Brinkman and Margaria [1931], who showed that blood or hæmoglobin, even when diluted to 1 part in 20,000, accelerated markedly the evolution of CO_2 from mixtures of bicarbonate with buffers of physiological *p*H. Finally in 1932 the present writers isolated from ox blood a white substance of which 1 part in 10,000,000 is active in accelerating the reaction. This solid extract, which is quite free from hæmoglobin and apparently also from hæmatin compounds, has the typical properties of an enzyme, and since it can be shown to be distinct from the other enzymes known to be present in blood, it has been given a special name: CARBONIC ANHYDRASE. Preliminary reports on its biochemical properties have already been given by us elsewhere [Meldrum and Roughton, 1932 *a*, *b*, *c*], and a more complete account of its preparation and properties is given below.

With such large amounts of carbonic anhydrase in the red blood corpuscles it no longer seems difficult to account for the rate of CO_2 evolution in the expired air on the simple bicarbonate theory. This does not, however, prove that the bicarbonate mechanism is the sole mode of physiological transport; a definite conclusion as to the rôle of the hypothetical CO_2 protein compounds is indeed needed, not only for the sake of its own interest and for possible importance, but also to enable us to assess precisely the rôle of carbonic anhydrase in blood. For reasons to be given later, we do not regard either the recent experiments of Henriques [1928, 1931 *a*, *b*, 1933] or of Margaria [1931] as decisive or complete, and we have therefore devised and carried out a new set of experiments, which seem to us less equivocal in their indications. Our results not only point to appreciable amounts of direct CO_2 protein compounds especially at low temperatures (the amount diminishing very much as the temperature and/or the acidity is increased), but also suggest strongly that these compounds are of a carbamate type, as has indeed been hinted at by previous workers. The whole question is dealt with in a separate paper by us [Meldrum and Roughton, 1933*b*] in the present *Journal*.

Scope of the present paper.

We shall start by describing the method of measuring the catalytic activity, and pass thence to the various methods of separating the enzyme from hæmoglobin and the means adopted for its further purification. After summarizing the evidence that carbonic anhydrase is distinct, not only from hæmoglobin, but also from some other enzymes present in the blood corpuscles, we then proceed to some of the biochemical properties of the enzyme. Here we deal, in a preliminary way, with the nature of the enzyme and some of the factors which influence its stability and activity. We have made a fairly detailed study of the effect of various poisons and inhibitors, including metallic salts from most groups in the periodic table. In the last pages we give some further notes about the distribution of the enzyme, especially in fœtal blood as compared with maternal blood; we also compare the carbonic anhydrase activity of unlaked corpuscles with that of laked corpuscles from the same blood. This last matter is obviously of great physiological importance as regards the action of the enzyme in circulating blood: our preliminary experiments show that there is need for much further study on these lines.

Section I. The method of estimating the catalytic activity of the various preparations.

The technique.

A simple means of measuring the amount of enzyme present in the various media and preparations is clearly an indispensable preliminary to a study of the isolation and properties of the enzyme. The method adopted was to measure the catalytic effect upon the rate of evolution of CO_2 from sodium-bicarbonate solutions when mixed with phosphate buffer of *p*H 6·8, with the aid of the boat apparatus of Brinkman, Margaria and Roughton [1933] as used by them in their work on the kinetics of the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ in the absence of catalyst.

In one compartment of the glass boat-shaped trough (Fig. 1) are placed 2 c.c. of phosphate solution made by mixing equal volumes of $M/5$ Na_2HPO_4 and $M/5$ KH_2PO_4, whilst in the other compartment are placed 2 c.c. of $M/5$ $NaHCO_3$ dissolved in $M/50$ NaOH. The boat is attached by a well-fitting stopper and length of pressure tubing to a manometer, open at the other end to the air. Most of the experiments in this paper

were thus done at atmospheric pressure, instead of at reduced pressure as in the earlier paper.

After allowing time for temperature equilibration the boat is then shaken rapidly in a constant temperature water bath (usual temperature about 15° C.) and the evolution of CO_2 followed by noting the reading of the manometer at 0, 5, 10, 15, 30, 45, 60, 90 and 120 sec. and thereafter at minute intervals if necessary. Provided that the first half of

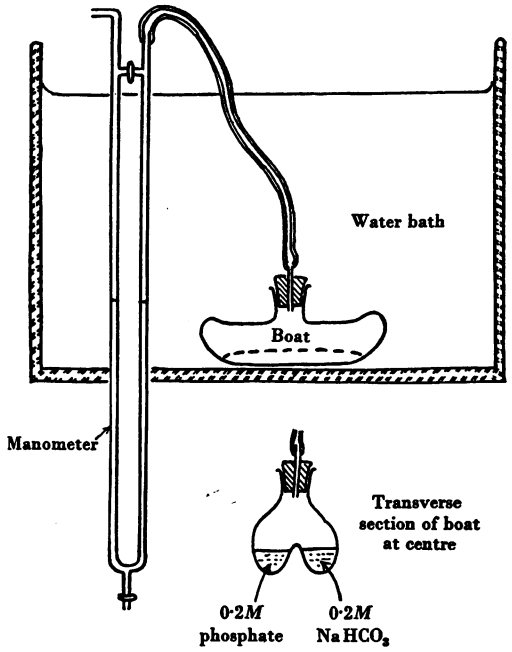

Fig. 1. Boat technique for measurement of enzyme activity.

the total CO_2 is evolved in not less than about 15 sec., diffusion from liquid to gas phase does not prove to be a limiting factor, and the rate of CO_2 evolution as observed is a true measure of the velocity of the chemical reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. A typical set of curves obtained with various amounts of goat's blood added to the phosphate-bicarbonate mixture is shown in Fig. 2 *a*.

Calculation of the activity.

In Fig. 2 *b* is plotted the average rate of CO_2 evolution during the second quarter of the process (as given by the reciprocal of the time

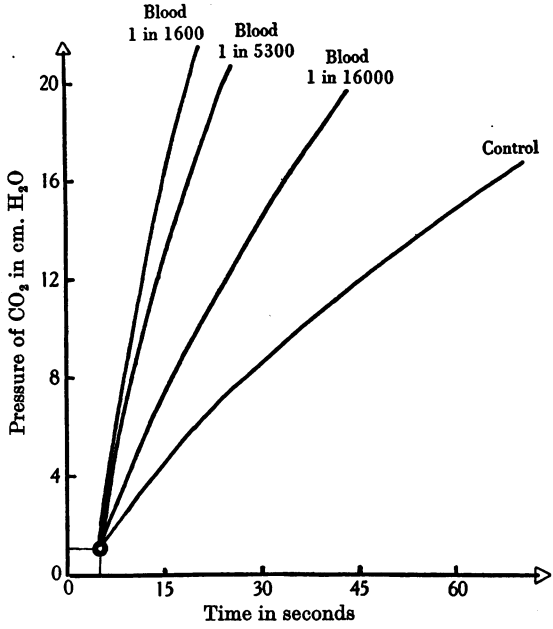

Fig. 2 *a*. Effect of various amounts of goat's blood on rate of CO_2 output from phosphate-bicarbonate mixture.

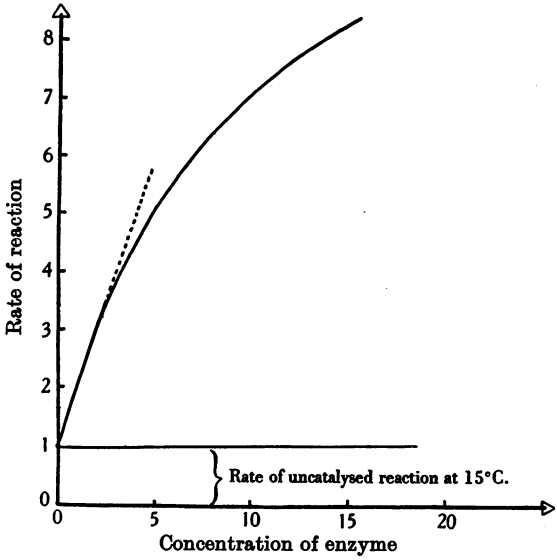

Fig. 2 *b*. Relation between concentration of enzyme and effect on rate of reaction. [The rate of the reaction in absence of catalyst being taken as unity.]

taken) against the concentration of catalytic material added, the samples of the latter ranging from crude blood to relatively pure material.

Let R_0 be the rate in absence of catalyst, R be the rate in presence of catalyst. Then it is clear from the curve that the fraction $(R-R_0)/R_0$ is a suitable measure of the catalytic activity, for:

(*a*) It is proportional to the amount of catalyst added, to within ± 5 p.c., provided that the rate of the process is not too fast, *i.e.* the time is not less than 10 sec.

The breakdown of the linear relation at faster rates is certainly in part due to diffusion becoming a limiting factor in addition to chemical reaction velocity.

(*b*) It is independent of the nature of the added catalyst, *e.g.* whether it contains hæmoglobin or not.

(*c*) It is independent of the dimensions of the apparatus, which need not even be known.

(*d*) It eliminates initial errors due to the time taken for the bicarbonate and phosphate to mix after the shaker starts.

(*e*) The range of the *p*H change during the period is small and constant.

In ordinary cases it makes no difference whether the catalytic material is added to the phosphate or to the bicarbonate, or if it is distributed between both solutions.

It seems premature to define the unit of carbonic anhydrase activity until a full study of the kinetics of the enzyme reactions has been made. For the present it is proposed to assign the value E to that amount of enzyme which, when dissolved in 4 c.c. of the phosphate bicarbonate mixture used above, gives a value of $(R-R_0)/R_0$ equal to 1 at 15° C. If the temperature differs slightly from 15° C., a correction can be made by applying a temperature coefficient (*i.e.* Q_{10}) of 3·6 per 10° C. for the uncatalysed reaction and a Q_{10} of 2·1 for the catalysed reaction. This temporary unit E has the convenience that ox blood (the main type of blood used in our work so far) contains on the average about 1 E per c.mm.

Whenever a test was made of the catalytic activity of any solution, a control experiment with phosphate and bicarbonate solutions alone was done as well. To get the most accurate result the amount of catalytic material added should be such as to increase the rate of the process about three to four times, since the relation between enzyme concentration and rate of reaction is still linear up to this point. In some tests, unfortunately a much larger amount of catalyst was used; in view of the shape

of the curve in Fig. 2 *b* the results were correspondingly less accurate, even though the necessary corrections were applied.

The percentage yield in any given preparation from blood was calculated from a comparison of the activity of the preparation with that of the original blood diluted with an amount of water equal to the amount of aqueous fluid added to the blood in the course of the preparation.

Section II. Separation and purification of the enzyme.

Separation from hæmoglobin.

The finding of Brinkman, Margaria, Meldrum and Roughton [1932] that not all hæmoglobins are catalytically active, but that native globin prepared from an active hæmoglobin is fully active, suggested strongly that the active principle is not hæmoglobin itself, but some substance or some system which frequently, but not invariably, accompanies the hæmoglobin. That the latter is the case was settled very quickly by applying the classical methods of removing hæmoglobin with but little modification. Three methods, *A*, *B*, *C*, were tried, and all were successful. Of these the crude product of method *C* was used for nearly all the further work in this paper.

Method A. Mammalian erythrocytes (previously washed three times with isotonic saline) are lysed with water and diluted till the hæmoglobin is about 1 p.c. The aqueous solution is then saturated with chloroform and allowed to stand at room temperature in presence of excess $CHCl_3$ with intermittent shaking till all the hæmoglobin is denatured. The usual time required is about 24 hours: a temperature of 37° C. hastens denaturation but destroys the enzyme. The protein and excess chloroform are centrifuged down and the enzyme solution poured off. It usually contains of the order of 50 p.c. of the carbonic anhydrase originally present, much catalase, and traces of hæmoglobin ($\ngtr$ 1 in 5000). This method is similar to that of Tsuchihashi [1923] for preparing catalase.

Method B. By following the method of Warburg and Christian [1931] for preparing the co-enzyme for the system which oxidizes glucose-*b*-phosphate, carbonic anhydrase can readily be prepared. 200 c.c. washed horse erythrocytes are lysed at 0° C. with an equal volume of water and 350 c.c. of ice-cold alcohol added. Then 15 c.c. $CHCl_3$ are added to the homogeneous mixture and the whole shaken for a few minutes at 0° C. The suspension is centrifuged and the centrifugate poured into 450 c.c. alcohol and 1200 c.c. ether, when a whitish precipitate gradually falls.

The supernatant liquid is sucked off, the solid collected on a filter and washed with absolute alcohol.

This enzyme preparation is very stable, it does not readily dissolve in water, but gives a highly active and fairly clear solution when 10 mg. are ground in a mortar with 1 c.c. of $M/5$ Na_2HPO_4.

The yield is only about 7 p.c., and the activity per mg. dry weight is about 350 *E* as measured by the method of Section IV, assuming that the whole of the enzyme is in solution. The latter assumption is, however, probably unfair.

Method C. To 10 c.c. washed ox corpuscles in a centrifuge tube are added 6 c.c. H_2O and 4 c.c. ethyl alcohol. The mixture is then shaken at room temperature for about a minute with 5 c.c. $CHCl_3$. On centrifuging for about 10 min. at 3500 r.p.m. a three-phasic system is formed, consisting of a top layer of enzyme solution, a central layer of denatured protein, and a bottom layer of chloroform.

The enzyme solution contains traces of hæmoglobin and much catalase. The yield is on the average about 50 p.c. of the maximum. In different cases there were considerable variations from this figure both in upward and downward directions. The cause of this is not known but may be in part explained by varying amounts of enzyme adhering to the hæmoglobin coagulum. Rubbing up the latter with water gave a solution of high activity on the one occasion when the test was made. On evaporating the enzyme solution to dryness in a vacuum desiccator a brown solid enzyme preparation is obtained containing methæmoglobin and hæmochromogen compounds. The yield is about 1 g. from 100 c.c. corpuscles.

The enzyme is extremely stable in this form and can be kept for many months even if exposed to the air. The brown solid readily dissolves in water or salt solutions to yield a clear solution, having the properties of the original (unevaporated) solution. In aqueous solution the enzyme keeps for many weeks in the ice chest. The rate of destruction varies with different samples, but in general the enzyme loses $\not>$ 20 p.c. activity in 5–6 weeks.

The solution of enzyme thus prepared is known in the succeeding parts of this paper as "crude chloroform" preparation, and is used as the basis for further purification.

Some two dozen preparations were made by this method, which was found to work for the blood of other mammals besides ox. In one case with horse blood an 80 p.c. yield was obtained. In the earlier cases of the series a lower proportion of alcohol was used, but this led to much

slower precipitation of the hæmoglobin, several shakings with $CHCl_3$ often being necessary before a colourless (*i.e.* hæmoglobin-free) supernatant fluid was obtained. The yield, however, was as great as when the proportion of alcohol mentioned above was used, the great advantage of the latter lying in the quickness of the method. The difference in speed of coagulation of the hæmoglobin forms, indeed, a striking demonstration: for the quickest results the proportion of 6 parts water to 4 parts alcohol should be adhered to closely.

Purification.

The further purification of the enzyme is best carried out starting from the crude chloroform preparation. Such a preparation contains two types of impurity: (*a*) dialysable; (*b*) non-dialysable.

The dialysable impurities are readily removable by ultra-filtration. For this purpose Bechhold's 6 p.c. and 4½ p.c. ultra-filtration membranes were purchased from Carl Schleicher and Schüll, and ultra-filtration by vacuum suction was found quite satisfactory, provided the fluid on the membrane is gently stirred.

The non-dialysable impurities: Attempts were first made to precipitate either the enzyme or the accompanying protein material with ammonium sulphate, but these were unsuccessful. In the case of crude hæmoglobin solutions most of the enzyme is carried down with the precipitated proteins, while in the case of the crude chloroform preparations a large and variable amount of enzyme is removed, probably by adsorption on precipitated material.

A series of adsorbents was next tried. Kaolin, permutit, four different sorts of charcoal, silk, cellulose and chalk were unsatisfactory. Very good results, however, were obtained with Willstätter's C-γ alumina cream, and with a suspension of $Ca_3(PO_4)_2$ (roughly 1 p.c. prepared by precipitation in the customary manner), filtering after each treatment with adsorbent. These adsorbents are both effective in removing selectively the other non-dialysable impurities, especially the traces of colouring (hæmatin) matter, without at the same time taking up serious amounts of carbonic anhydrase. The extent of the treatment must be adjusted for each sample.

The following is a protocol by N. U. M. of a purification by the $Ca_3(PO_4)_2$ method:

100 c.c. ox corpuscles were washed three times with isotonic saline and then treated with 80 c.c. water, 20 c.c. alcohol and 150 c.c. $CHCl_3$. The mixture was shaken for some minutes, stood overnight at 2° C.,

centrifuged, shaken and centrifuged again. The supernatant fluid was filtered, volume of filtrate $A = 108$ c.c. 25 c.c. A were treated with 20 c.c. of $Ca_3(PO_4)_2$ suspension, and the mixture filtered. Volume of filtrate $B = 40$ c.c.

30 c.c. of filtrate B were ultra-filtered on a 6 p.c. Bechhold membrane, being washed three times with about 13 c.c. water.

The residue on the membrane was made up to 40 c.c. with water = solution C.

The volume of the ultra-filtrate was 67 c.c. = solution C'.

0·05 c.c. of solution C was tested and found to have an activity = 6 E.

5 c.c. of solution C evaporated to dryness at 130° C. gave a dry weight of 1·2 mg. Therefore the activity per mg. of the solid in solution $C = 500$ E.

N. U. M.'s purest preparation was made by $Al(OH)_3$ adsorption, the steps of the process being otherwise almost the same as in the previous example. In this case details are available as to the activity per mg. dry weight at three different stages of the preparation.

Activity of original ox corpuscles per mg. dry weight = 4·6 E (assuming water content of corpuscles = 60 p.c. by weight).

Crude chloroform preparation shows 48 mg. dry weight per 5 c.c.

Activity per mg. dry weight = 90 E.

The dialysable impurities were removed by ultra-filtration and the final solution after three treatments with an equal volume of $Al(OH)_3$ cream had an activity per mg. dry weight = 1730 E. 5 c.c. of this final solution evaporated at 100–150° C. contained 1 mg. dry weight, and was thus almost the same in concentration as the final solution of the previous case, although the activity was about 3·5 times greater.

Comparison of the activity per mg. dry weight of the final preparation with that of the original corpuscles shows that a concentration of about 400-fold was attained.

The figures given show that 1 part of the pure solid (by weight) in 7,000,000 parts of solution is sufficient to double the rate of CO_2 evolution, and that during the first 15 sec. 1 g. of enzyme is responsible for the production of 825 g. of CO_2. This means a rate of 1·24 mols CO_2 per sec. per g. of enzyme.

If the enzyme is pure, and is a protein (*vide* Section III) of the minimum molecular weight found by Svedberg, viz. 34,000, then 1 mol enzyme would catalyse the formation of 4×10^4 mols CO_2/sec.

Further purification of the enzyme.

The fact that our best preparation has the same order of activity as the most active enzymes of other workers makes it perhaps unlikely that much further purification will be possible. We have not yet, however, used in a systematic way one of the classical weapons of enzyme purification, namely adsorption of the enzyme, followed by elutriation of the enzyme from the adsorbent. Preliminary work suggests that the enzyme is to some extent adsorbable by alumina C-γ cream, especially if the impurities adsorbed by the latter are first removed. Thus in two cases we were able by appropriate treatment to adsorb as much as 40 p.c. of the enzyme on alumina. We also found on one occasion that from the purest solution the enzyme was partly adsorbed by kaolin, and could be in part elutriated therefrom by means of $M/15$ Na_2HPO_4.

Fractional ultra-filtration is another possible line. Thus in one experiment the crude chloroform preparation was shaken with alumina, filtered and the filtrate then ultra-filtered through a 6 p.c. Bechhold membrane. The solution which came through the ultra-filter had an appreciable activity. In the two other cases, the crude chloroform preparation was ultra-filtered directly through a $4\frac{1}{2}$ p.c. Bechhold membrane. The solution which came through had respectively 13 and 25 p.c. of the activity of the crude chloroform preparation in the two cases.

Section III. The "individuality" and some properties of the enzyme.

The evidence that carbonic anhydrase is distinct from hæmoglobin.

Earlier workers on the CO_2 catalyst in blood inclined to the view that it was identical with hæmoglobin. Against this there are now three strong arguments:

(*a*) There is no correlation between the distribution of hæmoglobin and of carbonic anhydrase in lower organisms. Examples of this lack of correlation have already been given by Brinkman, Margaria, Meldrum and Roughton [1932], and further instances occur in Brinkman's [1933] paper in the present number of this *Journal*. A striking case of the same kind will be found in a later section of this paper, where it is shown that in fœtal blood of goats the ratio carbonic anhydrase content/hæmoglobin concentration, *i.e.* E/Hb, may be only 1–2 p.c. of the value in the mother blood of the same animal.

(*b*) It is now possible by the simple methods of Section II to prepare highly active preparations of the enzyme, which are completely free from hæmoglobin.

(*c*) We have been able in the case of two species of blood of which the hæmoglobin crystallizes easily—namely, horse and rat—to effect a considerable separation of hæmoglobin from the enzyme merely by crystallization.

Thus in the case of horse hæmoglobin, a single crystallization gave a value of E/Hb about three times greater in the mother liquor than in the crystals. In the case of the rat sample the first mother liquor, after crystallization at 0° C., had only one-seventh the hæmoglobin concentration of the original blood, but yet had nearly the same concentration of carbonic anhydrase. Two further crystallizations gave a preparation of hæmoglobin with an E/Hb value only 7 p.c. of the E/Hb in the original blood. It seems likely therefore that by very many recrystallizations hæmoglobin might be obtained free from carbonic anhydrase as well as from catalase, as has already been done as regards the latter only.

The properties of such hæmoglobin, if not too largely contaminated with "inactive material," would be interesting to compare with those of hæmoglobin "purified" by the usual means.

Freedom from hæmatin compounds.

No trace of hæmatin compounds could be detected in the purified alumina preparations, even when examined in very thick layers by spectroscopic methods.

Distinction of carbonic anhydrase from other enzymes of the blood, etc.

(*a*) *Catalase.* There is no correlation between the distribution of catalase and carbonic anhydrase: thus, for example, there is much catalase in yeast, but usually no detectable carbonic anhydrase. Furthermore we found that our crude chloroform preparations, which contained abundant catalase as well as carbonic anhydrase, were freed entirely of catalase by means of the $Al(OH)_3$ adsorption technique. Thus the filtrate obtained after the usual alumina treatment still contained a high proportion of carbonic anhydrase, but no catalase.

(*b*) *Peroxidase.* Both the guaiacum and benzidine tests are negative, when applied to our pure preparation.

(*c*) *Oxidase.* Preliminary observations failed to detect blueing of tetramethylparaphenylenediamine, nor was any catalysis of oxidation of reduced cytochrome *c* to be observed.

So far then we feel justified in believing that carbonic anhydrase is a new enzyme, distinct from any previously described enzyme or system.

Protein tests, etc.

The simple characteristics of the solid obtained by evaporating the crude chloroform preparation to dryness have already been described. The active solid obtained by evaporating the purest solutions to dryness at low temperatures is colourless and almost transparent.

The presence of proteins in the various preparations was looked for by the usual tests. The results are shown in Table I, which also includes the α-naphthol test.

TABLE I.

A = Crude chloroform preparation.
B = Solution of solid prepared as in Section II, method *B*.
C = Purest solution ($Al(OH)_3$ filtration treatment, etc.).

Test	Result *A*	*B*	*C*
Millon's	+ +	+ +	+ +
Xantho-proteic	+ +	+ +	+ +
Sulphur	+ +	+ +	?
Glyoxylic	+	+	+
Biuret	.	.	+
α-Naphthol	+	+	+

+ + = Marked reaction.
+ = Moderate to slight reaction.
? = Doubtful.

Since in relatively pure solution the enzyme is only partially and variably precipitated by saturation with ammonium sulphate, and tends furthermore to pass through ultra-filtration membranes to an appreciable extent, it is probable that the enzyme, if a protein, is a rather small molecule.

Further work on such matters as the chemical composition of the enzyme, etc., its molecular weight, solubility and acid and base-binding properties, has been postponed until the efforts at maximum purification shall have been exhausted.

SECTION IV. THE STABILITY, SCOPE OF ACTION, AND KINETICS OF THE ENZYME.

Stability.

(*a*) *To temperature.* The stability of the enzyme, in three different grades of purity, was tested by exposing the solutions to various tempera-

tures for 30 min., and then measuring the activity as in Section I. The result was as follows:

	20° C.	55° C.	65° C.
Lysed corpuscles (1 part in 80 parts water)	No loss	30 p.c. loss	Completely destroyed
Crude $CHCl_3$ preparation	,,	—	,, ,,
Very pure $Al(OH)_3$ preparation (in water)	,,	No loss	60 p.c. loss

(*b*) *To* *p*H. The same three preparations were taken to various *p*H's for 30 min. periods, and then tested for activity.

	Citrate phosphate buffer			Borate buffer	*N*/100 NaOH	*N*/10 NaOH
	*p*H 3	*p*H 4	*p*H 6–10	*p*H 11	*p*H 12	*p*H 13
Lysed corpuscles	Completely destroyed	No loss	No loss	—	—	Completely destroyed
Crude $CHCl_3$ preparation	,,	,,	,,	No loss	No loss	,,
$Al(OH)_3$ preparation	,,	,,	,,	—	30 p.c. loss	,,

The scope of the reactions catalysed by carbonic anhydrase.

The reactions $CO_2+H_2O \rightarrow H_2CO_3$ *and* $CO_2+OH^- \rightarrow HCO_3^-$. These reactions, *i.e.* the reverse to that so far dealt with in this paper, are of course also catalysed by the enzyme. The boat technique was again used, but in this case a two-compartment vessel was not necessary. 4 c.c. of a suitable buffer were placed in the boat, the latter connected by one lead through its cork to the manometer, and by a second lead through a very fine capillary and a rubber tube with pinchcock to a vessel containing CO_2 gas at about 1½ atmospheres. About 25 sec. before shaking was due to begin the pinchcock was opened and CO_2 allowed to stream into the boat until a suitable pressure, as shown by the manometer reading, was reached. This usually took 15 sec.

After 10 sec. more, shaking was started and the uptake of CO_2 recorded by the manometer readings at 0, 5, 10 and 15 sec., etc.

For higher CO_2 pressures than 50 mm. Hg, the water in the manometer was replaced by mercury and the technique slightly altered.

Fig. 3*a* shows the catalytic effect of 1 part crude $CHCl_3$ enzyme in 40,000 parts solution upon the rate of uptake of CO_2 by 0·2 *M* phosphate buffer *p*H 7·6 at 17° C. The reaction is accelerated about eight-fold. This may be compared with the fourteen-fold acceleration of CO_2 output produced by the same concentration of this sample of enzyme when tested by the method of Section I. At *p*H 7·6 the uptake of CO_2 occurs almost entirely through the reaction $CO_2+H_2O \rightarrow H_2CO_3$. Fig. 3*b*, however, gives the uptake of CO_2 by a solution containing 0·7 *M* $NaHCO_3$ and 0·57 *M* Na_2CO_3. The *p*H of this solution being about 10, the reaction

$CO_2 + OH^- \rightarrow HCO_3^-$ is also occurring to a marked extent according to Faurholt [1925], confirmed by Brinkman, Margaria and Roughton [1933]. One part of crude $CHCl_3$ enzyme in 40,000 parts of solution accelerates in this case, but to a less extent, viz. 1·7-fold.

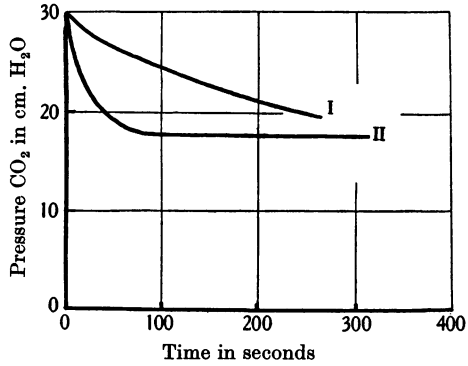

Fig. 3*a*. I = Rate of uptake of CO_2 by 0·2 *M* phosphate buffer *p*H 7·6; II = Rate of uptake of CO_2 by 0·2 *M* phosphate buffer *p*H 7·6, with crude $CHCl_3$ enzyme 1 in 40,000 added. Temp. 17° C.

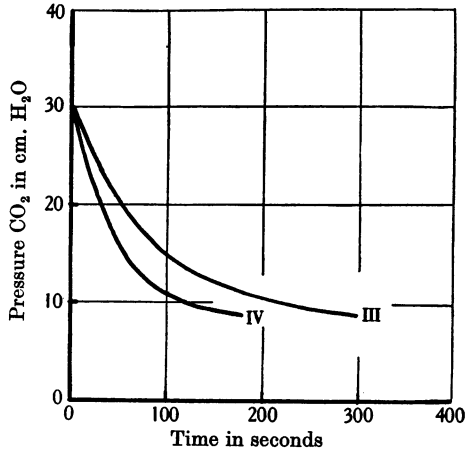

Fig. 3*b*. III = Rate of uptake of CO_2 by 0·71 *M* $NaHCO_3$—0·57 *M* Na_2CO_3; IV = Rate of uptake of CO_2 by 0·71 *M* $NaHCO_3$—0·57 *M* Na_2CO_3, with crude $CHCl_3$ enzyme 1 in 40,000 added. Temp. 17°C.

The enzyme can also be shown to catalyse CO_2 uptake by *p*H 11 solutions of Na_2CO_3, in which case the uncatalysed process occurs almost entirely *via* the reaction $CO_2 + OH^- \rightarrow HCO_3^-$.

Some more composite processes catalysed by carbonic anhydrase.

Any complex process, in which the reaction $CO_2+H_2O \rightarrow H_2CO_3$ or $CO_2+OH^- \rightarrow HCO_3^-$ is a link and is a limiting factor in the over-all speed of the whole, should be accelerated by carbonic anhydrase. Four cases of this kind were investigated, all of possible physiological bearing.

(*a*) *The solution of* $CaCO_3$ *by weak acids other than carbonic acid,* i.e. $CaCO_3+2HA \rightarrow CaA_2+H_2CO_3$, *followed by* $H_2CO_3 \rightarrow CO_2+H_2O$. If the chalk is shaken violently enough, the reaction $H_2CO_3 \rightarrow CO_2+H_2O$ tends to become a limiting factor.

A 2 p.c. suspension of $CaCO_3$ in water was shaken at 0° C. with an equal volume of acetate buffer *p*H *circa* 5, total acetate conc. 0·7 *M*. One part of crude $CHCl_3$ enzyme per 10,000 parts of suspension accelerated the solution of $CaCO_3$ three times.

(*b*) *The solution of* $CaCO_3$ *by carbonic acid to form calcium bicarbonate.* Exp.: 20 c.c. of saturated $Ca(OH)_2$ were shaken in a 70 c.c. boat with pure CO_2 at $1\frac{1}{2}$ atmospheres initially.

The uptake of CO_2 was followed manometrically. There was at first a rapid uptake of CO_2 occasioned by conversion of the $Ca(OH)_2$ into $CaCO_3$ with precipitation of the latter. This was followed by a smaller slow uptake due to the partial conversion of insoluble $CaCO_3$ into slightly soluble $Ca(HCO_3)_2$, *i.e.* $CO_2+H_2O \rightarrow H_2CO_3$, followed by $H_2CO_3+CaCO_3 \rightarrow Ca(HCO_3)_2$.

That the reaction $CO_2+H_2O \rightarrow H_2CO_3$ is to some extent limiting was shown by addition of crude $CHCl_3$ enzyme 1 in 16,000.

The slow process was then accelerated about twelve times at 0° C.

The rates of solution and deposition of $CaCO_3$ are of biological interest in regard to the formation of shells and other calcareous deposits.

(*c*) *The uptake of* CO_2 *by ammonia solution.* This was also measured manometrically. There is first of all a very rapid uptake of CO_2, due to the formation of ammonium carbamate, *i.e.* $2NH_3+CO_2 \rightarrow NH_2COONH_4$. This is followed by a long slow process in which ammonium carbonate and bicarbonate are formed, the final proportions of carbamate, carbonate and bicarbonate depending upon the initial amounts of NH_3 and CO_2. Of these two processes, the rapid one, *i.e.* the carbamate formation, does not seem to be affected by carbonic anhydrase, but the slow one is much speeded up. Thus with 4 *M* ammonia, 1 part of crude $CHCl_3$ enzyme in 6000 parts accelerated the slow process ten times and even in "Ammon. Fort." (sp. gr. 0·880) the enzyme still showed appreciable activity. This is a striking instance of its robustness.

(*d*) If an equilibrium mixture of ammonium carbamate, carbonate and bicarbonate is shaken with $CaSO_4$ suspension, the carbonate and bicarbonate react very quickly with the $CaSO_4$ to form $(NH_4)_2SO_4$ and $CaCO_3$ (which is precipitated): calcium carbamate is, however, relatively soluble and does not precipitate, but with the removal of the carbonate from the sphere of action, the carbamate changes over slowly into carbonate which then precipitates. Faurholt [1925] has shown that the conversion of ammonium carbamate into bicarbonate and carbonate occurs, not by a simple addition of water as has often been thought, but by a two stage process.

$$NH_2COONH_4 \rightarrow 2NH_3 + CO_2,$$

$$CO_2 + OH^- \rightarrow HCO_3^-, \text{ followed by } HCO_3^- + OH^- \rightarrow CO_3^{--} + H_2O.$$

If so, then the precipitation of $CaCO_3$ in the slow stage should be accelerated by carbonic anhydrase. This was confirmed by estimating the amount of $CaCO_3$ precipitate formed after various periods of shaking. In the slow stage 1 part crude $CHCl_3$ enzyme in 1000 parts accelerated the process about 3·5 times at 19° C.

Examples (*c*) and (*d*) are of physiological bearing in regard to the rôle of carbonic anhydrase in the interaction of carbamates and bicarbonates. In our adjoining paper [1933 *b*] we give new evidence for the existence of protein-carbamate compounds in physiological fluids especially at low temperatures.

Some notes on the kinetics of the enzyme.

Only preliminary observations have been made so far upon the effect of varying enzyme concentration, substrate concentration, temperature, *p*H and neutral salts upon the kinetics of the reaction.

(*a*) *Enzyme concentration.* All the data so far available have been given in Section I.

(*b*) *Substrate concentration.* Experiments have been done upon the effect of varying the pressure of CO_2 upon the rate of CO_2 uptake by 0·2 *M* phosphate buffer *p*H 7·6, and by 0·7 *M* $NaHCO_3$ + 0·57 *M* Na_2CO_3 *p*H *circa* 10. In Fig. 4 the rate is plotted against CO_2 pressure both for the uncatalysed and the catalysed reaction in both cases. For the catalysed experiments 1 part crude solid enzyme per 40,000 parts solution was used at both *p*H's. From 20 mm. Hg to 380 mm. Hg the rate of the catalysed reaction is proportional to the CO_2 pressure, but falls off at higher pressure in the *p*H 7·6 case. A similar result was found for rate of uptake of CO_2 by 4 *M* NH_3 almost saturated with NaCl; in this

case, owing to the depressing action of the strong NaCl, 1 part of the same crude solid enzyme per 800 parts of solution had to be used. This indicates that the Michaelis constant of the enzyme [see Haldane, *Enzymes*, 1930] is small, both in conditions where the activity of the enzyme is high, and also where the activity is low.

The uptake of CO_2 is a far more suitable case for studying the effect of varying the concentration of substrate than is the output of CO_2, for with the boat technique the pressure of CO_2 can be varied from 10 mm. Hg to 1500 mm. (*i.e.* 150-fold), whereas the concentration of $NaHCO_3$

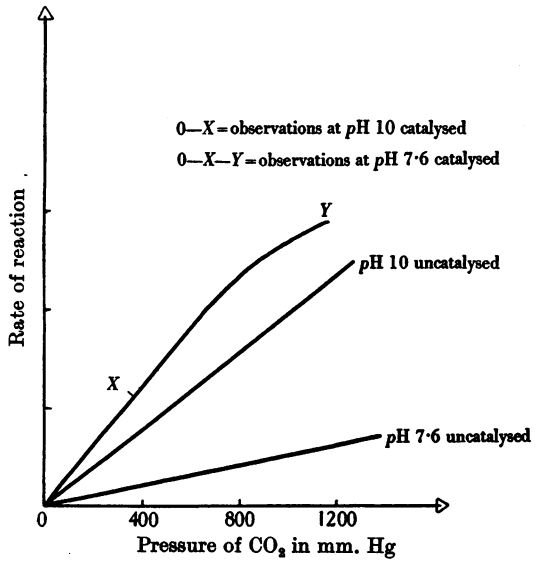

Fig. 4. Relation between substrate concentration and accelerating effect of enzyme.

cannot be conveniently varied more than about fifteen-fold. Furthermore, in the latter case with high concentrations of $NaHCO_3$ activity effects and other "neutral salt" effects, cause serious disturbances, but with high pressures of CO_2 in uptake experiments these troubles do not arise.

Only one experiment has so far been done on CO_2 output with varying substrate concentration. In this it was found that 1 part crude $CHCl_3$ enzyme in 8000 accelerated CO_2 output from $1{\cdot}0M$ $NaHCO_3 + 1{\cdot}0M$ phosphate buffer *p*H 6·8 three times, whereas 1 part of the same enzyme in 40,000 accelerated CO_2 output from the usual $0{\cdot}2M$ $NaHCO_3 + 0{\cdot}2M$ phosphate ten times.

(*c*) *Effect of temperature.* The rate of CO_2 output by the standard technique of Section I at 0·2° C. and 19·2° C. showed a temperature coefficient per 10° C. of 2·1 for the catalysed part of the reaction, the enzyme used being a purified $Ca_3(PO_4)_2$ preparation. Much further work is needed here also.

(*d*) *Effect of* *p*H. This has not yet been studied systematically, but the impression gained is that the enzyme has an optimum in the physiological range. For CO_2 uptake experiments above *p*H 11, and for output experiments below *p*H 5·5, it will be necessary to resort to Hartridge and Roughton's rapid reaction methods, or perhaps to some variant of the processes discussed under the heading "Scope of enzyme action" in this section.

(*e*) *Neutral salts.* High concentrations of salt, especially NaCl, have been found to exert a very depressing effect upon the enzyme activity both in CO_2 output and CO_2 uptake experiments.

(*f*) *A special advantage of carbonic anhydrase for kinetic studies.* Brinkman, Margaria and Roughton [1933] have measured the rates of the reactions $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ under the conditions where the time for the reaction to be half completed ranged from 15 min. to 0·01 sec., *i.e.* a 100,000-fold variation. These same methods are applicable to the catalysed reaction, which it is therefore hoped to follow over a much wider range of enzyme concentration than has been tried in the case of any other enzyme reaction.

Section V. The action of various inhibitors, and possible activators.

The substances which have been tried may be divided into four classes:

(*a*) Those which might act by poisoning some active centre in the enzyme of a metallic type (*e.g.* a hæmatin, iron or copper nucleus), viz. carbon monoxide (in dark and in light), cyanides, sulphides, azides, pyrophosphates.

(*b*) Other anions which have been shown to inhibit particular enzyme systems, viz. iodoacetate, fluoride.

(*c*) An extensive series of metallic salts, representative of most of the groups of the periodic table.

(*d*) Miscellaneous substances.

The general method used for testing the activity of the enzyme in presence of these various substances has been that described in Section I.

Classes (a) and (b).

Carbon monoxide. The boat technique was used with the following modification: the phosphate solution and bicarbonate solution, with enzyme contained in one or other of them, were saturated with pure CO in Thunberg tubes. Controls in which the enzyme was added subsequently showed that this shaking did not itself cause inactivation of the enzyme.

2 c.c. of each CO saturated reagent were then placed in the boat, the air in the latter replaced by pure CO, and after temperature equilibration the boat was shaken and CO_2 output followed in the ordinary way. The CO was prepared by acting on sodium formate with conc. H_2SO_4 and was stored under pressure over alkaline sodium hydrosulphite. It thus contained no O_2 or CO_2. Controls in which hydrogen was stored over alkaline sodium hydrosulphite under the same conditions showed that the gas did not thereby acquire any traces of inhibitory substances (*e.g.* SO_2, H_2S or particles of liquid spray). For experiments in the dark the water in the bath was replaced by a deeply coloured dye solution, whilst for light experiments a mirror was placed at the bottom of the bath, and a 100 watt lamp was placed just above the surface of the clean water in the bath.

The results of some experiments are given in Table II:

TABLE II.

Nature of enzyme preparation	Date	Activity expressed in p.c. of activity in air: In CO dark	In CO light	In CO dark / In CO light
Dilute human blood	29. iv. 32	—	—	80·0 p.c.
Crude $CHCl_3$ enzyme	16. ix. 32	36·0	32·0	112·0
$Al(OH)_3$ purified enzyme	20. iv. 32	36·0	70·0	53·0
$Ca_3(PO_4)_2$ purified enzyme	23. vi. 32	56·1	59·5	94·5
,, ,, ,,	22.viii. 32	23·3	30·5	77·0
,, ,, ,,	29. vi. 32	25·0	22·7	110·0

In all instances carbon monoxide produced a large inhibition in the dark; in half the cases, viz. the crude blood, the $Al(OH)_3$ preparation and one of the $Ca_3(PO_4)_2$ preparations, this inhibition was partially reversed by light, though in the other cases the light rate and the dark rate in CO were almost the same within experimental error. In none of the four purified preparations was there any visible trace of hæmatin present, but all these showed marked inhibition by CO in the dark, and in two cases this inhibition was partially reversed by light. Further work,

however, is needed to discover why the effect of light is variable, and in positive cases to investigate its action in detail.

Cyanides. The action of HCN (prepared by neutralization of KCN to *p*H 7·4) was studied upon crude blood and upon the enzyme in the various stages of purification described in Section II, methods *B* and *C*. The effect of various concentrations, ranging from *M*/800 to *M*/16,000 HCN, upon a $Ca_3(PO_4)_2$ purified enzyme solution, is shown in Fig. 5. Practically complete inhibition is produced by *M*/800 HCN. The latter

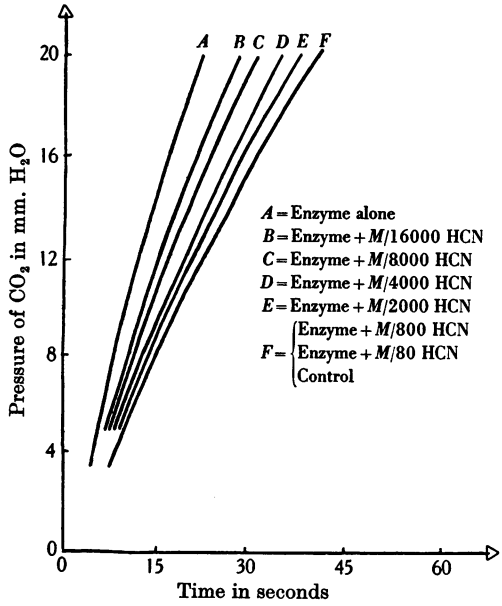

Fig. 5. Effect of various concentrations of HCN upon activity of enzyme.

was also found to be the case with four different samples of crude ox blood, one sample each of horse blood and whale blood, and one sample of enzyme purified by method *B* of Section II.

Considerable inhibition by *M*/4000 HCN was noted in the case of one sample of $Al(OH)_3$ purified enzyme, including the ultra-filtrate from the latter through a 6 p.c. Bechhold membrane, and also in the case of one sample of human blood. These two cases were not tested with *M*/800 HCN.

At the outset of our research we met with a solution of electro-dialysed ox hæmoglobin, which showed slight activation by *M*/1000 HCN instead of inhibition. Brinkman [1932] also has met with such cases.

Unfortunately we threw away all our material before we realized its value, and since then we have met with no other such case, though we have been continually on the look-out. The detailed investigation of an anomalous sample of such a kind might well throw important light on the nature of the enzyme.

Sulphides. The same group of enzyme preparations as those studied for cyanide inhibition (with the exception of human and whale bloods and the $Ca_3(PO_4)_2$ purified enzyme, none of which were tested at all) also showed complete inhibition by $M/1000$ Na_2S. At lower concentrations than $M/1000$, sulphide appears to be a somewhat more effective inhibitor than cyanide. Sodium sulphide was also shown to inhibit the catalysis of CO_2 uptake by $4{\cdot}0\,M$ aqueous ammonia.

Sodium azide (NaN_3). A $M/500$ solution of this reagent was tested on one sample of $Al(OH)_3$ purified enzyme and caused a partial inhibition.

Pyrophosphate, sodium fluoride ($M/1000$), *sodium iodoacetate* ($M/1000$), were all negative as regards inhibition.

Class (c). Metallic salts.

Salts of twenty-three different metals were tried, the final concentration in most cases being $M/1000$ or $M/2000$. A $Ca_3(PO_4)_2$ purified enzyme preparation was used in every case except Na, K and Mg. In the latter a crude $CHCl_3$ preparation was used. Negative results were found in the following groups of the periodic table.

Group 1 A. Na, K.
Group 2 A. Be (as sulphate), Mg (as sulphate), Ca.
Group 2 B. Cd (as sulphate and as chloride).
Group 3 A. Ce (as chloride).
Group 3 B. Al.
Group 4 A. Ti (as chloride).
Group 4 B. Pb (as nitrate).
Group 6 A. Cr (as chloride).
Group 7 A. Mn.
Group 8. Fe (as ferrous sulphate + ferric chloride), Co (as nitrate), Ni (as chloride), H_2PtCl_6.

Positive results were found for:

Group 1 B.	Cu (as $0{\cdot}0005\,M$ $CuSO_4$ recrystallized)	About 50 p.c. inhibition
	Ag (as $0{\cdot}001\,M$ nitrate)	Large inhibition
	Au (as $0{\cdot}001\,M$ $HAuCl_4$)	,, ,,
Group 2 B.	Zn (as $0{\cdot}001\,M$ $ZnSO_4$ not recrystallized)	About 50 p.c. inhibition
	Hg (as $0{\cdot}0005\,M$ $HgCl_2$ recrystallized twice)	Large inhibition
Group 5 A.	Va (as $0{\cdot}001\,M$ vanadyl sulphate not recrystallized)	,, ,,

With the exception of vanadium, metals which act as poisons to carbonic anhydrase are those of Groups 1 B and 2 B only. Such also have

been observed for a number of other enzymes, viz. xanthine oxidase [Wieland and Mitchell, 1932], saccharase [Myrbäch, 1926], papain, kathepsin [Krebs, 1930], and may indeed be an empirical principle of wide application for enzymes. The present case, however, cannot yet be quoted as a satisfactory example, because the reagents used in testing the enzyme activity, viz. phosphate and bicarbonate at *p*H 9·2, both form precipitates with many of the metals tested. Some of the inactive metals may have been ineffective on account of this purely inorganic factor, and we cannot be sure that if it were controlled there would any longer be such a sharp demarcation between them and the active group Cu, Ag, Au, Zn and Hg.

Such control would be very difficult to effect. At all events there is no doubt that Cu, Ag, Au, Zn and Hg are strong poisons for carbonic anhydrase.

Class (d). Some other substances.

Indifferent gases: N_2, O_2, H_2. The activity of the crude $CHCl_3$ enzyme in nitrogen, oxygen and hydrogen was found to be the same as in air.

Phenylurethane. This was chosen as an example of a commonly used cell narcotic. The phosphate and bicarbonate solutions were saturated with phenylurethane by shaking in Thunberg tubes for an hour or more and were then allowed to stand. Horse hæmoglobin, crude $CHCl_3$ enzyme, and $Ca_3(PO_4)_2$ purified enzyme all showed about the same inhibition, *i.e.* roughly 50 p.c.

Saponin. A 4 p.c. solution of ox blood dissolved in 1 p.c. saponin showed the same activity as a 4 p.c. solution of the same ox blood in water.

Hydrazine sulphate. A 0·002 *M* solution had no effect upon the activity of a crude $CHCl_3$ preparation.

Ferricyanide. Blood dissolved in 0·001 *M* K_3FeCy_6 has the same activity as blood dissolved in water.

Section VI. The distribution of the enzyme and some physiological considerations.

Distribution.

During this work, many estimations of the enzyme content of goat, ox and human blood have been made. These are collected in Table III, which also gives the content of some other mammalian bloods in single instances.

TABLE III.

Animal	No. of cases	*E*/c.mm. blood	Average *E*/c.mm. blood
Goat (anæsthetized)	11	1·30–6·10	2·80
Ox (defibrinated)	6	0·60–1·80	1·10
Man	8	0·37–0·68	0·55
Rabbit	2	0·71–1·72	1·21
Goat (normal)	1	1·35	1·35
Horse	1	0·64	0·64
Rat	1	1·70	1·70
Whale	1	1·40	1·40

The values were the same, whether clotting was prevented by defibrination or by oxalate. In any one species there is a two- to four-fold variation or more in normal animals; this suggests that the normal carbonic anhydrase content of mammalian blood is abundantly in excess of minimum physiological requirements. It seems doubtful whether there is any significance in the higher average content of goat's blood as compared with ox, and man.

During the last four months of this research we were lucky enough to have the opportunity, through the kind offices of Prof. Barcroft and his colleagues, of studying an exceptionally interesting case of distribution.

About a dozen female goats were "stocked" during the latter part of January 1933 and were operated on in roughly successive weeks from March 20 to June 1. Samples of the fœtal blood and maternal blood (treated with oxalate and fluoride) were given to us in each case and the carbonic anhydrase content of each estimated. The results are shown in Table IV, together with a single case of whale mother and fœtus, the blood of which was kindly supplied by Mr A. Laurie.

TABLE IV.

Date of operation	Date stocked	*E*/c.mm. mother blood	*E*/c.mm. fœtal blood	Activity of fœtal blood expressed in p.c. of activity of mother blood
Mar. 23	Feb. 2	1·6	<0·010	<0·5
April 11	Jan. 30	3·6	0·022	0·6
,, 13	,, 24	4·2	0·140	3·3
,, 18	,, 23	1·8	0·060	3·4
,, 24	,, 21	6·1	0·110	1·8
May 1	,, 20	1·3	0·067	5·2
,, 5	,, 19	1·6	0·056	3·5
,, 12	,, 18	1·9	0·066	3·5
,, 22	,, 16	1·7	0·040	2·3
,, 31	,, 12	5·0	0·456	9·2
June 1	Unknown, probably 15 weeks ±	1·9	0·292	15·0
May 31	Whale fœtus of unknown age	1·4	0·350	25·0

In the very young fœtuses there is extraordinarily little enzyme and the amount does not begin to rise appreciably till very near the end of term. Since the fœtuses live in an all fluid environment there is perhaps not so much need for the enzyme, since it is possible for them to excrete HCO_3^- into the mother blood as well as CO_2. It is, however, indispensable for them to have a good supply before parturition occurs, otherwise when they begin to breathe they may not be able to excrete CO_2 gas into their expired air fast enough. The consequences of the low content of enzyme in fœtal blood must indeed be taken fully into account in working out quantitatively the gas exchange between maternal and fœtal blood in the placenta. Finally, since the hæmoglobin content of the fœtal blood was never less than 50 p.c. of that of the maternal blood, this series of experiments gives a clarion instance of the independence of the enzyme from hæmoglobin.

The distribution of the enzyme in the blood of lower organisms, *e.g.* earthworm, planorbis, etc., was reported in the preliminary communication of Brinkman, Margaria, Meldrum and Roughton [1932]. The measurements of activity were made by a portable, but much less accurate, modification of the boat technique, and are too rough for values of E/c.mm. to be worth calculating.

Tests on some other materials besides blood have been made with the following results:

Cow's milk, normal human urine. Both inactive even when 1 part in 10 is added to the phosphate solution in the boat test.

Rabbit's sperm. Showed a content of 0·0034 E/c.mm. This sperm was kindly supplied by Dr J. Walton and appeared to be quite free from blood.

Muscle extract. Water extracts of the hind limbs both of a dog and a cat were made, after the muscles had been perfused with isotonic saline until the issuing fluid was almost free of blood hæmoglobin. In both cases an activity was observed, and it was found that the enzyme activity of the muscle extract/content in muscle hæmoglobin (measured colorimetrically) was roughly equal to the enzyme content of the blood/hæmoglobin content of the blood of the same animal.

Plasma. No carbonic anhydrase could be detected in oxalated rabbit's plasma even when tested 1 part in 10, provided that allowance was made for the very slight amount of laking of the red corpuscles which occurred before the plasma was separated. This is confirmed by the experiments with unlaked goat's blood described below.

Yeast. No enzyme could be detected in an extract of 15 g. Belgian top yeast ground with 100 c.c. of a mixture of $M/5$ Na_2HPO_4 and $M/5$

K_2HPO_4 and allowed to stand for 24 hours at 1° C. before being centrifuged.

In one experiment with a zymin preparation a slight positive effect was noticed. This needs confirmation. No carbonic anhydrase has yet been found in green plant material.

Comparison between laked and unlaked blood.

In early experiments, confirmed recently, with defibrinated ox blood, obtained from the butcher, we noticed that the activity of unlaked blood was about 23–35 p.c. of that of the same amount of laked blood. This was also true if the unlaked blood corpuscles were washed three times with saline to eliminate the slight laking which is almost always present in defibrinated blood. We attributed the lower activity of the unlaked corpuscles simply to the fact that the substrate has to diffuse into the corpuscles before it can react with the enzyme, so that this is another case of chemical reaction velocity and diffusion being joint limiting factors [Roughton, 1932].

We were, therefore, surprised to find that in goat's blood, drawn in the laboratory by syringe from a vein, the activity of unlaked corpuscle suspensions was, in all cases but one, only 0·05–0·01 p.c. of the activity of the same amount of laked blood, when tested by the method of Section I. In each case the blood was put in the 0·2*M* phosphate, and the result was the same whether the goat was anæsthetized or not and independent of whether clotting was stopped by defibrination or by oxalate-fluoride solution.

The fact that whole blood was used for these "unlaked" experiments also confirms that plasma and serum are both practically inactive. The very slight activities observed may indeed be due to traces of laking.

The result must apparently be a permeability effect of the corpuscle membrane, which may depend upon the species of animal, the manner in which the blood is drawn, and the medium in which it is suspended. In any case, much further work on these lines is needed not only to clear up the subject for its own sake, but also to enable us to assess the activity of carbonic anhydrase in circulating blood *in vivo*.

Possible rôle of the enzyme in other processes than evolution of CO_2 *in the lung.*

Carbonic anhydrase may possibly have some effect in earlier stages of the metabolism, than the final one in which CO_2 is evolved from the body. The only one so far tested is the fermentation of glucose-phosphate

mixture *p*H 7·6 by zymin at 26° C. No effect was observed. Many other possibilities, such as decarboxylation, formation of urea in the liver, effect in muscular activity, etc., need to be explored, as do also the effects of injection of the purified enzyme. Brinkman [private communication] tells us that he has made a start at the latter problem. Further work, on such lines, is contemplated in collaboration with him and his co-workers.

Summary.

1. Carbonic anhydrase is an enzyme present in red blood corpuscles (but not in the blood plasma) which catalyses both phases of the reversible reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. It is thus of prime physiological importance in the formation of CO_2 from bicarbonate in the lung. Without it, CO_2 could not be excreted nearly fast enough for the needs of the body.

2. The activity of the enzyme can be estimated by its accelerating effect upon the evolution of CO_2 from a mixture of 0·2 *M* phosphate buffer *p*H 6·8 with 0·2 *M* bicarbonate. At low concentrations of enzyme, the acceleration is directly proportional to the amount of enzyme added.

3. The enzyme can be separated from the hæmoglobin of the red blood corpuscles by coagulating the hæmoglobin. Three different methods were tried with success. In the best of these, ox corpuscles are treated with an equal volume of 40 p.c. alcohol, and then with half their volume of chloroform. The mixture is then shaken and centrifuged. The supernatant fluid is practically colourless and contains roughly 50 p.c. of the total enzyme of the blood. This crude preparation is further purified by adsorption with $Al(OH)_3$ cream or $Ca_3(PO_4)_2$ suspension and by ultra-filtration. The purest product is about 1800 times as active as the original blood and seems to be free from hæmoglobin, hæmatin, catalase, peroxidase and oxidase. The enzyme is very stable when evaporated to a solid.

4. The enzyme is stable in solution over the range *p*H 3–12. Its solution in water is destroyed by 30 min. heating at 65° C. It shows most of the protein reactions. It accelerates a number of processes with over-all velocity dependent upon the rate of one or other of the reactions $CO_2 + H_2O \rightleftharpoons H_2CO_3$, or $CO_2 + OH^- \rightleftharpoons HCO_3^-$.

5. The enzyme is poisoned by CO, cyanides, sulphides, azides, Cu, Ag, Au, Zn, Hg, phenylurethane. A number of other substances were tested, but had no effect.

6. Preliminary work has been done on the kinetics of the enzyme action, and the effect of temperature, salts and *p*H thereon.

7. The fœtal blood of goats has far less enzyme than the maternal blood, but the content rises towards the end of gestation. A suspension of unlaked corpuscles is much less active than a solution of laked corpuscles.

8. Some other instances of the distribution of the enzyme are given.

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