# 138. CARBONIC ANHYDRASE. PURIFICATION AND NATURE OF THE ENZYME

By D. KEILIN and T. MANN[1]
*From the Molteno Institute, University of Cambridge*

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## I. Introduction

Under the name of carbonic anhydrase Meldrum & Roughton [1933][2] described a new enzyme present in red blood corpuscles which catalyses both phases of the reversible reaction: $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. They purified this enzyme from mammalian erythrocytes and gave a detailed account of its properties and the kinetics of the reactions which it catalyses. The greatly purified enzyme preparation obtained by them had a very high catalytic activity, was a colourless substance free from haematin and from other known enzymes, stable within *p*H 3–12, thermolabile, and very sensitive to KCN, $H_2S$, $NaN_3$ and to several heavy metals. The amount of the purified enzyme obtained by these workers was, however, very small and hardly sufficient to recognize it with certainty as a protein compound. The true nature of this enzyme and especially of its active group remained unknown until very recently.

While studying the metallo-protein compounds present in the red blood corpuscles we have found that the fractions of our preparations left after complete removal of haemocuprein [Mann & Keilin, 1938] had a very high content of Zn and a high carbonic anhydrase activity. By adapting our methods of purification to preparations on a larger scale, taking special care to increase the yield of the Zn fraction, we have obtained from the red blood corpuscles of the ox a highly active preparation of carbonic anhydrase as a colourless protein containing 0·31–0·34 % Zn. These results, together with some other considerations, have led us to the conclusion, put forward in a preliminary communication [Keilin & Mann, 1939], that the carbonic anhydrase is a Zn-protein compound.

In this paper we propose to give a detailed account of the methods of purification of this enzyme and to bring forward fresh evidence of its Zn-protein nature.

## II. Methods of identification and estimation of Zn

### A. *Qualitative tests*

In purified preparations of carbonic anhydrase Zn was identified by the following three tests:

(1) *As Zn quinaldinate*, $(C_{10}H_6NO_2)_2Zn, H_2O$. 25 mg. pure carbonic anhydrase in 5 ml. water are treated in a small centrifuge tube with 2 ml. 20 % trichloroacetic acid; after 15 min. standing the precipitate is centrifuged off and washed with dilute trichloroacetic acid. The combined clear, protein-free extracts are adjusted with ammonia to *p*H 6 and treated with one drop of concentrated acetic acid and

[1] Beit Memorial Research Fellow.

[2] An interesting historical account of the work by other investigators which led Meldrum & Roughton to the discovery of this enzyme can be found in their paper [1933] and in the two excellent reviews of the whole subject by Roughton [1934, and especially 1935].

0·2 ml. 1 % solution of sodium quinaldinate. The crystallization of Zn quinaldinate begins almost immediately and is completed within a day.

(2) *As Zn mercury thiocyanate*, Zn $Hg(SCN)_4$ [Jamieson, 1918]. The organic matter of 120 mg. pure carbonic anhydrase is destroyed by heating with 2 ml. conc. $H_2SO_4$ in a Kjeldahl flask over a small flame. The water-clear solution is diluted to 12 ml. with water and treated with 2 ml. Jamieson's reagent (ammonium mercury thiocyanate). The crystallization of zinc mercury thiocyanate begins immediately and is completed within an hour.

(3) *As "Rinmann's green"*. The crystalline Zn mercury thiocyanate precipitate obtained as described above is centrifuged off and suspended in 0·3 ml. 10 % $HNO_3$; two drops of the mixture are deposited on a small strip of potassium cobalticyanide paper [Feigl, 1937] which is dried on a platinum plate and ashed over a gas flame. The green colour of the ash which appears immediately is due to the formation of mixed crystals of zinc oxide and cobaltous oxide.

### B. *Quantitative estimations*

For quantitative estimation of Zn in blood and different fractions of carbonic anhydrase preparation two methods have been used:

(1) *Formation of Zn-diphenylthiocarbazone complex salt* or the red Zn-"dithizone" [Schwaibold *et al.* 1938]. Not less than 10 μg. and not more than 100 μg. Zn were used for a single determination. All glass containers must be carefully washed with sulphuric-dichromate mixture followed by HCl and rinsed in glass-distilled water. The reagents must be Zn-free, especially the sodium tartrate and dithizone. The solution of dithizone in $CCl_4$ is washed with dilute $H_2SO_4$ followed by several washings with glass-distilled water. The blank should not contain more than 2 μg. Zn. The samples for estimation are usually incinerated with $H_2SO_4$ in a Kjeldahl flask heated over a small flame. Samples containing a large amount of protein, as is the case with red blood corpuscles or very crude preparations, are evaporated to dryness and ashed for 5 hr. in an electric muffle.

Example of estimation: 2 ml. solution of carbonic anhydrase containing a known dry weight are incinerated with 1 ml. of pure Zn-free $H_2SO_4$. The colourless acid solution thus obtained is diluted with 50 ml. glass-distilled water and transferred quantitatively into a separating funnel. To make sure that the solution is free from copper, which combines with dithizone at acid *p*H, the solution is shaken with a few ml. of 0·006 % dithizone in $CCl_4$. The presence of Cu is revealed by the change in colour of dithizone from green to purple. If such change takes place successive portions of dithizone are added to the acid solution until the colour of dithizone remains unchanged. With a pure or highly purified sample of carbonic anhydrase the addition of dithizone at acid *p*H is unnecessary. The solution in the separating funnel is then treated with 3 ml. 20% sodium tartrate and rendered alkaline with ammonia, a 5 % solution of which is added slowly until a drop of the solution mixed on a spot-test plate with a drop of thymol blue gives a bluish colour. The solution is then shaken with a small amount of 0·006 % dithizone in $CCl_4$ which turns red or purplish red. The treatment is repeated several times with small portions of dithizone until the latter remains distinctly bluish-green in colour. The combined red Zn-dithizone fractions are diluted with $CCl_4$ to a definite amount, transferred into a clean separating funnel and washed several times with small portions of a freshly prepared 0·04 % solution of $Na_2S$ which turns yellow and the washing is continued until the solution of sodium sulphide is left colourless. The red Zn-dithizone solution is then compared colorimetrically with a standard solution of Zn treated with dithizone in a

similar way. It is important to note that the Zn-dithizone solution should not be exposed to light more than necessary as it changes colour from red to brown.

(2) *Formation of potassium-Zn-ferrocyanide* estimated iodimetrically [Sahyun & Feldkamp, 1936]. This method of estimation requires, however, a much larger amount of Zn than that with dithizone and was only used with some of the fractions at the earlier stages of purification.

## III. Estimation of catalytic activity of carbonic anhydrase

The activity of carbonic anhydrase in different preparations was estimated by two methods.

(1) *Manometric method* with the aid of the boat apparatus of Brinkman *et al.* [1933] based on the rate of evolution of $CO_2$ from sodium bicarbonate solution when mixed with phosphate buffer at *p*H 6·8.

In this method one side of the glass boat receives 2 ml. of phosphate solution prepared by mixing equal volumes of $M/5$ $Na_2HPO_4$ and $M/5$ $KH_2PO_4$, whilst in the other side are placed 2 ml. of $M/5$ $NaHCO_3$ dissolved in $M/50$ NaOH. In catalysed reaction a known amount of enzyme preparation is added to the side of the boat containing the phosphate solution. The experiments are carried out as described by Meldrum & Roughton [1933]. We have adopted also their definition of enzyme unit (E.U.), namely as the "amount of enzyme which, when dissolved in 4 ml. of the phosphate bicarbonate mixture used above, gives a value of $(R-Ro)/Ro$ equal to 1 at 15°" where $Ro$ and $R$ are respectively, the rates of reaction in the absence and in the presence of the catalyst.

The activity ($A$) of an enzyme preparation is expressed as the number of enzyme units (E.U.) per 1 mg. of dry weight of the preparation. The greater is this activity the smaller is the dry weight per enzyme unit. Thus, the enzyme preparation of which $A = 2000$ has 0·5$\mu$g. dry weight per enzyme unit which can be expressed as 0·5$\mu$g./E.U.

(2) *Colorimetric method* of Philpot & Philpot [1936] slightly modified. 10 ml. 0·00263$M$ solution of $NaHCO_3$ with a few drops of phenol red are put into a wide reaction tube kept at 0°. The current of $CO_2$ is bubbled through the solution and a few minutes later, while the current is still on, 2 ml. of 0·3$M$ $Na_2CO_3$ containing 0·206$M$ $NaHCO_3$ are added to the mixture. The reaction which is required to change the colour of phenol red from purplish red to pale yellow is carefully timed and is found to be from 65 to 75 sec. in uncatalysed reaction. The addition of carbonic anhydrase shortens this time and an enzyme unit (E.U.) by this method of estimation is defined as the amount of enzyme which is required to shorten the time of the reaction from 65–75 sec. to 25–30 sec. The enzyme unit determined in this way is about ten times larger than the unit of Meldrum & Roughton. The colorimetric method requires less manipulation and is much more rapid than the manometric method. It was found more convenient for quick estimations of enzyme activities and was therefore used as the main routine method for testing different fractions during the purification of the enzyme. The final values for the activities ($A$) of all the enzyme fractions described in this paper were estimated by Meldrum & Roughton's method.

## IV. Material and general lines of purification

The highest concentration of carbonic anhydrase in nature is found in the red blood corpuscles [Meldrum & Roughton, 1933] and in the mucous membrane of the stomach [Davenport, 1939] of mammals.[1]

[1] The distribution of this enzyme in different animals, vertebrates and invertebrates was recently reviewed by Goor [1940].

The purification of the enzyme from erythrocytes of ox blood was carried out by two different methods which can be applied with the same success to the blood of sheep and with somewhat less success to blood of man and other mammals.

In both methods of purification the first step consists in treating the washed and plasmolysed red blood corpuscles with a mixture of alcohol and chloroform [according to Tsuchihashi, 1923] which denatures and precipitates the whole of the haemoglobin, leaving a clear yellow extract containing the enzyme. The similarity of these two methods does not go beyond this first step. The next step of purification in one method (I) consists in precipitation of the alcohol-chloroform extract, with lead acetate, elution of the precipitate with alkaline phosphate and further purification with $Ca_3(PO_4)_2$ and alumina $C\gamma$. In the second method (II) the alcohol-chloroform extract is dialysed, precipitated with ammonium sulphate redissolved in $H_2O$ and fractionated with ammonium sulphate and alumina $C\gamma$.

The main advantages of method I consist in omitting the dialysis of a very large bulk of alcohol-chloroform extract and in securing a larger variety of enzyme fractions together with the fraction containing the haemocuprein. The defect of the method is in its comparatively smaller yield of the purified enzyme.

The advantages of method II are in its shortness and the greater yield of the final product. Its main defect is the necessity to dialyse a large amount of alcohol-chloroform extract.

The preparation of the enzyme from sources other than erythrocytes was carried out only from the mucous membrane of pig's stomach. The method used in this purification is, however, entirely different from the previous two methods applied to blood and will be described later on.

## V. Purification of carbonic anhydrase from red blood corpuscles of the ox

### A. *Method I*

(1) *Washing and plasmolysis of erythrocytes.* The red blood corpuscles of 7 l. of defibrinated ox blood are separated from serum and washed with an equal volume of 0·9 % NaCl. The washed erythrocytes are plasmolysed with an almost equal amount of distilled water which brings the volume of the solution to 7 l. This solution contains 0·14 g. dry weight per ml. and 0·0005 ml. contains 1 E.U.; 70 $\mu$g./E.U.; $A = \frac{1000}{70} = 14{\cdot}3$; 7 l. contain 14,000,000 E.U.; 0·0024 % Zn. Zn present in this solution is not dialysable but can easily be liberated by trichloroacetic acid.

(2) *Extraction with alcohol and chloroform.* The solution of 7 l. of laked erythrocytes is cooled in ice and treated with ice-cold mixture of alcohol and chloroform [Tsuchihashi, 1923]. The best results are obtained by treating it in two or three successive lots in the following way: 2 l. of laked corpuscles are mixed with 1·8 l. 90 % alcohol and 130 ml. chloroform. The mixture, which is made in a wide cylindrical jar, is rapidly stirred with a thick glass rod until the denatured haemoglobin sets into a large spongy mass which can be removed from the rest of the solution, pressed out and put aside for further washing. The fluid is mixed with kieselguhr and filtered on a large Büchner funnel (30 cm. diameter) through a filter paper covered with kieselguhr.

The residual cakes from the successive lots are collected, broken into small pieces, washed with about 2 l. of water and filtered in the same way.

The joint filtrates consist of 12 l. of clear yellowish fluid containing 2·5 mg. dry weight per ml.; 7·6 $\mu$g./E.U.; $A = 133$; 3,947,000 E.U.; 0·027 % Zn. The yield at this stage varies from 26 to 46 %.

(3) *Precipitation with lead acetate.* 12 l. of cooled alcohol-chloroform solution are treated with saturated solution of lead acetate until no further precipitate is formed by the addition of a drop of this reagent to a few ml. of the filtrate. Usually, 25–35 ml. of saturated lead acetate per l. are required for this purpose. After 10–15 min. standing, the precipitate is centrifuged off, suspended in about 300 ml. of $M/5$ $K_2HPO_4$ and after 2–3 hr. standing again centrifuged. The brown fluid collected is dialysed for 24 hr. against distilled water, giving 750 ml. of brownish fluid.

(4) *Purification with $Ca_3(PO_4)_2$.* 750 ml. of the above fluid are mixed with 2 g. of $Ca_3(PO_4)_2$ gel, acidified to $p$H 6·8 and after 10 min. standing filtered through Chardin folded filter. The clear and faintly coloured fluid obtained has 2·13 mg. dry weight per ml.; 2$\mu$g./E.U.; $A=500$; 800,000 E.U.; 0·12 % Zn.

(5) *Fractional adsorption on alumina $C\gamma$.* The above fluid was cooled and mixed with 35 ml. of alumina $C\gamma$ gel containing 30 mg. dry substance per ml. The alumina gel is centrifuged off, giving a yellow-green cake $A$. The supernatant fluid is mixed with another 35 ml. of alumina gel, adjusted to $p$H 7 and the gel centrifuged off, giving a bluish green cake $B$. To the supernatant colourless fluid is added cooled alcohol, bringing its concentration to 30 % and 30 ml. of alumina gel. The mixture is adjusted to $p$H 6·7 and the gel centrifuged off, giving the colourless cake $C$. The supernatant fluid which is completely inactive is discarded.

(6) *Elution of alumina $C\gamma$ gels.* Each of the above three alumina gel cakes is eluted twice with $M/10$ alkaline phosphate, the first time with 40 ml. and the second time with 20 ml. The combined elutions of each cake are dialysed separately against distilled water, giving three phosphate-free clear solutions with the following properties:

$A$. 60 ml.; 6·6 mg. dry weight per ml.; 5$\mu$g./E.U. $A=200$; 79,200 E.U.

$B$. 60 ml.; 4·7 mg. dry weight per ml.; 1·2$\mu$g./E.U. $A=833$; 235,000 E.U.; 0·15–0·16 % Zn.

$C$. 60 ml.; 3·8 mg. dry weight per ml.; 0·6$\mu$g./E.U. $A=1666$; 380,000 E.U.; 0·264 % Zn.

Of these fractions, $A$ and $B$ contain large concentrations of copper (0·04–0·1 % Cu or more) and are therefore suitable for the isolation of haemocuprein, giving 50–80 mg. of this compound in the crystalline state. Fraction $C$, which contains only traces of Cu but is rich in Zn, is most suitable for further purification of carbonic anhydrase.

(7) *Further purification on alumina $C\gamma$.* Fraction $C$ was treated once more with alumina $C\gamma$ added in sufficient amount to adsorb the whole enzyme. The gel is centrifuged off, eluted with alkaline phosphate and dialysed. This step gave 150 mg. of the final product with the following properties: 0·45$\mu$g./E.U.; $A=2222$; 333,000 E.U.; 0·31–0·33 % Zn. The nitrogen content of this preparation was 14·95 %.

### B. *Method II*

(1) *Extraction with alcohol and chloroform.* The red blood corpuscles of 2 l. of defibrinated ox blood are washed as in the previous method. 1150 ml. of corpuscles collected are plasmolysed with 575 ml. distilled water, cooled and treated with 575 ml. alcohol and 575 ml. chloroform, both previously cooled in ice. The mixture is carefully stirred, left standing for 15 min. and centrifuged. The cake of denatured protein is discarded and the fluid filtered through a Chardin filter.

(2) *Dialysis of alcohol-chloroform extract.* The collected clear fluid is dialysed for 24 hr. against running water giving 2250 ml. of clear yellowish solution which has 1·72 mg. dry weight per ml.; 2·6$\mu$g./E.U.; $A=384$; 1,488,000 E.U.

(3) *Total precipitation with ammonium sulphate.* The above fluid is saturated with ammonium sulphate (750 g./l.), the precipitate is collected on a Büchner filter, suspended in a small amount of water, dialysed against running water until free from ammonium sulphate and centrifuged. This gives 110 ml. of brown opalescent fluid containing 17 mg. dry weight per ml.; 1·7 μg./E.U.; $A=588$; 1,100,000 E.U.

(4) *Fractional precipitation with ammonium sulphate.* The fluid is mixed with 31 g. ammonium sulphate giving 45 % saturation. The precipitate (*a*) is filtered off, the filtrate is saturated with ammonium sulphate and filtered again. The second precipitate (*b*) is collected while the filtrate is discarded.

Both precipitates are dissolved in water and dialysed, giving two solutions:

(*a*) 55 ml. of brown and opalescent solution containing 275,000 E.U.

(*b*) 85 ml. of clear and slightly coloured solution containing 9 mg. dry weight per ml.; 1–1·2 μg./E.U.; $A=830$–1000; 765,000 E.U.; 0·15–0·17 % Zn.

(5) *Purification with alumina Cγ gel.* The fraction (*b*), which is still slightly coloured, is treated three times with successive portions of 5 ml. (= 100 mg.) of alumina Cγ at *p*H 6·8 and centrifuged, the cakes being discarded each time. The solution becomes completely colourless although it contains most of the enzyme.

(6) *Fractional precipitation with ammonium sulphate.* The colourless solution is treated with ammonium sulphate to 50 % saturation and the precipitate is filtered off and discarded. The filtrate is saturated with ammonium sulphate, the precipitate collected, dissolved in water and dialysed against distilled water until completely free from salt. 50 ml. of the fluid collected have 4·5 mg. dry weight per ml.; 0·45 μg./E.U.; $A=2220$; 500,000 E.U.; 0·33 % Zn. 2 l. of ox blood by this method give therefore 225 mg. of this highly purified enzyme preparation. The yield of this fraction from 2 l. of ox blood in other preparations varied from 96 to 415 mg.

## VI. Other preparations of carbonic anhydrase from erythrocytes

Other preparations of carbonic anhydrase from ox blood gave the fractions with the following activities and Zn contents:

(1) $A=1000$; 0·162 % Zn;
(2) $A=1375$; 0·2 % Zn;
(3) $A=1825$; 0·295 % Zn.

As regards other animals the best results were obtained with the red blood corpuscles of sheep which give, if anything, a better yield than the erythrocytes of the ox and a final preparation which had 0·42 μg./E.U.; $A=2380$; 0·34 % Zn.

Attempts at the purification of carbonic anhydrase from the red blood corpuscles of man gave so far a preparation containing only 0·164 % Zn which corresponds to about 50 % of our purest enzyme.

## VII. Purification of carbonic anhydrase from gastric mucosa

It was recently shown by Davenport [1939] that the parietal cells of the gastric mucosa of cats and rats are very rich in carbonic anhydrase which probably plays an important role in the mechanism of formation of HCl. This discovery made possible an attempt at the purification of carbonic anhydrase from a new and unexpected source, thus checking some of our results obtained from the study of the enzyme isolated from erythrocytes. After a rapid survey of the gastric mucosa of different animals we found that by far the best material for

isolation of this enzyme is presented by the mucosa of the pig's stomach. The greatest difficulty in handling such a material is due to the presence of a very large amount of mucus which prevents in this case the application of methods used for isolation of carbonic anhydrase from erythrocytes. We had, therefore, to devise for this material an entirely different method which will be described presently.

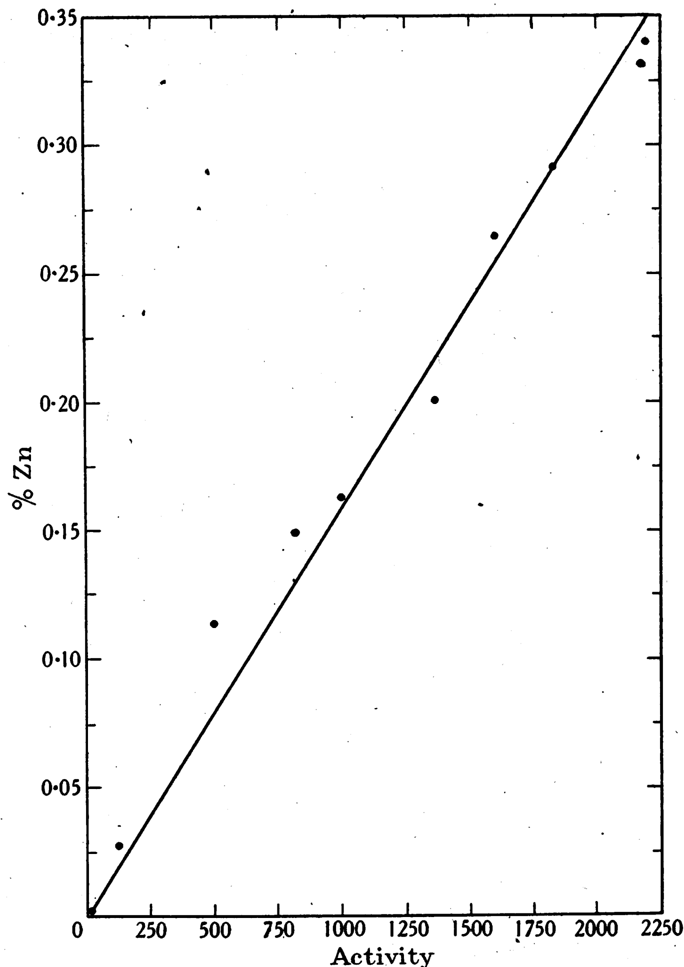


Fig. 1. Relationship between activities (as defined by Meldrum & Roughton) and Zn contents of different fractions of carbonic anhydrase obtained from blood.

(1) *Extraction of enzyme from gastric mucosa.* The stomachs of five pigs are split open and the mucosa is separated from the rest of the tissue by means of sharp scalpels. The mucous membranes thus obtained are minced, giving 1 kg. of wet tissue which is mixed with sand and 150 g. $Na_2SO_4$, $10H_2O$ and ground in a mechanical mortar for 15 min. The thick mass is mixed with 2 l. of 15% sodium sulphate and the grinding continued for another 30 min. The fluid collected is centrifuged, sand and coarse particles of tissue are discarded, leaving 2 l. of turbid

solution containing 500,000 E.U. of carbonic anhydrase. From the haemoglobin content of this solution we could easily calculate that the amount of enzyme in this solution which derives from the erythrocytes represents less than 3750 E.U. —in other words, about 0·75 % of the total enzyme content.

(2) *Treatment with barium acetate.* The above solution is treated with 30 % barium acetate until all sulphate is precipitated; the precipitate is centrifuged off, leaving 2 l. of a pink, clear fluid containing 400,000 E.U.

(3) *Fractional precipitation with acetone.* 2 l. of cold solution are mixed with 1 l. of cold acetone and 10 ml. 50 % $CaCl_2$. The precipitate is centrifuged off, while the supernatant fluid is treated with 2 l. of cold acetone. The precipitate is collected and dissolved in 500 ml. of water, giving a distinctly pink solution which contains 11 mg. dry weight per ml.; 18 $\mu$g./E.U.; $A = 55$; 305,000 E.U.

This solution is completely free from haemoglobin and its strong pink colour is due entirely to cytochrome *c*.

(4) *Adsorption on tricalcium phosphate.* The solution is mixed with 2 g. tricalcium phosphate gel adjusted to *p*H 6·8 and centrifuged. The cake is eluted with *M*/10 alkaline phosphate and dialysed, giving 360 ml. of clear solution containing 2·2 mg. dry weight per ml.; 7·1 $\mu$g./E.U.; $A = 141$; 111,500 E.U.

(5) *Fractional adsorption on alumina C$\gamma$.* The above solution is mixed with 20 ml. alumina C$\gamma$, adjusted to *p*H 7·2, the gel is centrifuged off and discarded, while the supernatant pinkish yellow fluid is mixed with 60 ml. alumina gel adjusted to *p*H 6·8 and centrifuged. The gel is collected, eluted with *M*/10 alkaline phosphate and dialysed, giving 160 ml. of solution containing 2·6 mg. dry weight per ml.; 4 $\mu$g./E.U.; $A = 250$; 104,000 E.U.

(6) *Precipitation with acetone.* The above solution is cooled in ice and treated with two volumes of cold acetone. The precipitate is redissolved in 17 ml. water, giving a clear solution containing 7·5 mg. dry weight per ml.; 1·5 $\mu$g./E.U.; $A = 667$; 85,000 E.U.; 0·12–0·13 % Zn.

Further purification of the enzyme from this preparation was not successful although this stage was reached several times. This difficulty may be partly due to some impurities which cannot easily be removed, partly to the fragility of the enzyme caused by the initial stages of purification.

If we consider our best preparation of carbonic anhydrase from erythrocytes as a pure enzyme the preparation obtained from gastric mucosa will be only 30 % pure. It shows, nevertheless, the corresponding high concentration of Zn, and this gives additional evidence supporting the view that carbonic anhydrase is a Zn-protein compound.

## VIII. Tests for other metals in preparations of carbonic anhydrase

In addition to Zn the concentration of several other metals was estimated in various fractions of carbonic anhydrase and this gave the following results:

*Magnesium* was estimated by the titan yellow method. 80 mg. of enzyme showing 0·57 $\mu$g./E.U. and $A = 1750$ were found to contain only 0·006 % Mg which indicates that it is present only as an impurity.

*Manganese*, estimated colorimetrically by the permanganate method in a sample of 30 mg. of the same enzyme preparation, was found to be present only in traces.

*Iron*, estimated by $\alpha\alpha'$-dipyridyl in preparations of different activities, gave the results summarized in Table 1.

Table 1

| Preparations | Activity | % Fe |
|---|---|---|
| I | 250 | 0·007 |
| II | 1000 | 0·005 |
| III | 2250 | 0·003 |

These results show clearly that the very small amounts of Fe found in different preparations can only be considered as impurities.

*Copper*. Very interesting results were obtained with the estimation of copper in different fractions of carbonic anhydrase preparations. As is shown in Fig. 2,

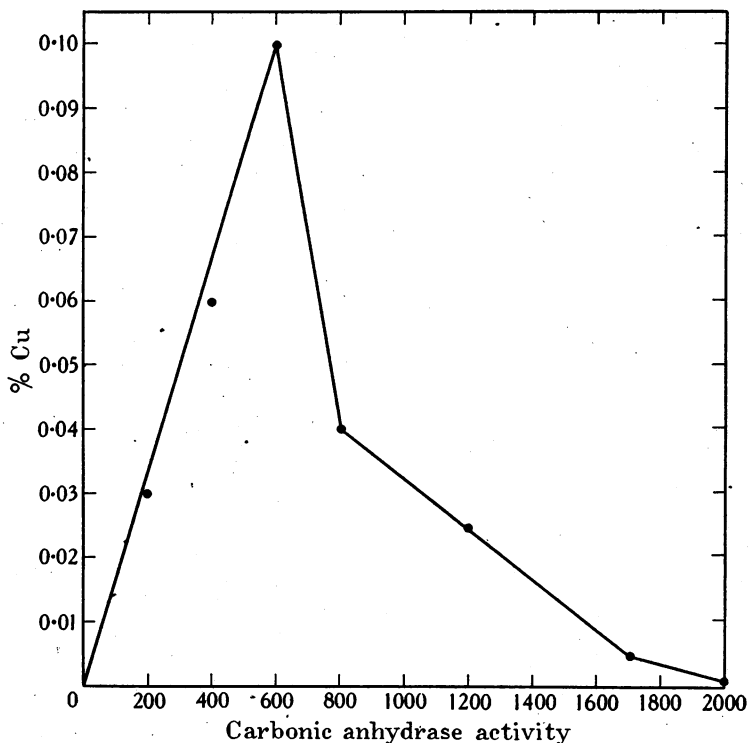


Fig. 2. Relationship between activities and Cu contents of different fractions of carbonic anhydrase.

the concentration of Cu rises gradually with the increase in the activity of the enzyme, reaching almost 0·1% and then suddenly drops almost to vanishing point. The initial increase in Cu concentration is due to haemocuprein which accompanies carbonic anhydrase during the first few steps of purification. A point is, however, reached when haemocuprein is almost quantitatively separated from carbonic anhydrase and this point is indicated by the sudden drop in Cu concentration in the fraction containing carbonic anhydrase. This observation may serve as a warning against any rash conclusion as to the nature of an enzyme based on a parallelism between the activities and concentrations of one of the substances found in some fractions during the first steps of purification.

*Lead*. 35 mg. of pure enzyme tested with the dithizone method was found to be completely free of lead.

The above tests show that purified carbonic anhydrase which contains about 0·33 % Zn is either devoid of other metals such as Fe, Cu, Mn, Pb and Mg or contains them as impurities in very low concentrations.

## IX. Some properties of carbonic anhydrase

### A. *Effect of inhibitors*

It was already shown by Meldrum & Roughton [1933] that carbonic anhydrase is strongly inhibited by KCN, $H_2S$ and $NaN_3$[1]. We have confirmed this observation and found that a concentration of cyanide even as low as $4 \times 10^{-6}M$ inhibits carbonic anhydrase to the extent of almost 85 %. The inhibitory effect of cyanide in spite of being so very marked is completely reversible. For instance, 37 mg. of half-purified enzyme preparation ($A = 1100$) in 10 ml. water are mixed with 1 ml. $M$ KCN at $p$H 6·5 and after standing 30 min. at room temperature the mixture is saturated with ammonium sulphate, the precipitate centrifuged off, dissolved in water and dialysed. The preparation after this treatment recovers at least 90 % of its original activity.

### B. *Stability of the enzyme*

Enzyme preparations when purified keep well in solution. It is, however, important to note that when the enzyme is inactivated by long standing or by various treatments Zn remains bound to the protein and cannot be removed by dialysis. While the separation of Zn from enzyme protein by acids irreversibly destroys its activity, the inactivation of the enzyme does not necessarily liberate the metal. This shows that the surplus of Zn found in certain fractions of enzyme preparations is due to the presence in these fractions of a certain amount of inactivated enzyme which still retains its complete share of Zn. The activities of these fractions when plotted against their Zn contents always give points lying above the line of strict proportionality (see Fig. 1).

## X. Evidence of Zn-protein nature of carbonic anhydrase

The evidence upon which our main conclusion is based, namely that carbonic anhydrase is a Zn-protein compound, can be summarized as follows:

(1) The facts that the enzyme is very strongly inhibited by KCN, $H_2S$ and $NaN_3$, that this inhibition is instantaneous and perfectly reversible, clearly indicate that the active group of the enzyme must be a metal.

(2) The highly purified enzyme preparation contains 0·31–0·33 % Zn. Such concentration of metal in a protein is already of the same magnitude as that of Fe in haemoglobin or of Cu in polyphenol oxidase, haemocyanin or in crystalline haemocuprein.

(3) There is a distinct proportionality between the activities of enzyme preparations and their Zn contents (Fig. 1).

(4) Purification of carbonic anhydrase by two entirely different methods gives final products of the same high activity and the same Zn content.

(5) Enzyme preparations obtained from two very different sources such as the red blood corpuscles and the gastric mucosa have Zn contents corresponding to their catalytic activities.

[1] Since this paper went to press we have been able to demonstrate (Mann & Keilin [1940], *Nature, Lond.*, **146**, 164) that carbonic anhydrase is strongly and reversibly inhibited by sulphanilamide and some other sulphonamide compounds. These inhibitors, unlike KCN, $H_2S$ and $NaN_3$, are highly specific and therefore will render it possible to determine experimentally the function of carbonic anhydrase in respiration and in the secretion of HCl by gastric mucosa.

(6) Other metals which are known to occur in biological material, such as Fe, Cu, Mn, Pb, Mg and others, are either absent from the highly purified enzyme preparation, or found only in negligible concentrations.

Zn in carbonic anhydrase forms therefore an active part of its prosthetic group stoichiometrically combined with the protein. That this Zn does not derive from outside as a result of a selective accumulation of this metal during purification of the enzyme is shown by the fact that several other proteins (serum- and egg-albumin, haemoglobin, haemocuprein and laccase) submitted to somewhat similar purifications have been found to be either free from Zn or to contain less of it than their crude preparations.

It is important to note that carbonic anhydrase is the only Zn compound which has characteristic catalytic properties. Other substances such as inorganic and simple organic Zn compounds, Zn-porphyrin, crystalline Zn-insulin and Zn-protein compounds of serum and different tissues are completely devoid of this catalytic property. This is not surprising considering that other metals such as Fe and Cu form with different proteins compounds either acting as carriers of molecular oxygen or capable of catalysing a series of completely different reactions.

## XI. Degree of purity of the final product

The best fraction of carbonic anhydrase obtained by Meldrum & Roughton [1933] was already a highly active preparation corresponding probably to about 65–75 % pure enzyme.

The claim by Kiese & Hastings [1940] to have obtained an enzyme preparation which is several times more active than the above preparation cannot, therefore, be accepted. In fact, the high activity values obtained by these workers are misleading and probably due to the low temperature (5 instead of 15°) at which their estimations were carried out. Considering that the temperature coefficient of the non-catalysed reaction is much higher than that of the catalysed reaction [Meldrum & Roughton, 1933] the relative activities of enzyme preparations can be compared only when determined at the same temperature. In fact, while at 15° about 0·45 $\mu$g. of our purest enzyme preparation doubled the velocity of the non-catalysed reaction, at 5° this effect is already obtained by 0·12 $\mu$g. of the same preparation.

If, however, the activity values are obtained at different temperatures, they should be supplemented by values for the catalytic activity of the plasmolysed red blood corpuscles, obtained under exactly the same conditions. The ratio between these values determines independently of the temperature the stage of purification reached by the enzyme preparation.

Such data show for instance that the activity of our purest enzyme preparation is only about 150 times higher than that of the red blood corpuscles, which suggests that the concentration of the enzyme in the red blood corpuscles is very high.

It is difficult to determine with certainty the degree of purity of our most active enzyme preparation. Several considerations, however, indicate that our final product is either pure or almost pure enzyme. Thus, the purification of the enzyme from different sources and by different methods gives the same final product of which neither the activity nor Zn content could be increased by any further attempt at purification. The high Zn content of the final product leaves very little room for the presence of another protein not combined with Zn. Finally, the cataphoretic experiments in the Tiselius apparatus carried out at the Low Temperature Research Station (see Appendix, p. 1176) show that our

purified preparation behaves like a homogeneous protein. The examination of different fractions collected after these experiments reveals, moreover, a distinct proportionality between their activities and their nitrogen and Zn contents.

The experiments in an ultracentrifuge carried out by Dr F. Eirich of the Colloid Science Laboratory, Cambridge, show that the enzyme is of approximately half the size of a haemoglobin molecule.[1] This would indicate that the protein in each molecule of enzyme is combined with two atoms of Zn.

## XII. The role of Zn in organisms

The presence of Zn in tissues of plants and animals was discovered by Lechartier & Bellamy [1877]. Since this discovery a large amount of work has been done on the distribution, localization and estimation of Zn in tissues of different organisms under normal, experimental and pathological conditions. It is now well established that Zn is a true and general microconstituent of living organisms. Its concentration in higher animals has a constant value for each organism and for each of its tissues[2] and this value is not much affected even by a marked change in the amount of Zn taken with food. Although the presence of Zn in living organisms has been known for more than 60 years there was no indication as to its biological significance, nor even a suspicion as to its possible connexion with a definite physiological function of an organism.

In fact Eichholtz [1934], after reviewing the literature on Zn in connexion with living organisms, concluded that "we do not know of any substance in organisms of warm-blooded animals the biological significance of which is due to the presence of zinc, and we do not know of any physiological function which requires the co-operation of zinc."

The discovery of Zn in carbonic anhydrase thus establishes for the first time the physiological function of this metal in organisms.

Iron, copper and zinc are three widely distributed metals, the biological significance of which is now definitely established.

The fact that the distribution of Zn in nature is much wider than that of carbonic anhydrase shows only that Zn, like iron and copper, forms with proteins several compounds having different properties and functions.

## XIII. Concentration of three metallo-protein compounds in erythrocytes

The comparison of the catalytic activities and Zn contents in the red blood corpuscle and in the highly purified enzyme shows that probably the whole of the Zn present in the red blood corpuscles belongs to carbonic anhydrase (Table 2).

Table 2

| | Pure enzyme *a* | Red blood corpuscle *b* | *a/b* |
|---|---|---|---|
| Activity | 2220 | 14·3 | 154 |
| % Zn | 0·33 | 0·00245 | 137 |

It is therefore possible to determine the concentration of carbonic anhydrase in the red blood corpuscles and to compare it with those of the other two metallic constituents of erythrocytes. The results thus obtained, summarized in Table 3,

[1] The detailed account of these experiments will be published separately.

[2] The data concerning the distribution of Zn in the red blood corpuscles and plasma in man and higher animals are given in a paper by Burstein [1929].

Table 3

| Constituents of the red blood corpuscles | Amount in g. (dry wt.) per 100 ml. corpuscles | Metal in the compound % |
|---|---|---|
| Haemoglobin | 28·00 | 0·34 Fe |
| Carbonic anhydrase | 0·21 | 0·33 Zn |
| Haemocuprein | 0·06 | 0·34 Cu |

show that the concentration of carbonic anhydrase in the red blood corpuscles is three to four times higher than that of haemocuprein and about 133 times lower than that of haemoglobin. One litre of mammalian blood contains approximately 1 g. of carbonic anhydrase.


## Summary

1. Carbonic anhydrase is an enzyme discovered by Meldrum & Roughton [1933] in the red blood corpuscles of mammals, which catalyses both phases of the reaction $H_2CO_4 \rightleftharpoons CO_2 + H_2O$. In mammals high concentration of this enzyme is also found in the gastric mucosa [Davenport, 1939].

2. Methods are described for purification and isolation of this enzyme from the red blood corpuscles and the gastric mucosa of mammals.

3. The purest enzyme preparation obtained from erythrocytes of ox and sheep by two distinct methods is a colourless protein containing 14·95 % N and about 0·33 % Zn. It is devoid of haematin, Fe, Cu, Mn, Mg and Pb. Evidence is brought forward that this preparation is either pure or almost pure enzyme. Its catalytic activity is about 150 times higher than that of the red blood corpuscles.

4. The maximum yield of this purified product is about 200 mg./l. of ox blood.

5. That carbonic anhydrase is a Zn protein compound where Zn forms the active part of the enzyme molecule is strongly supported by the following considerations:

(a) An immediate, strong and completely reversible inhibition of this enzyme by small concentrations of KCN, $H_2S$ and $NaN_3$ which are known to react in this way only with metals.

(b) The presence of 0·33 % Zn in the purest preparations obtained from erythrocytes of ox or sheep by two very different methods.

(c) The presence of Zn in carbonic anhydrase obtained from gastric mucosa.

(d) The absence of other metals from purified enzyme preparation.

(e) The proportionality between the enzyme activity and Zn content of different fractions obtained from erythrocytes and gastric mucosa.

6. The concentration of carbonic anhydrase in red blood corpuscles is very high. 100 ml. of erythrocytes contain about 0·21 g. of the purified product, which is three to four times higher than the concentration of haemocuprein and about 133 times lower than that of haemoglobin.

7. The presence of Zn in carbonic anhydrase establishes for the first time the physiological function of this metal in organisms.


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## APPENDIX

## CATAPHORETIC BEHAVIOUR OF CARBONIC ANHYDRASE

By E. C. Bate Smith

*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research, Cambridge*

A preparation of purified carbonic anhydrase was submitted by Prof. Keilin and Dr Mann for examination in the Tiselius apparatus. The solution, containing 0·5 % of protein (0·33 % of Zn, 0·45 μg./E.U., $A = 2222$) was dialysed for 18 hr. against 0·05$M$ phosphate buffer, $p$H 7·0. In the cataphoresis cell the preparation showed a *single* boundary moving at a velocity of $-1{\cdot}9 \times 10^{-5}$ cm./sec./volt/cm. Dialysed against buffer at $p$H 8·0 the velocity was $-2{\cdot}2 \times 10^{-5}$.

A second preparation (0·55 μg./E.U., $A = 1820$) was examined at $p$H 5·8. This was less pure than the first preparation and, in fact, showed two boundaries. The main boundary, corresponding to the carbonic anhydrase component, moved at this $p$H with a velocity of $-0{\cdot}85 \times 10^{-5}$ cm./sec./volt/cm. Extrapolating these values to zero velocity, the enzyme would appear to be isoelectric in the neighbourhood of $p$H 5, confirming the observation of Kiese & Hastings [1939].