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Some Observations on the Zinc in Carbonic Anhydrase

BY R. TUPPER, R. W. E. WATTS AND A. WORMALL

Departments of Biochemistry and Physiology, Medical College of St Bartholomew's Hospital, London

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Keilin & Mann (1939, 1940) showed that carbonic anhydrase is a zinc protein and this has been confirmed by many authors (Hove, Elvehjem & Hart, 1940; Scott & Fisher, 1942, 1944; see also the reviews by Vallee & Altschule, 1949*a, b*). The nature of the linkage between the metal and the protein is unknown (cf. the review by Roughton & Clark, 1951), but the zinc is undoubtedly firmly bound and is not removed by dialysis, even when the enzyme has become inactivated by long standing (Keilin & Mann, 1940).

Red blood cells and leucocytes can readily be labelled *in vitro* with ^{65}Zn by adding a solution of a ^{65}Zn -containing zinc-glycine complex to the cell suspension and keeping the mixture at 37° and pH 7.4 for 2 hr. (Tupper, Watts & Wormal, 1951). A further study of the distribution of the ^{65}Zn in these labelled red cells has shown that, although about 94% of the ^{65}Zn which has become fairly firmly attached to the cells is associated with the cell proteins, little or none is present in the carbonic anhydrase. In view of these results we decided to find out, using ^{65}Zn , whether *in vitro* exchange of zinc between zinc salts and carbonic anhydrase is possible if the mixtures are kept for a long time. The results of these and some closely related investigations are described in this paper.

EXPERIMENTAL

Materials

Carbonic anhydrase. The enzyme was prepared from washed ox erythrocytes by 'method II' of Keilin & Mann (1940). Two different batches (*A* and *B*) were used, corresponding, respectively, to solution (*a*) and the most highly purified preparation described by these authors. *A* and *B* contained, respectively, 383 and 158 μg . of protein, based on 14.95% N in the enzyme (Keilin & Mann, 1940), and 120 and 126 enzyme units/ml. Assuming that the purer batch (*B*) contained no other nitrogenous compound, this enzyme preparation contained 0.33% of Zn. Batch *A* was used for experiments 1 and 2 (Table 1) and *B* for Exp. 3.

Zinc-glycine complex. Glycine (4.0 mg.) was added to a ^{65}Zn -containing ZnCl_2 solution (0.5 ml. containing 105 μg . Zn and giving 8225 counts/min. with our counting system), and 0.01N-NaOH was added dropwise until the pH was about 7.4. The solution was diluted to 1.0 ml. with glass-distilled water and used for Exps. 1 and 2. For Exp. 3 double the above amounts of glycine and ZnCl_2 solution were used and the mixture was diluted to 4.5 ml.

Ammonium sulphate. Since the purest available (Analar) samples of $(\text{NH}_4)_2\text{SO}_4$ contain significant amounts of Zn, it was necessary to remove this metal before the salt was used in these 'exchange' investigations. A saturated aqueous solution of the salt was made alkaline to phenol red with NH_3 , and the solution was shaken with a solution of dithizone (diphenylthiocarbazone) in CCl_4 until no more Zn

could be extracted. The dithizone remaining in the aqueous solution was then extracted with CCl_4 , and the $(\text{NH}_4)_2\text{SO}_4$ solution evaporated to dryness in an evaporating basin which had previously been washed, successively, with conc. HNO_3 , glass-distilled water, a solution of dithizone in CCl_4 and finally CCl_4 . The Zn-free $(\text{NH}_4)_2\text{SO}_4$ obtained was used for the final precipitation in the preparation of the enzyme, and also for precipitation of the enzyme from the Zn-glycine-enzyme mixtures.

Distilled water. For dialysis and for the preparation of all the solutions used in this investigation, glass-distilled water was used. This was found to contain not more than $0.44 \mu\text{g}$. in contrast to a value of $32 \mu\text{g}$. Zn/l. for the water from a 'Manesty' still.

Methods

Carbonic anhydrase activity. The enzyme activity was determined by the method of Meldrum & Roughton (1934) and expressed in terms of the carbonic anhydrase unit defined by these authors. The boat used was essentially that described by Brinkmann, Margaria & Roughton (1933) and Meldrum & Roughton (1934).

Zinc determinations. The dithizone method as modified by Gettler & Bastian (1947) was used. The material was digested with acid and the digest was brought to pH 8.5–9.0 with NH_3 as described by Banks, Tupper & Wormall (1950). The zinc was then extracted by repeated shaking with dithizone solution (40 mg./l. of CCl_4), and the amount of Zn present calculated by comparing the amount of dithizone solution required with that needed to give complete extraction of the metal from standard ZnSO_4 solutions under comparable conditions. Blank determinations were carried out and allowance was made for the small amount of Zn in the digestion mixture and other reagents used.

Radioactivity determinations. All radioactivity determinations were made with the liquid counter described by Veall (1948); solutions with a relatively low salt content, e.g. the purified carbonic anhydrase preparations and solutions of zinc-glycine complexes, were 'counted' without further treatment. Solutions containing appreciable amounts of $(\text{NH}_4)_2\text{SO}_4$, e.g. undialysed solutions of $(\text{NH}_4)_2\text{SO}_4$ -precipitated carbonic anhydrase, were digested with acid and the ^{65}Zn extracted and measured as described by Banks *et al.* (1950).

RESULTS

Attempted exchange of zinc between carbonic anhydrase and a zinc-glycine complex

A solution of ^{65}Zn -containing zinc-glycine complex was added to solutions of carbonic anhydrase and the mixtures were kept in the refrigerator for varying lengths of time, after which the enzyme was precipitated by ammonium sulphate. The enzyme activity and the ^{65}Zn content of the precipitates were determined to find out whether any exchange of ^{65}Zn had occurred. A typical experiment (Exp. 2, Table 1) was carried out as follows.

The carbonic anhydrase solution (15 ml. of batch A) was mixed with a solution of labelled zinc-glycine complex (0.5 ml. containing $52.5 \mu\text{g}$. of Zn) and the mixture kept at $0-4^\circ$. Radioactivity and enzyme activity determinations were made at the start and after 8 days. At the end of this storage period a sample (10 ml.) of the mixture was saturated with $(\text{NH}_4)_2\text{SO}_4$, and the precipitated carbonic anhydrase was filtered off, washed with five 2 ml. portions of saturated $(\text{NH}_4)_2\text{SO}_4$ solution and dissolved in glass-distilled water (10 ml.). Radioactivity and carbonic anhydrase determinations were made on this solution, and, as a check, the ^{65}Zn contents of the filtrate and washings were determined.

In another similar experiment the mixture was kept for 32 days at $0-4^\circ$, and then divided into two equal portions for precipitation of the enzyme by $(\text{NH}_4)_2\text{SO}_4$. In this experiment the yield of carbonic anhydrase in the precipitate was unexpectedly low, but sufficient was obtained for the purposes of this investigation.

The results of these experiments (Table 1) show that no significant amount of ^{65}Zn entered the carbonic anhydrase molecule even when the mixtures were kept for 32 days. Calculations based on the total zinc contents of the enzyme and the zinc-glycine complex, and the ^{65}Zn content of the latter, indicated that considerable amounts of ^{65}Zn would have been present in the precipitated

Table 1. *Attempt to obtain in vitro exchange of ^{65}Zn between a ^{65}Zn -containing zinc-glycine complex and carbonic anhydrase*

(Experimental details are given on p. 430.)

Exp. no.	Period available for exchange (days)	Mixture				(NH ₄) ₂ SO ₄ -precipitate		
		Total zinc		⁶⁵ Zn (counts/min.)	Carbonic* anhydrase (units)	Carbonic anhydrase (units)	⁶⁵ Zn	
		In the mixture (μg.)	In the carbonic anhydrase (μg.)				Found (counts/min.)	Predicted† (counts/min.)
1	1	73	22	4114	1710	780	0‡	565
2	8	49	15	2732	1360	690	0‡	425
3	32	47	6.2	2555	756	(a) 130 (b) 142	0‡ 0‡	58 63

(a) and (b) refer to separate precipitations of the enzyme.

* Determined at the end of the period of storage.

† Calculated on the assumption that there would be free exchange between the zinc of the zinc-glycine complex and that in the carbonic anhydrase.

‡ Counts within the variation of the background (i.e. less than 3).

enzyme if there had been free exchange of zinc between the enzyme and the complex (see last column of Table 1).

'Availability' of the ^{65}Zn in the zinc-glycine complex

In these experiments with carbonic anhydrase, and in other investigations on the labelling of blood cells with ^{65}Zn , we have kept the mixtures at about pH 7.4. The direct addition of ordinary inorganic zinc salts to the enzyme solution or cell suspension was thus precluded, since the metal is readily precipitated at this pH even from very dilute solutions. We have therefore added sufficient glycine to the zinc chloride solution to prevent the formation of a precipitate when alkali was added to bring the mixture to pH 7.4.

It might be contended that the zinc in this zinc-glycine complex may be relatively firmly bound and thus unable to undergo exchange reactions. To test this possibility we have studied the availability of the zinc in the complex, and have found that the metal is readily freed from its combination with glycine. For example, the addition of phosphate (as 0.5M-phosphate buffer of pH 7.4) to a solution of the zinc-glycine complex at pH 7.4 leads to almost complete precipitation of the zinc as phosphate; all the glycine remains in the supernatant solution, and no significant change occurs in the pH of the solution. In other tests we have found that the zinc of the zinc-glycine complex is readily extracted at pH 7.4 when the solution, or a mixture of blood plasma and zinc-glycine solution, is shaken with a solution of dithizone in carbon tetrachloride. Thus the ^{65}Zn of the zinc-glycine complexes used here was freely available for exchange with other zinc compounds in the systems studied. Furthermore, it is known that the stable complexes of zinc and other heavy metals with amino-acids 'are in equilibrium with a (usually exceedingly minute) proportion of inorganic ions from which they are formed' (Albert, 1950).

Zinc in the carbonic anhydrase of ^{65}Zn -labelled red blood cells

Most of the ^{65}Zn which enters or becomes attached to red blood cells when they are exposed to a solution of ^{65}Zn -containing zinc-glycine complex and then washed with 0.9% NaCl (w/v) is relatively firmly bound (Tupper *et al.* 1951), and experiments were therefore carried out to determine the proportion of the bound ^{65}Zn which is associated with the carbonic anhydrase in these labelled erythrocytes.

In a preliminary experiment to determine how much of the ^{65}Zn is attached to the total protein of red cells, washed rabbit erythrocytes (5 ml.) were treated with a ^{65}Zn -containing zinc-glycine solution at pH 7.4 and kept for 2 hr. at 37°. The cells were then washed thrice with 0.9% NaCl (5 ml. each time) and then lysed by adding distilled water (25 ml.). The mixture was centrifuged and the deposited

stroma was washed with 0.9% NaCl until the washings were colourless. (This washed stroma contained less than 1% of the ^{65}Zn of the laked erythrocyte solution.) The stroma washings (20 counts/min.) were added to the haemoglobin solution and the mixture (4310 counts/min.) was saturated with $(\text{NH}_4)_2\text{SO}_4$. The mixture was kept overnight in a refrigerator, and then warmed gently in a water bath (to aid flocculation of the precipitate) and filtered. The precipitate was washed well with saturated $(\text{NH}_4)_2\text{SO}_4$ solution, and radioactivity determinations were made on the filtrate, the washed precipitate and the washings.

The results showed that the washed precipitated proteins contained about 94% and the filtrate only about 1% of the total ^{65}Zn of the labelled red cells. It is of interest that the insoluble protein (stroma) of the lysed red cells contained only a minute proportion of the total ^{65}Zn , since this apparently excludes the possibility that the radioactivity of the labelled red cells is due to adhering precipitated inorganic zinc salts.

In view of these results, other experiments were carried out to determine what fraction of the cell-bound ^{65}Zn is attached to the carbonic anhydrase of the *in vitro* labelled erythrocytes.

Table 2. ^{65}Zn content of the carbonic anhydrase of ox erythrocytes labelled with ^{65}Zn

(The values given below are those for 20 ml. of labelled ox erythrocytes, or fractions obtained from this amount. For further details see p. 431.)

	Carbonic anhydrase (units)	^{65}Zn content (counts/min.)
Lysed erythrocytes	28,800	20,300
Separated crude carbonic anhydrase*	17 900	40
Denatured protein	—	21,500

* Separated by adding ethanol- CHCl_3 to the lysed erythrocytes.

Fresh defibrinated ox erythrocytes (20 ml.) were washed well with 0.9% NaCl and then treated with 4.5 ml. of a zinc-glycine solution (pH 7.4 and with NaCl added to make the solution isotonic with ox erythrocytes; total counts, 26,900/min.). The mixture was kept at 37° for 2 hr. and then centrifuged. The labelled cells were washed thrice with 0.9% NaCl (20 ml.) and lysed by adding distilled water (10 ml.). ^{65}Zn and carbonic anhydrase determinations were made on samples of this solution, and the remainder (26 ml.) was cooled to 0° and treated with 17 ml. of an ice-cold ethanol- CHCl_3 mixture (1 vol.: 1 vol.). The mixture was stirred well, kept at 0° for 15 min. and then centrifuged for 30 min. at 0°. The supernatant solution was dialysed against running water for 24 hr. and the radioactivity and carbonic anhydrase activity of the product (' CHCl_3 -enzyme') were determined. The precipitate from the water-ethanol- CHCl_3 mixture ('denatured protein') was dried at 120°, boiled down to a small volume after adding conc. HNO_3 (30 ml.) and the solution filtered through glass wool and then diluted to 50 ml.; a radioactivity determination was made on this solution.

The results (Table 2) showed that the separated carbonic anhydrase fraction (representing about two-thirds of the original enzyme of the lysed red cells) contained less than 0.25 % of the ^{65}Zn present in the labelled cells. Essentially the whole of the ^{65}Zn was present in the 'denatured protein' fraction. In view of the very low ^{65}Zn content of the relatively crude ' CHCl_3 -enzyme' preparation, it was decided that it was unnecessary, for the purposes of this experiment, to continue with the purification of the enzyme, since the small amount of purified enzyme which would have been obtained, would almost certainly have contained a negligible amount of ^{65}Zn .

DISCUSSION

The results of these investigations with ^{65}Zn are in complete agreement with the observations of Keilin & Mann (1940) that the zinc in carbonic anhydrase is firmly bound to the protein. The absence of exchange *in vitro* of zinc between the enzyme and solutions containing available ^{65}Zn emphasizes the similarity between the stability of this metal-protein complex and that of certain other metallo-proteins. For example, Ruben, Kamen, Allen & Nahinsky (1942) have found that there is no significant exchange of iron between methaemoglobin ('ferrihaemoglobin' and 'ferrihaem') and radioactive

ferric salts when kept in contact for 2 months, and Ruben, Frenkel & Kamen (1942) observed no exchange *in vitro* of magnesium between chlorophylls *a* and *b* and magnesium ions.

SUMMARY

1. Carbonic anhydrase kept as long as 32 days at 0° and pH 7.4 in a solution of ^{65}Zn -containing zinc salts (plus glycine to prevent precipitation of the zinc) takes up no significant amount of ^{65}Zn .

2. When red blood cells are labelled *in vitro* with ^{65}Zn little or none of the zinc which enters the red cell is firmly attached to the carbonic anhydrase.

3. The inability of carbonic anhydrase to exchange its zinc with zinc ions and simple zinc compounds supports the view of Keilin & Mann (1939, 1940) that the metal which is an integral part of the enzyme molecule is firmly bound to the protein.

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