Some factors affecting the in vitro binding of zinc by isolated soya-bean protein and by α-casein

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It is well established that soya-bean protein contains some compound rendering zinc in the food unavailable and increasing the turkey's apparent requirement for it (Supplee, Combs & Blamberg, 1958; Kratzer, Vohra, Allred & Davis, 1958) and the chick's (O'Dell & Savage, 1960; Zeigler, Leach, Norris & Scott, 1961).

Kratzer, Allred, Davis, Marshall & Vohra (1959) showed that the dietary Zn requirement of turkey poults was slightly reduced when autoclaved isolated soya-bean protein was used in place of untreated protein, but that a much greater reduction in the Zn requirement occurred when the disodium salt of ethylenediamine tetraacetic acid (EDTA) was added to the diet.

Subsequently, O'Dell & Savage (1960) suggested that the presence of phytic acid in isolated soya-bean protein may be a factor contributing to the low availability of Zn from diets containing this protein, for it is known that phytate accounts for about 70% of the phosphorus in soya-bean meal and that during the extraction of soya-bean protein phytic acid forms a complex with protein (Smith & Rackis, 1957). Such complexes are resistant to digestion by pepsin (Barré, 1956), so that by combining with Zn they could render it unavailable. O'Dell & Savage (1960) showed that the addition of phytic acid to chick diets containing isolated soya-bean protein or casein caused a marked reduction in the growth of the birds unless additional supplies of Zn were also given.

The object of the investigation described here was to develop a method for measuring directly the in vitro binding of Zn by isolated soya-bean and other proteins and to study some factors that affect it. A brief preliminary report of this work was given by Kratzer, Allred & Porter (1961). In the method used the protein was shaken with a solution containing $^{65}ZnCl_2$, and the residual ^{65}Zn in the protein-free solution was determined. This procedure was found to be rapid and convenient and to give consistent results over a wide range of concentrations of either Zn or protein. Subsequently studies were made of the binding of Zn by different proteins and of the effect of pH and of the addition of EDTA and of phytic acid on binding activity.

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The first part of the experimental work was carried out at Davis, and the remainder at Shinfield during the sabbatical leave of one of us (F.H.K.).

EXPERIMENTAL

Materials

Isolated soya-bean proteins. ADM assay protein C-1 (Archer-Daniel-Midlands Company, Cincinnati) and α-protein (Glidden Company, Chicago) were dried overnight at 90°.

Autoclaved ADM assay protein C-1. The undried protein was heated on open trays in an autoclave at 15 lb/in^2 for 30 min and then dried overnight at 90°.

α-Casein. This, prepared by the urea method of Hipp, Groves, Custer & McMeekin (1952), was kindly supplied by Dr R. Aschaffenburg of the National Institute for Research in Dairying.

Low-phytate soya-bean protein. ADM assay protein C-1 (30 g) was suspended in water (800 ml) and stirred mechanically during addition of sufficient 2N-NaOH to bring it into solution. The solution was adjusted to pH 7 with N-HCl and then dialysed against three changes of distilled water. The salt-free solution was treated with two successive portions of 25 ml of the acetate form of De-acidite resin (Permutit Co. Ltd) in a settled suspension. The supernatant solution was adjusted to pH 4·6 with N-HCl, and the resulting precipitate of protein was collected, washed five times with distilled water and dried over P_2O_5. The product contained 0·18% of phytic acid phosphorus, approximately one-third of the amount present in the original ADM assay protein, as determined by the method of Gerritz (1935).

$^{65}ZnCl_2$. The specific activity of $^{65}ZnCl_2$ used at Davis was 0·17 μc/mg and of that used at Shinfield 0·33 or 0·56 μc/mg.

Methods

Effect of different concentrations of ADM assay protein C-1 and of Zn on amount of Zn bound. Six amounts of protein (3·9, 7·8, 15·6, 31·3, 62·5 and 125 mg) were treated in turn with five amounts of Zn (40, 80, 160, 320, and 640 μg) as $ZnCl_2$. The appropriate amount of protein was weighed out into a test tube, 3 ml of water were added, and the protein was stirred into suspension before adding the required amount of an aqueous solution of zinc chloride (0·04%, w/v) containing a tracer amount of $^{65}ZnCl_2$. Distilled water was added to give a total volume of 8 ml. The tubes were then stoppered and shaken mechanically for 2 h at room temperature. The solutions were unbuffered, but in each the pH remained at 4·6. After allowing the insoluble protein to settle, 2 ml portions of the supernatant liquid were taken for measurement of ^{65}Zn activity in a well-type scintillation counter.

General procedure for determining the amount of Zn bound by proteins. The protein (50 mg) was suspended in water or, when required, 0·1 M-acetate or 0·1 M-citrate buffer. A solution containing 400 μg Zn (as $ZnCl_2$) with a tracer amount of $^{65}ZnCl_2$ was added and the volume made up to 8 ml with water, containing, when required, appropriate amounts of EDTA or sodium phytate.

Subsequent operations were as described above. The pH of the supernatant solution was determined after the period of shaking.

Stability of Zn–protein complexes to repeated washing. The individual proteins were dissolved in dilute NaOH and brought to pH 7 with aqueous HCl. Portions containing 50 mg protein were placed in test tubes or small flasks; in certain instances duplicate portions were measured out, and 5 mg sodium phytate were added to each, the solutions being kept at room temperature for 24 h. The 0·1 M-acetate buffers at pH 4·0 or 5·4 were then added to the tubes, precipitating the protein. A solution containing 400 μg Zn (as $ZnCl_2$) with a tracer amount of $^{65}ZnCl_2$ was added, the volume was made up to 8 ml with water, and the whole was shaken for 50 min at room temperature. The protein residue was then separated by centrifuging. The residue was washed four times by suspending it in 4 ml of the appropriate 0·1 M-acetate buffer, allowing to stand for 45 min and then centrifuging. The ^{65}Zn activity was measured in the original supernatant solution, in the solution from each wash and in the final protein residue.

RESULTS

Effect of different concentrations of ADM assay protein C-1 and of Zn on amount of Zn bound

The amount of Zn bound by the protein proved to be roughly proportional to the concentrations of both Zn and protein. Thus at each level of protein the proportions of added Zn bound, and hence the ratio of protein-bound Zn to free Zn, were relatively constant. This would accord with the concept that during binding equilibrium is being established according to the system: free Zn + protein $\rightleftharpoons$ protein-bound Zn, and for which the binding constant, k, is

$$\frac{[\text{protein bound Zn}]}{[\text{free Zn}]\,[\text{protein}]} \quad \text{or} \quad k\,[\text{protein}] = \frac{[\text{protein bound Zn}]}{[\text{free Zn}]}.$$

The results are summarized in Fig. 1, in which the mean values of the ratio [protein-bound Zn]:[free Zn] at each level of protein are plotted against the concentration of protein. (The range of values for the ratio at each protein level is also shown.)

It is apparent from Fig. 1 that at first the ratio increased linearly as the amount of protein increased, but that it tended to deviate from a straight-line relationship at the two highest levels.

Effect of autoclaving and of addition of EDTA on amount of Zn bound by ADM assay protein

The proportions of added Zn bound in unbuffered suspensions at pH 4·6 by ADM assay protein, before and after autoclaving, and after the addition of different quantities of EDTA are shown in Table 1. It is clear that autoclaving had a small effect, and the stepwise addition of EDTA an increasing effect, in reducing the amount of added Zn bound.

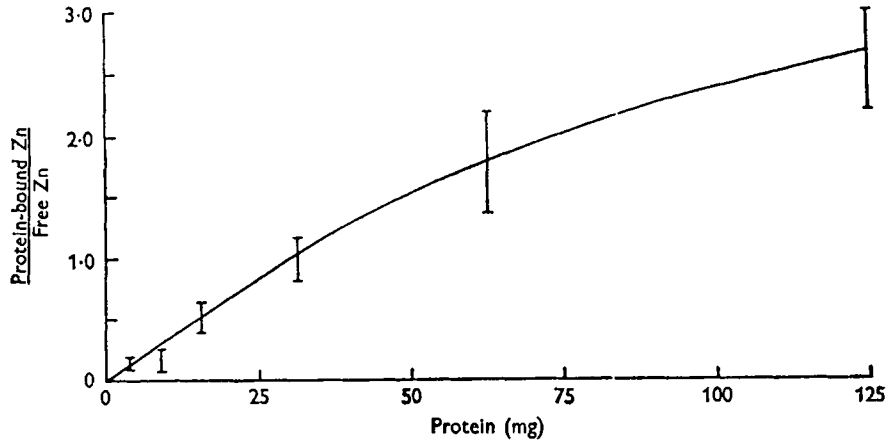

Fig. 1. Effect of amount of protein on ratio, protein-bound zinc:free zinc. The steps indicate the range of values about the mean.

Table 1. *Effect of autoclaving and of adding EDTA on the binding of zinc by 50 mg ADM assay protein*

(Mean values with their standard errors)

Material	Percentage of added Zn bound
ADM assay protein	62·3 ± 1·8
Autoclaved ADM assay protein	57·2 ± 2·0
ADM assay protein + 23 μg EDTA	50·8 ± 4·1
ADM assay protein + 46 μg EDTA	47·7 ± 4·0
ADM assay protein + 125 μg EDTA	16·7 ± 0·9
ADM assay protein + 250 μg EDTA	12·3 ± 3·3

Effect of pH and nature of the buffer on the amount of Zn bound by isolated soya-bean proteins and by α-casein

Less than 20% of the added Zn was bound by ADM assay protein when 0·1 M-sodium citrate buffer was used at either pH 3·7 or 4·8. This was probably due to the citrate ions having a stronger chelating action than do soya-bean protein molecules. However, when 0·1 M-sodium acetate buffer was used, the percentage of Zn bound increased from around 10 at pH 3·7 to around 65 at pH 4·8 and thus agreed well with that for unbuffered protein at pH 4·6. Accordingly, in all subsequent trials the pH was controlled by 0·1 M-sodium acetate buffer.

The results illustrated in Fig. 2 show that both ADM assay protein and α-protein had little binding activity at pH 3·7, but that the percentage of Zn bound increased markedly as the pH was increased to 5·3; a similar, though less marked, increase in binding activity was shown by α-casein. Low-phytate ADM assay protein bound little Zn in the pH range 3·7–4·3, but the percentage of Zn bound increased as the pH increased further, though at all pHs studied the binding activity was less than that of the original ADM assay protein.

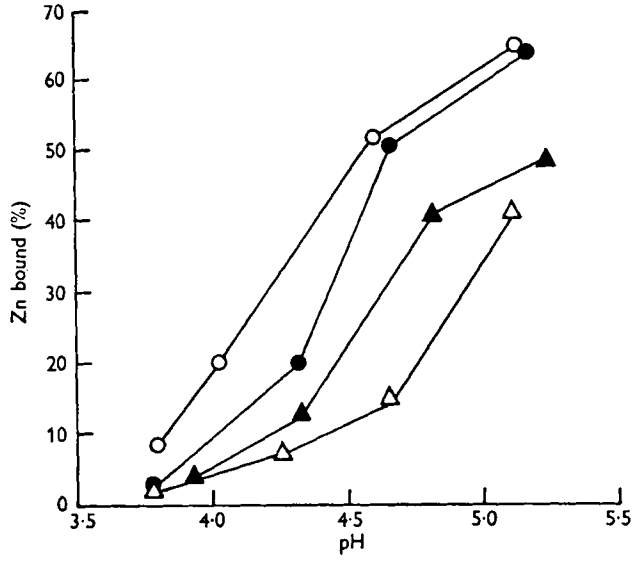

Fig. 2. Effect of pH on the binding of zinc by ADM assay protein (○——○), α-protein (●——●), low-phytate ADM assay protein (△——△) and α-casein (▲——▲).

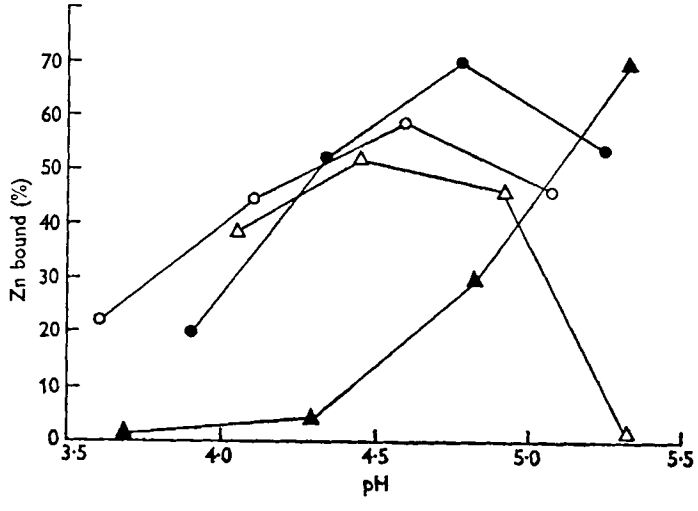

Fig. 3. Effect of pH on the binding of zinc by phytate complexes with ADM assay protein (○——○), α-casein (●——●) and low-phytate ADM assay protein (△——△), and by phytate alone (▲——▲).

Effect of addition of sodium phytate on the amount of Zn bound by isolated soya-bean protein and by α-casein

At pHs below 4·3 no precipitation of zinc phytate occurred when an aqueous solution of 5 mg sodium phytate was shaken with 0·1 M-sodium acetate buffer containing 400 μg Zn as $ZnCl_2$, but at higher pH values a precipitate was formed and the percentage of Zn bound increased markedly, as shown in Fig. 3.

The results in Fig. 3 also show that, when 5 mg sodium phytate were added to 50 mg ADM assay protein, α-casein or low phytate ADM assay protein, the proportion of Zn bound increased as the pH of the mixture was increased to about 4·5, and it is apparent from Figs. 2 and 3 that in all instances the amount of Zn bound was appreciably greater than in the absence of sodium phytate. At higher pHs the amount of Zn bound appeared to decrease, presumably owing to increasing solubility of the phytate–protein complex.

Table 2. *Effect of washing with buffer at pH* 4·0 *or* 5·4 *on stability of zinc complexes with* 50 *mg of ADM assay protein, low-phytate ADM assay protein and* α*-casein.* (5 *mg of sodium phytate were added where indicated,* 24 *h before reaction except when marked with an asterisk* (*) *when it was added immediately before reaction*)

Material	pH	% of added Zn bound	% of bound Zn remaining bound during successive washes	Mean	% of original Zn in residue after four washes	% of Zn originally bound and remaining bound after four washes
ADM assay protein	4·0	14	26, 22, —, —	24	0·1	0·7
ADM assay protein	5·4	33	62, 68, 74, 83	72	8·3	25
ADM assay protein + sodium phytate	4·0	33	50, 44, 41, 50	46	1·4	4·2
ADM assay protein + sodium phytate	5·4	35	63, 59, 58, 69	62	4·9	14
ADM assay protein + sodium phytate*	5·4	51	84, 82, 82, 85	83	24·6	48
Low-phytate ADM assay protein	4·0	9	20, —, —, —	20	0·02	0
Low-phytate ADM assay protein	5·4	34	53, 59, 64, 73	62	4·7	14
α-casein	4·0	6	16, —, —, —	16	0·05	0
α-casein + sodium phytate	4·0	28	56, 52, 49, 59	54	2·0	7

Stability of Zn-protein complexes to repeated washing

It is evident from the results given in Table 2 that the Zn bound by ADM assay protein at pH 4·0 passes into solution during washing at this pH more rapidly than that bound and washed at pH 5·4. In fact, after four washes at pH 4·0 little Zn was still bound by either ADM assay protein or by α-casein, even in the presence of added sodium phytate. However, washing at pH 5·4 caused only a slow solution of bound Zn from ADM assay protein, either in the presence or the absence of sodium phytate, and from low-phytate ADM assay protein.

A greater amount of Zn was bound and the complex appeared to be more stable to washing in the one experiment in which sodium phytate was added to the protein immediately before reaction with Zn instead of 24 h earlier.

DISCUSSION

The method described proved rapid and convenient and gave reproducible results for the amount of Zn bound by proteins under various conditions of test, but it will be

apparent that it is only applicable to determination of the amount of Zn bound by insoluble proteins. Its use is restricted, therefore, to the measurement of binding activity at pH values near the iso-electric point of the protein being studied. Thus, for isolated soya-bean protein and for α-casein the working range of the method was from pH 3·4 to pH 5·3. The observed increase in the amount of Zn bound and in the stability of the complexes formed as the pH was increased within this range may indicate that there was competition between Zn and hydrogen ions for common binding sites on the protein. As the hydrogen ion concentration was decreased, more of these binding sites became available to Zn.

The possible influence of the size of the protein particles on the amount of Zn bound was not investigated in detail, though in a single experiment it was found that a sample of α-protein ground as finely as possible in a mortar bound exactly the same amount of Zn as the unground material. ADM assay protein and α-protein were supplied as fine powders passing through 80- and 120-mesh sieves, respectively. Such powders are suitable for use in experimental diets, and it was considered apposite to use them for the binding tests in the form supplied. It was to preserve this analogy with feeding experiments with poultry that the binding tests were carried out on suspensions rather than on solutions of the protein, though we appreciated that the use of solutions would eliminate possible difficulties that might arise with protein suspensions of different particle sizes.

As ADM assay protein that had been autoclaved bound less Zn than the untreated protein, it seems probable that the heat treatment destroyed binding sites on the protein and thus reduced the Zn-binding activity. The addition of EDTA greatly reduced the amount of Zn bound by the protein. This was presumably because EDTA is a strong chelating agent and, in forming the soluble Zn-EDTA complex, it prevented much of the Zn from being bound by the soya-bean protein. The slight reduction in the amount of Zn bound by autoclaved ADM assay protein and the greater reduction in the presence of EDTA accord with the increases found in the availability of Zn to turkey poults given diets containing these materials (Kratzer *et al.* 1959).

The addition of sodium phytate increased the amount of Zn bound by either isolated soya-bean protein or α-casein. Contrariwise, the removal of part of the phytic acid normally present in ADM assay protein reduced the amount of Zn bound by the protein. These findings support the suggestion by O'Dell & Savage (1960) that phytic acid is involved in making Zn unavailable, though the nature of the protein–phytic acid complex and the way in which it can bind Zn require further investigation.

SUMMARY

1. The zinc-binding activities of isolated soya-bean proteins and of α-casein were determined by shaking the protein with a solution of $^{65}ZnCl_2$ and measuring the ^{65}Zn activity of the supernatant solution.

2. The amount of Zn bound was proportional to the concentration of both Zn and protein.

3. Autoclaved ADM assay protein bound less Zn than the original protein.

4. The amount of Zn bound by ADM assay protein was markedly reduced in the presence of EDTA.

5. The amount of Zn bound by isolated soya-bean proteins and by α-casein was relatively low at pH 4 but increased markedly as the pH was increased to 5·3.

6. The amount of Zn bound by ADM assay protein and by α-casein increased markedly, particularly at pH values below 4·5, in the presence of sodium phytate.

7. ADM assay protein from which part of the phytic acid had been removed bound less Zn than the original protein.

8. The significance of these findings is discussed in relation to the availability of Zn in diets for chicks and turkey poults.

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