It is believed that chelates in addition to those of iron may be effective sources of plant nutrients. In many instances zinc, manganese, and molybdenum cannot be applied effectively to the soil. Foliage sprays are often undesirable because the residue causes an increase in insect infestation. Studies are now in progress at this station to determine the usefulness of various metal complexes for plants.

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Manuscript received May 12, 1952.

The Binding of Metal Ions by ACTH: A Property Correlated with Biological Activity

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A survey of the polarographic behavior of a number of metal ions in solutions containing ACTH led to the discovery that the hormone binds zinc and copper (II) ions in acid solution, and that a relationship exists between the extent of binding under certain fixed conditions and the adrenocorticotropic activity as judged by rat assay. Manganese (II), cobalt (II), and nickel (II) were also observed to bind in acid solution, but without any definite relationship to bioactivity; no binding occurred with antimony (III), bismuth (III), lead (II), and tin (II). The formation of complexes between certain metal ions and amino acids has been the subject of numerous publications, and has been shown to involve the carboxyl group—i.e., to be favored by high pH. The unique characteristic of the binding of zinc ion by ACTH is that it is favored by *low* pH, but is substantially unaffected by buffer composition (Table 1). It is not practicable to carry out polarographic zinc ion measurements in buffers of pH much less than 4.5, because of interference by the hydrogen discharge wave; on the other hand, the copper (II) wave is usually affected by its nearness to the anodic wave of the supporting electrolyte. Practical considerations have led to the adoption of the zinc wave for study. The visible effect of ACTH on the zinc depolarization wave involves (1) the suppression of the zinc diffusion current; (2) distortion of the wave, which increases progressively with the extent of binding to the point where, above 50% diminution, the diffusion current loses all definition; and (3) a progressive shift of the zinc half-wave potential to more negative values.

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[2] The authors wish to acknowledge their indebtedness to T. J. Webb, who suggested the experimental approach which led to the above findings, and to N. Brink, K. Folkers, F. Kuehl, and J. Richter for their interest and cooperation.

TABLE 1

EFFECT OF pH AND BUFFER COMPOSITION ON THE BINDING OF ZINC ION BY ACTH*

Buffer composition	pH	Diminution of standard zinc wave by ACTH (8 *x* Armour) 0.24 mg/ml (%)
0.1 *M* NaOAc · HOAc	4.64	18
0.1 *M* NH_4OAc	6.46	8
0.1 *M* N-ethyl morpholine acetate	8.18	0
0.1 *M* Na glutamate	4.63	22

* Corticotropin B of Brink *et al. J. Am. Chem. Soc.,* **74**, 2120 (1952).

In order to correlate the zinc-binding reaction with biological activity, a standard procedure for carrying out the measurement was adopted, and numerous samples of ACTH (the potency of which had been determined by the hypophysectomized rat method [*1*]) were subjected to zinc-binding determination. The data were assembled in the Klotz (*2*) function of pm/x versus

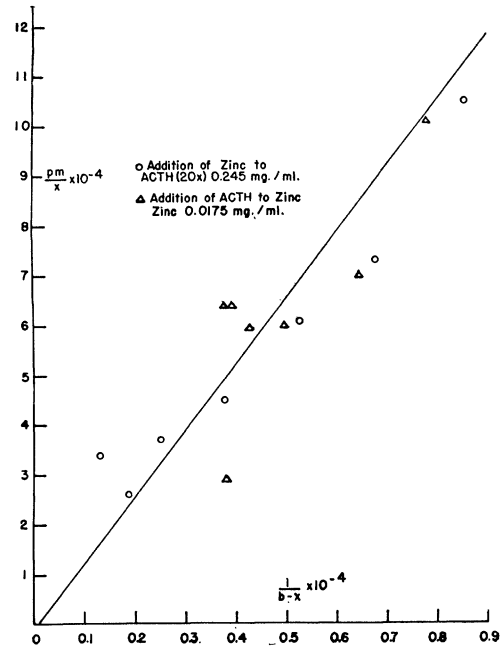

FIG. 1. Binding of zinc ion by ACTH in 0.1 *M* acetate buffer, pH 4.64.

$1/b\text{-}x$, where p represents the potency in multiples of Armour standard and m the concentration in mg/ml, or more conveniently as their polarographic equivalents of $pm/(I_d^0 - I_d)$ and I_d^{-1}, where I_d^0 and I_d are the diffusion currents of the standard zinc solution and the same with added ACTH. A straight line was fitted by the method of least squares, and the resulting equation for p in terms of m and I_d was employed for further work. The standard zinc solution at present in use contains 0.0175 mg/ml zinc in 0.1 M acetate buffer, pH 4.64, to which is added 0.001% gelatin as zinc wave maximum suppressor. Typical data are plotted in Fig. 1 for a single sample, and for several samples in Fig. 2.

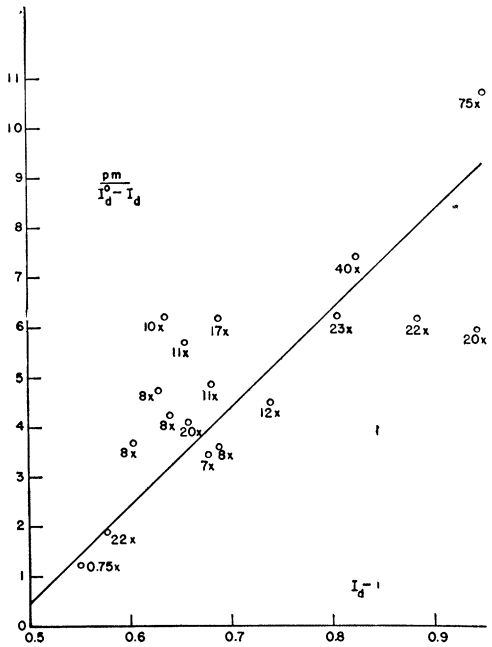

FIG. 2. Binding of zinc ion by ACTH calibration curve.

Since it has become evident that there is more than one possible molecular entity displaying adrenocorticotropic activity (*3, 4*), certain decisive limitations must be borne in mind when applying zinc binding as an assay method: samples must have the same origin and manipulative history. The calibration of Fig. 2 was performed on preparations derived from swine ACTH protein by pepsin digestion. Illustrations of results are given in Table 2. The scattering of points in Fig. 1 is a result of the high dilutions that had to be employed to conserve material; those of Fig. 2 are in part accountable by the uncertainty of the bio-assay, and in part by experimental variation in the preparative method. Indeed, it is for many purposes preferable to prepare a calibration curve from a single standard sample (having a preparative history in common with other samples under study), to assign to the standard an arbitrary index (of, say, 100), and to compare all other samples with it.

The procedure is to dissolve the sample in the standard zinc solution at a concentration such that the diminution of the zinc wave lies somewhere between 10–50% (usually not more than 0.3 mg/ml) and to polarograph in the voltage range −0.8 to −1.4 (*vs* SCE) and current range 5 μamp (25°). The observed diffusion current is read and entered into the calibration equation. Ten or more samples can be run per day with ease.

TABLE 2

A COMPARISON OF BIOLOGICAL AND POLAROGRAPHIC ASSAYS FOR ACTH

Sample	Potency (X standard)		Comments
	Bio-assay	Polarographic assay	
54	38	35	Swine ACTH, pepsin digest
286	12	16	" " " "
455	2.5	4	" " " "
125–4	12	16	" " " "
125–7	18	18	" " " "
125–9	20	24	" " " "
125–11	22	19	" " " "
468	15	19	" " " "
459	10	11	" " " "
511	15	18	" " " "
621	8.5	9	" " " "
H	12–15	100	Swine ACTH, HCl hydrolysate
B	12	22	Beef ACTH

It is at least of theoretical interest to locate the site of zinc binding in the ACTH molecule. No amino acid has been found which shows any evidence of zinc binding under the conditions employed for ACTH; the peptides L-prolyl-L-valine and glutathione likewise

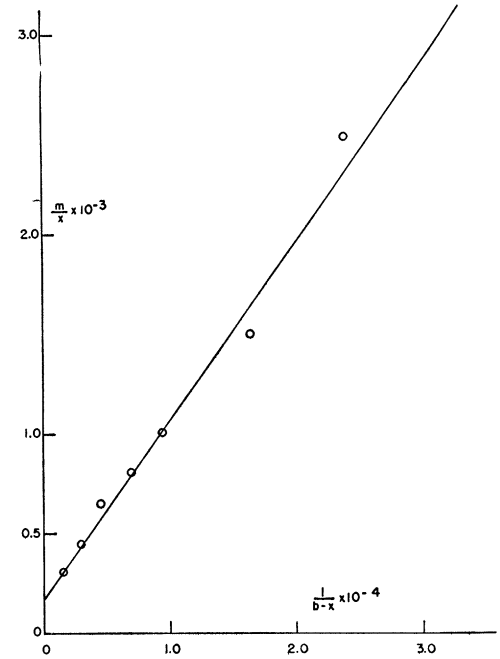

FIG. 3. Binding of zinc ion by salmine.

gave negative results. On the other hand, salmine and dihydrostreptomycin bind zinc ion strongly, the former with an apparent ACTH potency of 275 x on the basis of the calibration of Fig. 2. The binding of zinc ion by salmine is illustrated in Fig. 3. From the m/x intercept of the line, it is estimated that the binding approaches a limit of about 2 zinc ions/arginine residue. Analysis of the polarographic data on the dihydrostreptomycin-zinc complexing reaction by the De-Ford-Hume method (*5*) indicates an equilibrium of the type:

$$(DHS)^{3+} + Zn^{2+} \rightleftarrows (DHS\ Zn)^{5+},$$

with a constant of 43,000 at 25°; this result is, however, subject to a demonstration of reversibility of the reaction.

The only obvious common features of ACTH, salmine, and dihydrostreptomycin are (1) they are all cations under the conditions of study, and (2) they contain guanidine residues, present in the first two as arginine and in the third as streptidine. On the other hand, arginine alone gives no evidence of zinc binding; hence, if guanidine residues are indeed involved, some large common structural feature must be exerting influence. Further speculations do not appear justified at this time.

The binding of zinc by salmine has interesting electrophoretic consequences. The cathodic mobility of salmine (7.5 mg/ml in 0.1 *M* acetate buffer, pH 4.64) was observed to be 29×10^{-5} cm^2 sec^{-1} v^{-1}; addition of zinc at a concentration of 3.3×10^{-3} *M lowered* the mobility to 15×10^{-5} cm^2 sec^{-1} v^{-1}. Since the binding of zinc in these circumstances is favored by low pH, it is necessarily accompanied by an increase in net positive charge of the complexing ion; a *lowering* of cathodic mobility thus suggests a clumping of ions into large aggregates, at least in the case of salmine. The multiplicity of components in the relatively crude ACTH preparations at hand rendered electrophoretic data difficult to interpret, but a general qualitative lowering of cathodic mobilities was likewise observed in the presence of zinc ion.

It is of interest that, while this publication was in process, Holtermann and Heier (*6*) have reported the presence of abnormal amounts of zinc in crude whale corticotropin, and have suggested that the metal may be an inherent and significant constituent. However, in view of the observed binding of zinc ion by substances displaying no adrenocorticotropic activity (salmine and dihydrostreptomycin), there is not necessarily any direct connection between the two properties of ACTH. This gives further force to the warning against attempting any application of a given calibration curve to preparations of history different from those from which the curve was constructed.

Inherent in the application of the Klotz equation to the binding of zinc by ACTH is the assumption that the equilibrium concentration of free zinc ion is being measured; in reality, this may not be entirely the case, since a negative shift of half-wave potential is observed. Thus the wave may be in part due to the reduction of the ACTH-zinc complex; this is of little concern from the practical standpoint, however, provided that measurements are restricted in binding levels of less than 50%.

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Manuscript received April 16, 1952.

"Mixed" Skeletal Muscle Fibers[1]

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In the posterior part of the lingual septum of rabbits, muscle fibers are found which are "mixed" in the sense that they show portions of nonmuscular material. These parts stain pink with hematoxylin-eosin, red with van Gieson's method, and take the aniline blue in Masson's trichrome stain. From these reactions it may be concluded that they are composed of collagen or reticulin, or some closely related substance. Morphologically, they appear finely reticular or, more often, fibrous, not unlike ordinary collagenous fibers. They may occupy only one part of the muscle fiber (Fig. 1) or mingle with the myofibrillae to a lesser or greater extent. This pattern can best be studied in cross sections. In longitudinal sections, the mixed fibers appear to have a core of sarcoplasm surrounded by a coat of collagenlike material of varying thickness. Such pictures are difficult to interpret, however.

When sections of skeletal muscle are stained by the McManus periodic acid–Schiff method (*1*) the sarcoplasm and myofibrillae show only faintly, if at all, but the sarcolemma stands out distinctly, just like basement (limiting) membranes of other structures. In "mixed" fibers of the lingual septum of the rabbit this method brings out the nonmuscular component very clearly, confirming the results obtained with other staining procedures. In cross sections, where the "collagenization" is slight, one can observe that it starts from the sarcolemma (Fig. 2), in that small septa or trabeculae penetrate into the muscle fiber proper. These partitions are either very fine, which may be a first stage, or coarse, until most of the fiber has been "collagenized" (Figs. 3, 4). The diameter of these "mixed" fibers is usually less than that of the ordinary muscle fibers in the tongue.

Since the replacement of the sarcoplasm starts apparently from the sarcolemma, it may be worth while to consider what sarcolemma really is. According to some authorities, it is composed of collagenous or

[1] This is part of a project supported by the Medical Division, National Research Council of Canada.