northern call type is the easternmost record for that call type and extends the range approximately 975 km from the nearest locality previously reported (*1*) in South Dakota. The Normal record for the western call type is the easternmost and northernmost record for that call type and extends the range approximately 580 km from the nearest record in Kansas (*1*). The Bath record for the eastern call type represents the northernmost and easternmost record for that call type and extends the range some 210 km from the nearest record in Missouri (*1*).

In conclusion, our finding of three call types of the *R. pipiens* complex in Illinois supports evidence from the western United States (*1, 3–5, 10*) which indicates that "*R. pipiens*" can no longer be regarded as a single, widely distributed species.

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8. When calls consisted of two or more series of pulses, we analyzed only the first series to allow comparison with the data of Littlejohn and Oldham (*1*).
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Zinc in Entamoeba invadens

Abstract. *Atomic absorption spectroscopy, electron microprobe analysis, and dithizone staining of trophozoites and cysts of* Entamoeba invadens *demonstrate that these cells have a high concentration of zinc (approximately 10^{-6} microgram per cell or 1 percent of their dry weight). In the cysts of this organism, the zinc is confined to the chromatoid bodies, which previous work has shown to contain crystals of ribosomes. The chemical state and function of this zinc are unknown.*

Cysts of the intestinal parasite *Entamoeba invadens* have the remarkable ability to condense nearly all their ribosomes into crystals, the chromatoid bodies (*1, 2*). Attempts to isolate these crystals into conventional buffers were hampered by the fragility of the crystals and by the extreme sensitivity of their component ribosomes to nuclease (*3*). These experiments left the suspicion that magnesium-containing buffers, though usually sufficient for the isolation of ribosomes, were not reproducing the intracellular environment of *Entamoeba*. The demonstration that zinc was an essential element for the formation of ribosomes in *Euglena gracilis* (*4*) led us to investigate the role of this element in *Entamoeba invadens*. The results we present here allow us to conclude that zinc is a major constituent of chromatoid bodies. We will also describe the estimation of the amount of zinc per cyst by atomic absorption spectroscopy of the ash of counted numbers of cells, the demonstration that this zinc is actually in the cysts by electron microprobe analysis, and finally the localization of the zinc within the cysts by means of histochemical staining with dithizone.

Entamoeba invadens, strain TRM [see (*5*)], was cultured as described by Myer and Morgan (*6*). Cells were collected at room temperature by centrifugation, washed quickly three times with twice-distilled water, and resuspended in a known volume of water. The concentration of cells in this suspension was measured in a hemocytometer. (Although the fragile trophozoites are ultimately lysed by such a washing schedule, they will survive as recognizable objects long enough to be counted.) Water was then removed by drying at 95°C, and cells were ashed in an oven at 550°C for 12 hours. The ash was dissolved in a known volume of either 1*N* HCl or H_2O, and the concentration of zinc in this solution was measured with an atomic absorption spectrometer (Perkin-Elmer, model 303), by reference to a standard curve of the absorbancies of known solutions of $ZnCl_2$. The cells from a total of 11 cultures were prepared in this manner. In the four of these in which the number of cysts exceeded the number of trophozoites, the average zinc per cell was found to be 0.5×10^{-6} μg. In the remaining seven (which contained trophozoites almost exclusively), the average zinc per cell was found to be 0.8×10^{-6} μg. In view of the experimental uncertainties attending these measurements (which we estimate to be perhaps 20 percent), we do not feel that this apparent difference in zinc content between cyst and trophozoite is significant.

The most direct evidence that this zinc is in the cysts was provided by the electron microprobe. For these measurements, cysts were washed in water and then suspended in 0.1*M* ammonium acetate. A drop of this suspension was placed on a polished plug of beryllium, was frozen by immersing the end of the plug in a mixture of acetone and solid CO_2, and was evaporated to dryness under vacuum. The plug was then examined with an electron microprobe (Applied Research Laboratories, model 20 EMX), using a KAP crystal set for ZnLα x-rays (wavelength, 12.28 Å). We found, as have others (*7*), that the use of Lα x-rays improved the peak to background ratios by a factor of at least 6 compared to the ratios found with Kα x-rays. Figure 1 shows the zinc x-rays recorded during a scan across a cyst that was visualized in the instrument's microscope. Of 21 cysts randomly encountered on the surface of the plug, eight gave zinc x-ray signals that were appreciably above (that is, two to five times) background. This fraction corresponds to the fraction of cysts containing visible chromatoid bodies in this particular preparation. In order to quantitate these zinc signals, a series of known proportions (from 0.1 to 10 percent) of $ZnCl_2$ in gelatin were made and smeared on quartz glass slides. Under the same instrumental conditions, the ZnLα counting rates of these mixtures were recorded. These rates were proportional to the zinc content of the mixture, thus the maximum rate of Fig. 1 corresponds to a zinc content of 1 percent dry weight. The microprobe results leave no doubt that the metal we are concerned with here is zinc and that this zinc is within the cysts. The resolution of this instrument

does not permit one to determine where within a cyst the zinc lies.

To localize the zinc, we stained cysts with dithizone. Our use of this reagent posed some problems: healthy cysts are impermeable to most ions and dyes, while dithizone is insoluble in water. We found the following method satisfactory. Washed cysts were fixed in 5 percent glutaraldehyde in phosphate buffer (0.35*M*, *p*H 7.5), containing 0.4 percent NaCl, for 2 hours at room temperature. They were then washed with twice-distilled water. The dithizone was dissolved in a mixture of 95 percent ethanol (nine parts) and concentrated NH_4OH (one part) at a concentration of 10 mg/ml. A drop of this orange solution was then mixed on a slide with a drop of the suspension of fixed cysts. Within a few minutes, a magenta coloration of the chromatoid bodies was seen in the light microscope, the rest of the cysts remaining unstained. Trophozoites stained diffusely throughout (see Fig. 2). Although this test by itself would not be specific for zinc as opposed to the other transition metals, it does indicate the presence of some transition metal in the chromatoid body. Taken in conjunction with the other evidence presented above, there can be no doubt that this metal is zinc.

The magnitude of these findings is unexpected. The only previous association of zinc with *Entamoeba* of which we are aware is the use of 1*M* solutions of $ZnSO_4$ as an aid in the finding of cysts in feces (*8*). This solution was selected because cysts would float in it without shrinking or losing their viability. No previous determination of the metal content of *Entamoeba* appears to have been made.

Our amoebas probably obtain their zinc principally from the serum that constitutes a third of their culture medium, since serum contains on the order of 1 μg of zinc per milliliter (*9*). The level of zinc which we find in these cells, about 10^{-6} μg per cell or approximately 1 percent of their dry weight, places these amoebas between the cells of the tapetum lucidum of carnivores, which contain solid zinc cysteine in amounts up to 10 percent of their dry weight (*10*), and human leukocytes, which contain on the order of 10^{-8} μg of zinc per cell (*11*). The mantle and gills of oysters may contain as much as 1 percent zinc, which they concentrate by a factor of 10^5 from seawater (*12*). In none of these cells, however, is the function of such

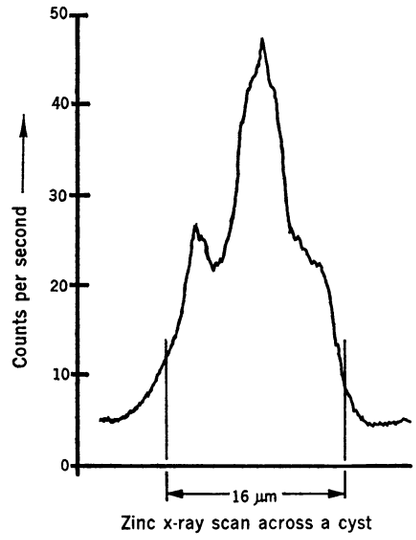

Fig. 1. Counting rate of zinc x-rays emitted from a cyst of *Entamoeba invadens* as the cyst is moved across the electron probe. Diameter of probe about 5 μm.

large amounts of metal known. If we take the volume of a typical chromatoid body as 8 μm^3, then the zinc present in this volume is concentrated by a factor of 10^5 compared to its level in the culture medium.

By dithizone staining, zinc has been found in the nucleolus of starfish oocytes (*13*). By absorption spectroscopy, trace levels of zinc have previously been reported in nucleic acids (*14*) and ribosomes (*15*), but this is the first finding of large amounts of this metal in conjunction with these substances. If

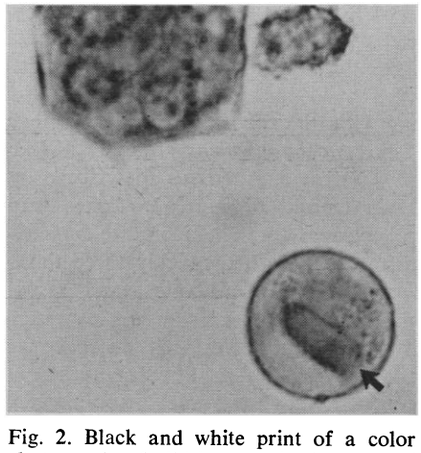

Fig. 2. Black and white print of a color photograph of dithizone-stained cyst and trophozoite of *Entamoeba invadens*. Arrow points to the single chromatoid body within the cyst. Both cells had been fixed with glutaraldehyde prior to staining. Blackness here is due to the magenta color of the stain. The appearance of a hexagonal outline to the trophozoite is an artifact of fixation.

we assume that a typical chromatoid body contains 2×10^6 ribosomes (*3*), and that each of these ribosomes has 2×10^6 daltons of RNA made of nucleotides of average weight 350, then a typical cyst has about 10^{10} ribosomal nucleotides. This is just the number of atoms of zinc that our analyses indicate a typical cyst contains. Thus a possible role for amoebal zinc is to neutralize the charge of the phosphates of the ribosomal RNA. Another possible role is to inhibit the ribonuclease of the cyst [which our previous work (*3*) has shown can destroy cyst ribosomes] for zinc ions can inhibit pancreatic ribonuclease (*16*). The actual role of zinc in these cells remains to be determined.

That zinc was concerned with the crystallization of a protein, insulin, was found by Scott in 1934 (*17*). The present finding of zinc in chromatoid bodies adds a new dimension to the problem of crystallizing ribosomes and to our understanding of the biology of the *Entamoeba*.

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