Summary. The calcium and phosphorus contents of the ash of femora, mandibles and teeth were determined in 230 fetuses, aged 5-9 months, in areas supplied by drinking water containing about 0.1 ppm F (low), 0.5-0.6 ppm F (medium) and about 1 ppm F (high). Significant differences in the mean calcium content of bone and tooth ash, depending upon fluoride intake of the mother during pregnancy from the drinking water, without any change in the mean phosphorus content, were demonstrated. In the low-fluoride area the mean calcium content of the bone and tooth ash of the younger fetuses was lower than was found in the older fetuses, whereas in medium- and high-fluoride areas no such difference was found. The observed differences in Ca/P ratios of the hard tissues of the fetus may possibly be due to the different constitution of the precipitated apatitic calcium phosphates.

1. Gedalia, I., Brzezinski, A., Portuguese, N., Bercovici, B., Arch. Oral Biol., 1964, v9, 331.

2. Gedalia, I., Zuckermann, H., Leventhal, H., J. Am. Dent. Assn., submitted for publication.

3. Harrison, H. E., Harrison, H. C., Fleishman, E., J. Lab. & Clin. Med., 1955, v46, 662.

4. Fiske, G. H., SubbaRow, Y., J. Biol. Chem., 1925, v66, 375.

5. Brudevold, F., Amdur, B. H., Messer, A., Arch. Oral Biol., 1961, v6, 304.

6. Kuyper, A. C., Kutnerian, K., J. Dent. Res., 1962, v41, 345.

7. Likins, R. C., Posner, A. S., Steere, A. C., J. Am. Dent. Assn., 1958, v57, 335.

8. Editorial: Bone density and fluoride ingestion, Nutr. Rev., 1961, v19, 198.

9. Gedalia, I., Frumkin, A., Zuckermann, H., Endocrinology, 1964, v75, 201.

10. Menczel, J., Schraer, R., Pakis, G., Posner, A. S., Likins, R. C., Proc. Soc. Exp. Biol. and Med., 1963, v112, 128.

11. Zipkin, I., McClure, F. J., Lee, W. A., Arch. Oral Biol., 1960, v2, 190.

12. Dallemagne, M. J., Phosphate and carbonate of mineralized tissues. First European Bone and Tooth Symposium, 1963.

13. MacGregor, J., Brown, W. E., Nature, 1965, v205, 359.

Received March 26, 1965. P.S.E.B.M., 1965, v119.

Ingestion of Soluble Ferritin-Antiferritin Complexes by Phagocytic Cells.* (30275)

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The ingestion of soluble antigen-antibody complexes by spleen cells in an *in vitro* system has been demonstrated by isotope tracer and immunofluorescent techniques(1). The previous investigation utilized albumin-antialbumin complexes prepared in varying degrees of antigen excess and the uptake of the complexes by spleen cells *in vitro* was related to the degree of antigen excess used in preparation of the soluble complexes.

Ferritin has been used similarly here to study the uptake of soluble ferritin-antiferritin complexes in an *in vitro* system. Ferritin, which can be identified readily in electron micrographs, is antigenic(2), readily purified by dextran gel chromatography(3) and can be trace labelled with I^{131}(3).

Materials and methods. Antigen and antibody. Horse spleen ferritin (HSF) was obtained commercially (Pentex Corp., Kankakee, Ill.) and further purified by chromatography through Sephadex G-200(3). Nitro-

* This investigation was supported in part by the Ernest S. Bazley Asthma Research Fund, Chicago Wesley Memorial Hospital, and USPHS Research Grants NB05170-01 and AM-06998-03.

† Research Career Development Award 3-K3-AI-19,460-02, USPHS.

TABLE I. Ingestion of I*HSF-Anti HSF Complexes Prepared in Antigen Excess by Guinea Pig Spleen Cells Using Rabbit Antibody.

Tube	1	2	3	4	5	6	7	8
Complex added*	1.5	3.0	1.5	3.0	None	None	1.5†	1.5†
I*HSF added§ to cells (μg N)	1.2	1.2	.012	.012	1.2‡	.012‡	1.2	.012
% radioactivity in cells	19.6	6.5	28.2	11.0	3.6	4.0	1.3	4.0

* Expressed as degree of antigen excess used in preparation of complex (see text).
† Cells heated to 56°C for 30 minutes prior to addition of complexes.
‡ I*HSF alone added to cells.
§ Expressed as μg N of ferritin added to appropriate dilution of antiferritin.

gen content of ferritin was determined by Markham's modification of the micro-Kjeldahl reaction(4). The ferritin was labeled with I^{131} (I*) as previously described(3). Rabbit antiferritin was prepared by immunization with HSF(3) and the anti HSF serum conjugated with fluorescein isothiocyanate (5,6). The antibody content of the anti HSF serum was determined by the precipitation of I^{131} (I*HSF)(3)and expressed as μg of ferritin N precipitated by 1 ml of anti HSF serum.

Soluble antigen-antibody complexes. HSF-anti HSF complexes in antigen excess were prepared in the manner previously described for preparation of bovine serum albumin-anti bovine albumin complexes(1). The preparation of the I*HSF-anti HSF complexes in antigen excess is shown in Table I. Antigen-antibody complexes in antibody excess were prepared from very dilute solutions of I*HSF and rabbit anti HSF. The solubility of the complexes in antibody excess was the result of the high dilutions of antigen and antiserum from which the complexes were prepared. The I*HSF-anti HSF complexes prepared in antibody excess could be demonstrated in solution by means of coprecipitation of the labeled complexes with unlabeled HSF and anti HSF.

Detection of uptake of complexes by cells. The ingestion of complexes by cells *in vitro* was determined using aliquots of cell suspensions in separate tubes. Cells were incubated with I*HSF-anti HSF complexes or I*HSF alone and washed as previously described(1). Some cells were heated to 56°C prior to addition of complexes. The radioactivity of washed cells, supernatant solutions, and the per cent uptake of complexes was determined and aliquots were prepared for immunofluorescent and electron microscopic examination. All *in vitro* experiments with phagocytic cells were conducted in the presence of complement provided by fresh normal guinea pig serum.

Electron microscopic preparations. Two per cent osmium tetroxide was added to cells suspended in tissue culture medium (pH 7.4) to make a final concentration of 1%. Following fixation for 30-45 minutes at 4°C, the cell suspensions were centrifuged and the osmium tetroxide solution decanted. The cells were rapidly washed with distilled water, dehydrated with a graded series of ethyl alcohol, and treated with propylene oxide prior to embedding in Araldite 502(7) or Maraglas (8). Sections were mounted without supporting film on copper grids, lightly stained with 2.5% aqueous uranyl acetate and examined with an Hitachi HU-11A electron microscope or a Siemens Elmiskop I electron microscope. Images were recorded on Cronar ortho litho film which was developed in D-19.

Results. Passive ingestion of soluble antigen-antibody complexes prepared in antigen excess. The uptake of I*HSF-anti HSF complexes by guinea pig cells (Table I) is similar to that described for bovine serum albumin-anti-bovine serum albumin complexes(1). A lower percentage of cell bound radioactivity was found when complexes were prepared in greater antigen excess. The cells to which I*HSF alone was added or cells heated to 56°C for 30 minutes prior to ingestion of complexes contained significantly less radioactivity than living cells exposed to the complexes. Preparations of heated or unheated cells after exposure to antigen or complexes studied by immunofluorescent microscopy

TABLE II. Ingestion of Homologous and Heterologus Complexes by Spleen Cells.

	Chicken spleen cells				Guinea pig spleen cells				
Antiserum*	Rabbit	Chicken		None†	Rabbit		Chicken		None†
% radioactivity in cells	16	22	3.2‡	10	22.8	2.9‡	4.9	4.2‡	4.6

* Complexes prepared from these antisera at 1.2 times equivalence and containing .05 μg of I*HSF were added to cells.
† .05 μg of I*HSF alone added to cells.
‡ Cells heated to 56°C for 30 min.

TABLE III. Ingestion of I*HSF-Anti HSF Complexes Prepared in Antibody Excess.

	Guinea pig spleen cells					Mouse peritoneal macrophages			
I*HSF added (μg N)	.002	.004	.002*	.004*	.002†	.002	.004	.002*	.004*
% radioactivity in cells	49.0	44.3	1.2	2.1	2.9	11.2	8.3	1.4	1.0

* Antigen alone added to cells.
† Cells heated to 56°C prior to addition of complexes.

confirmed the ingestion of complexes by demonstration of fluorescent material in the cytoplasm of macrophages, mononucleocytes and polymorphonuclear cells. Cells exposed to HSF alone and heated cells exposed to complexes were markedly less fluorescent and served as controls.

Separate experiments were consistent in the relative degree of uptake of complexes and free antigen by unheated or heated cells. While this proportionate uptake is consistent, it varies with different experiments (guinea pig spleen cells, Tables I and II). This is probably the result of variations in numbers of viable phagocytic cells in different spleen cell preparations. Cell counts do not accurately record numbers of viable phagocytic cells(1).

The uptake of soluble complexes by chicken spleen cells was similar using complexes prepared from chicken or rabbit antiserum (Table II). Chicken spleen cells showed a higher retention of HSF alone than did guinea pig spleen cells. Uptake of soluble complexes by guinea pig spleen cells appeared to be dependent upon source of antibody (Table II), being consistently less in the case of complexes prepared with chicken antibody than those prepared with rabbit antibody.

Immunofluorescent studies of spleen cells to which I*HSF-anti HSF complexes had been added confirmed the presence of HSF in the cytoplasm. Controls of heated cells were negative and preparations of cells to which HSF alone was added were only faintly positive or negative.

Ingestion of complexes by mouse macrophages. Because the normal guinea pig spleen cells without added complexes or HSF contain sufficient autologous ferritin to be readily visible in electron micrographs, phagocytic cells from another source were necessary for controlled electron microscopic studies. The use of mice in passive immune phagocytic experiments has been established and electron microscopic examination of mouse peritoneal macrophages did not demonstrate significant amounts of ferritin. Initial electron microscopic observations indicated that mouse peritoneal macrophages ingested both HSF-anti HSF complexes and HSF alone but failed to differentiate between these two types of preparations. A significant difference was demonstrated, however, by radioactivity or immunofluorescent techniques. To demonstrate a more marked difference, complexes were prepared in antibody excess using a 1:12,000 dilution of rabbit anti F. One ml of undiluted antiserum would maximally precipitate 990 μg of HSF N, and 0.02 μg HSF N was added to 1 ml of diluted antiserum. The high dilution of anti HSF was necessary to prevent precipitation of antigen and antibody. That the HSF-anti HSF complexes were soluble was demonstrated by the inability of additional antibody to precipitate the I*HSF. These complexes were ingested by guinea pig spleen cells *in vitro* and by mouse macrophages (Table III). The greater

percentage of radioactivity in spleen cells using these complexes compared to complexes prepared in antigen excess might reflect the larger size of complexes in addition to the decreased content of free antigen when complexes were prepared in antibody excess. The higher uptake of complex by guinea pig spleen cells over mouse macrophages (Table III) is the probable result of the greater number of spleen cells available for ingestion of complexes in these preparations. The mouse macrophages, stained for immunofluorescence by labeled rabbit anti HSF, are shown in Fig. 1. Controls of cells alone or cells to which HSF alone was added were negative after staining with fluorescent antiserum.

The intracellular distribution of ferritin, determined electron microscopically, was as follows: 1) apparently free, within the ground cytoplasm of the cell; 2) within pinocytotic vesicles; 3) and within cytoplasmic dense bodies, morphologically similar to lysosomes (Fig. 2). Additionally, ferritin was found adherent to cell membranes. In such cells an increased density of the cytoplasm was demonstrated immediately subjacent to membrane sites of ferritin binding, which appeared similar to structure described previously in other cells active in pinocytosis(9, 10,11).

In electron micrographs, it was not possible to differentiate HSF and HSF-anti HSF complexes morphologically. The intracellular distribution of ferritin, administered as HSF or HSF-anti HSF complexes did not differ significantly.

Discussion. The study of the dynamics of the transport of solutes or particles across cell membranes, differentially termed pinocytosis or phagocytosis depending on the solubility and size of the ingested materials, would be aided by a system of isotope labeled, electron dense material which can be traced by cell bound radioactivity and electron microscopic localization. The use of soluble I*HSF-anti HSF complexes provided such a system which could be evaluated further by the use of fluorescein isothiocyanate labeled anti HSF antibody. The I*HSF-anti HSF complexes are eliminated more rapidly from the circulation

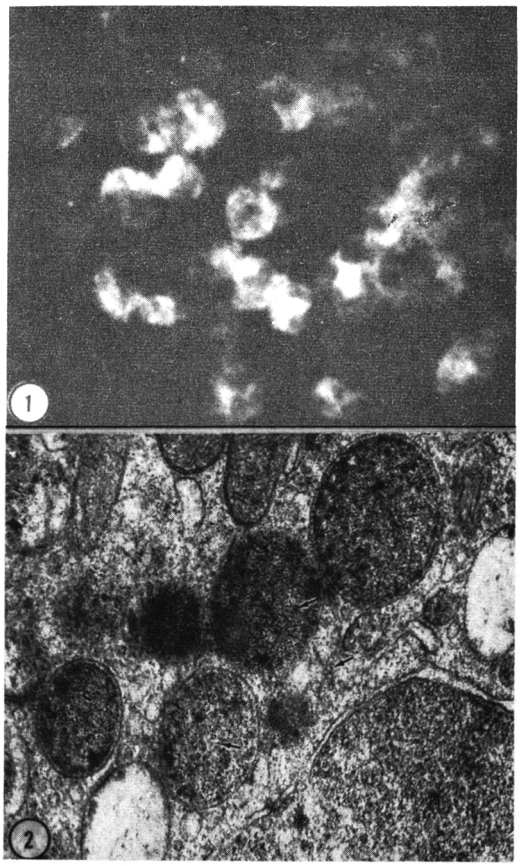

FIG. 1. Photomicrograph of mouse peritoneal macrophages stained for HSF-anti HSF complexes after exposure to complexes *in vitro* and staining by immunofluorescent techniques. Degree of staining varies with different cells. × 1,000.

FIG. 2. Electron micrograph of mouse peritoneal macrophage after exposure of macrophage to HSF-anti HSF complexes *in vitro*. Ferritin (arrow) is seen free within the ground cytoplasm and in cytoplasmic dense bodies. × 40,000.

of guinea pigs than I*HSF alone and more radioactivity is localized in the spleen in those animals receiving passive antibody than antigen alone, an analogous system to that described for bovine serum albumin-anti bovine serum albumin complexes(12).

Studies in which electron microscopy, isotope tracing and immunofluorescent microscopy could be used for joint evaluation provided a means of comparative examination. Isotope tracing indicates that mouse phagocytes ingest a significantly greater quantity of HSF when presented as HSF-anti HSF soluble complexes than free HSF unbound to

antibody. Because it was not possible to differentiate intracellular HSF and HSF-anti HSF complexes morphologically in this system and because there was no significant difference in the intracellular distribution of HSF, whether presented as the antigen alone or as a soluble complex, the explanation for these quantitative findings remains unknown. Whether the increased uptake in the presence of increasing amounts of antibody is the result of complex size or bears a specific relation to the antibody component of the globulin is uncertain.

Ferritin is known to enter cells readily by pinocytosis and perhaps also by a more rapid mechanism similar to pinocytosis(13). Presumably soluble ferritin-antiferritin complexes enter even more rapidly, as large molecular weight complexes. The possibility remains, however, that the complex, having activated the cell membrane, dissociates into component parts which separately are incorporated into the cell.

The difference noted between degree of uptake of rabbit or chicken complexes by guinea pig cells suggests the possibility of a specific difference in the recognition of complexes prepared with antisera of different species of phagocytic cells.

Summary. The ingestion of soluble ferritin-antiferritin complexes was studied by parallel immunofluorescent, electron microscopic and isotope trace labeled antigen methods. The complexes were ingested by guinea pig spleen cells or mouse peritoneal macrophages. The degree of cellular activity demonstrated by these techniques was dependent on the degree of antigen excess used in preparation of the complexes, the source of antisera and the particular methodology applied.

1. Patterson, R., Suszko, I. M., Pruzansky, J. J., J. Immunol., 1963, v90, 829.
2. Mazur, A., Shorr, E., J. Biol. Chem., 1950, v182, 607.
3. Patterson, R., Suszko, I. M., Pruzansky, J. J., Proc. Soc. Exp. Biol. and Med., 1965, v118, 307.
4. Kabat, E. A., Mayer, M. M., Experimental Immunochemistry, 2nd Ed., Charles C Thomas, Springfield, Ill., 1961.
5. Marshall, I. D., Eveland, W. C., Smith, C. W., Proc. Soc. Exp. Biol. and Med., 1958, v98, 898.
6. Peters, J. H., McDweitt, H. O., Pollard, A. W., Harter, J. G., Coons, A. H., Fed. Proc., 1961, v20, 17.
7. Glauert, A. M., Rogers, G. E., Glauert, R. H., J. Biophys. Biochem. Cytol., 1958, v4, 191.
8. Spurlock, B. O., Kattine, V. C., Freeman, J. A., J. Cell Biol., 1963, v17, 203.
9. Roth, T. F., Porter, K. R., ibid., 1964, v20, 313.
10. Rosenbluth, J., Wissig, S. L., ibid., 1964, v23, 307.
11. Fawcett, D. W., Anat. Rec., 1964, v148, 370.
12. Patterson, R., Weigle, W. O., Dixon, F. J., Proc. Soc. Exp. Biol. and Med., 1960, v105, 330.
13. Bondareff, W., Exp. Neurol., 1964, v10, 237.

Received March 29, 1965. P.S.E.B.M., 1965, v119.

Influence of Oligodeoxyribonucleotides on Early Events in Antibody Formation.* (30276)

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Oligodeoxyribonucleotides, present in enzymatic digests of DNA, can enhance bacterial population changes(1,2,3), can cause a selective stimulation of DNA synthesis in "resting" Gram-positive bacteria(4), and can restore immune responses in irradiated animals(5) as well as in animals made nonresponsive to a specific antigen by administration of 6-MP(6). These effects are independent of the source of DNA employed in preparation of the oligodeoxyribonucleotides. Oligonucleotides can also produce an occasional elevation of circulating antibody levels in normal, mature animals exposed to antigen plus DNA digest(7,8), or antigen plus RNA digest(8), and they can increase host-resistance to a variety of Gram-negative pathogens provided the oligonucleotides are ad-

* Supported by grants from U.S.P.H.S. and National Science Foundation.