STUDIES ON THE UPTAKE AND FATE OF HEMOGLOBIN PINOCYTOSED BY RAT EMBRYO FIBROBLASTS CULTURED IN NORMAL OR LOW MAGNESIUM MEDIA

by

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LIST OF ABBREVIATIONS

BME	- Basal medium Eagle
BSA	- Bovine serum albumin
BSS	- Balanced salt solution
Cl^-	- Chloride ion
DOC	- Deoxycholate
FlHb	- Fluorescein labeled hemoglobin
Hb	- Hemoglobin
HEPES	- N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid
HSA	- Human serum albumin
I^*-Hb	- Radioactive iodine labeled hemoblobin
I^*-HSA	- Radioactive iodine labeled human serum albumin
K^+	- Potassium ion
Li^+	- Lithium ion
Mg^{2+}	- Magnesium ion
Na^+	- Sodium ion
nbcs	- Newborn calf serum
NH^+	- Ammonium ion
NO_3^-	- Nitrate ion
PBS	- Phosphate buffered saline
SO_4^{2-}	- Sulfate ion
TCA	- Trichloroacetic acid

INTRODUCTION

The metabolism of a macromolecule by cells can be divided into three general steps. The first, pinocytosis, is the invagination of the cell membrane with the bound macromolecule, and subsequent vesicle formation. The second is the merger of this vesicle with a primary lysosome to form a secondary lysosome. It is within this secondary lysosome that digestion occurs. The final step is the release of undigestible fragments or unusable molecules to the exterior of the cell. The control of the coordinated processes which governs the vacuolar apparatus in mammalian cells has not been studied extensively. In particular, the influence of the medium composition is of interest in that the external medium will become the intra vacuolar medium following vesicle formation.

The purpose was to obtain supplemental baseline information on the behavior of this cell system under optimum conditions and in response to alterations in the Mg^{2+}[1] concentration of the culture medium. A study of these processes for the metabolism of a fluorescein labeled hemoglobin was undertaken utilizing rat embryo fibroblasts in cell culture. The fibroblasts, in secondary culture, were treated with medium containing fluorescein labeled hemoglobin. Various experiments were performed to measure the metabolism of FlHb and to determine any effect of the low Mg^{2+} (0.204mM) concentration. The control medium contained

[1]The abbreviations used in this text are defined in the List of Abbreviations, page viii.

Mg^{2+} (1.82mM) close to the physiological concentration (0.88mM) while the low magnesium medium contained only the Mg^{2+} present in the serum, which was about one tenth of the control concentration.

After harvesting, the fluorescence was measured and converted into the amount of FlHb accumulated by the cell. From this type of data any effect of the Mg^{2+} could be measured.

LITERATURE SURVEY

The process of cellular digestion is complex with many parts working together to reduce ingested material to the level of utilizable compounds.

The central part of this system is the lysosome which was discovered by de Duve and co-workers in late 1949. Liver lysosomes have a mean diameter of 0.4μm and an average specific gravity of about 1.15 with wide variations about these values (1). Christian de Duve has suggested that the term lysosomes is too general and should be further qualified. He has defined at least three types of lysosomes; primary lysosome, secondary lysosome, and a post-lysosome. The primary lysosome is a storage granule containing the characteristic hydrolytic enzymes. The secondary lysosome arises following combination of primary lysosomes with vacuoles, usually endocytic, containing material to be digested. The secondary lysosome is larger than the primary and it is within this organelle that digestion actually occurs. The secondary lysosome may be sub-divided into the heterolysosome which digests extracellular material or the autolysosome which digests endogenously derived material. The post or telolysosome, often called the residual body, is thought to contain the undigestible matter taken up by the cell (2).

The secondary lysosome contains a number of enzymes including nucleases, proteases, esterases, polysaccharidases, and glycosidases. All those studied to date have been found to have an acidic pH optimum suggesting that lysosomal digestion occurs at a low pH. Although each enzyme is rather specific in its point of attack, the combined action

can carry digestion of a wide range of ingested material to completion (2). Detailed studies have been conducted by Coffey and de Duve (3) *in vitro* on lysosomal enzymes. The digestion of acid denatured human or bovine globin by extracted rat liver lysosomal enzymes was about 70% complete at 24 hours with only a slight increase at 48 hours. The remaining undigested products were a mixture of peptides mostly dipeptides. The pH optimum ranged from about 4.0 to 5.7. The peptides were degraded further with a soluble fraction from rat liver. Thus only about 10% of the original protein bonds were not cleaved.

A theory of lysosome formation has been proposed by Novikoff, *et al*. (4). Proteins are synthesized at the ribosomes, pass into the rough endoplasmic reticulum and then to the smooth endoplasmic reticulum. The proteins then move to microbodies or to lysosomes. There are four possibilities suggested. The first is that protein travels to the Golgi saccules which then pinch off to form the Golgi vesicles. These vesicles then migrate to secondary lysosomes. An alternative pathway is from the Golgi saccules to the Golgi vacuoles. The third possibility is that material migrates from the smooth endoplasmic reticulum into the dense bodies. The fourth pathway is the migration of the protein from the smooth endoplasmic reticulum directly into autophagic vacuoles.

Endocytic vesicles or vacuoles are formed by the invagination and fusion of the cell membrane to form a vesicle. Although the size is quite varied, there are two major groups of vesicles. Phagosomes are formed by the incorporation of an insoluble particle such as a bacterium or a latex bead. Pinosomes are formed by the incorporation

of soluble material adhering to the membrane or free in the extracellular fluid. Pinosomes are usually smaller than the phagosomes (5).

Pinocytosis is a property of many types of cells other than macrophage and amoebae such as mouse sarcoma S-187 cells (6)(7), capillary endothelial and mesothelial cells (5), cells of the kidney tubules, thyroid cells, adipose cells, intestinal lumen cells, cultured strain-L-fibroblasts, and He La cells (2). Cultured hamster fibroblasts, osteoclasts, synovial cells (8), and parenchymal liver cells (9) have also been observed to carry out pinocytosis.

The factors which influence the selection and rate of uptake of proteins have been studied extensively by Ryser (6)(7). There is the same amount of binding at 1 minute as there is at 60 minutes when mouse sarcoma S-180 II cells are exposed to ^{131}I-albumin at 4°. This is a reversible binding which can be removed by several washings. As the temperature is raised, increased amounts of protein are pinocytosed with time, as it is not removable by continual washings. Electron microscopy studies of ferritin uptake indicate that protein absorbs to the cell surface and is localized in vesicles.

Pinocytosis of protein is a semi-selective process according to Ryser (6). Basic poly-amino acids such as poly-L-lysine and poly-L-ornithine not only enhance the uptake of albumin but they themselves are also taken up. Several generalizations can be made concerning the uptake of a polypeptide. As the molecular weight increases, all other factors remaining constant, the uptake also increases. Basic proteins are taken up more readily than the more acidic ones and can

also enhance the uptake of the acidic proteins. Factors other than charge and size must enter into the selection for protein uptake as poly-D-lysine is preferentially taken up over poly-L-lysine. In the macrophage, however, the basic poly amino-acids have no effect on pinocytotic vesicle formation (5). In Acanthamoeba castellanii (10) there appears to be very little if any surface binding prior to pinocytosis for several different substrates. In thin sectional material there appears to be no cell surface coat. The evidence does, however, support bulk transport of material in pinocytotic vesicles.

Pinocytosis in some species of fresh water amoeba has been studied extensively by Chapman-Andresen (11) over the past few years. The three kinds used were Amoeba proteus, Amoeba dubia, and Chaos chaos. These organisms can withstand a wide range of conditions, pH 4 to 10 and osmotic pressure from 0 to 250 m osmoles. These amoebae have a typical unit membrane with an exterior mucus coat.

Pinocytosis in the amoeba (11) lends itself for study under the microscope as the channel formation is quite visible and can be measured by counting. The actual pinocytotic vesicles form at the base of the channel and can only be seen on electron micrograph.

When an amoeba was placed in an inducing solution the largest number of channels formed at 10 to 15 minutes and then the number began to decrease. By 20 to 30 minutes very few channels remained. It is estimated that about 50% of the surface membrane was interiorized in one simple pinocytotic cycle. After each period of pinocytosis, a rest period was needed before another cycle began.

The induction of pinocytosis (11) in these amoebae was caused by a binding of an inducing solute to the mucus coat which was independent of temperature. The invagination of the membrane to form the channel was temperature dependent with a maximum at about 23°. Pinocytosis was relatively non-specific for a large number of different compounds. There seemed to be very little relationship between molecular weight or osmotic pressure of the compound and the extent of pinocytosis. It did seem to be somewhat dependent on ionic strength of the inducer. Uncharged compounds such as high and low molecular carbohydrates, urea, and alcohol have no effect in causing pinocytosis.

The three groups of compounds which were most effective in inducing pinocytosis in these amoebae (11) were the inorganic salts, proteins, and alcian dyes. Amino acid salts had the same effect as inorganic salts. The inorganic salts were inducers at neutral pH in a concentration range around 0.1 M. As the pH was raised or lowered from neutrality, pinocytosis decreased. The inducing solution must be present all during channel formation, for if the amoeba was removed to a non-inducing culture medium, pinocytosis ceased. This could indicate a very labile surface binding. Proteins could act as inducers at pH's where they exist as positively charged molecules. The surface binding appeared to be more stable since replacement with a non-inducing solution of the same pH caused no halting of pinocytosis. If, however, transfer was made to a solution in which the pH caused a change in the charge on the protein, the protein was released and pinocytosis stopped. Alcian dyes formed an irreversible binding to the mucus coat.

In a qualitative study of the effect of cations, Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, Li^{+}, and NH_4^{+}, Chapman-Andresen (12) used phase contrast microscopy to measure pinocytosis in Amoeba proteus. The Cl^{-}, NO_3^{-}, and SO_4^{2-} salts of Mg^{2+} were used in a range of 7.8 mM to 1 M. The method of measuring pinocytosis was similar to that in other studies (11). No pinocytosis was observed in any cation solution below 7 mM. The $MgCl_2$ solution induced slight pinocytosis at 15.6 mM and intense pinocytosis at 0.125 M. The amount of pinocytosis decreased to zero when the concentration was raised to 0.5 M. The nitrate salt caused pinocytosis from 0.062 M to 0.5 M with an optimum at 0.250 M. The sulfate salt caused pinocytosis in the same range as the chloride but with greater intensity from 0.0625 M to 0.250 M. Calcium salts had much less effect than the Mg^{2+} salts. The calcium sulfate was not measured above 15.6 mM due to its insolubility (12).

Pinocytosis versus ionic strength was measured (12) for three Mg^{2+} salts. The overall range of ionic strengths which caused effects were 0.2 to 1.0. The peak for $MgCl_2$ was 0.4, $Mg(NO_3)_2$ was 0.7, and $MgSO_4$ was 0.7. Both $MgCl_2$ and $Mg(NO_3)_2$ were toxic above 0.75 but this was considered an anionic effect. Although for Mg^{2+} salts pinocytosis began at a higher ionic strength, the range was greater and the intensity was somewhat higher than the other cations studied.

A study by Cooper (13) involving Amoeba proteus was conducted utilizing various cation concentrations in a 0.67mM phosphate buffer, pH 6.85 to 6.99. This time, channels per minute and percent forming channels were measured. If the Mg^{2+} concentration was raised from 0.30mM

to 200mM, the channel formation went from about 5 per minute to about 75 per minute. The percent forming channels went from 40% to 80% as the concentration was increased from 60mM to 200mM. At 85mM 50% of the amoebae formed channels. These channels formed from Mg^{2+} induction and were short-lived.

A biochemical study of pinocytosis, digestion, and release of material in mammalian cells was undertaken by Ehrenreich and Cohn (14). They measured the accumulation and digestion of iodinated human serum albumin in culture of macrophages. The macrophages were placed in medium with 50% nbcs containing I^*-HSA. After 24 hours the cells had a net uptake of 10^{-2}µg per million cells. The accumulation versus time curve shows an initial rapid rise with a leveling off beginning at about 10 hours. To determine if pinocytosis had actually been responsible for the ingested I^*-HSA, the net uptake was measured under conditions of 10%, 20%, and 50% nbcs. Pinocytic vesicle formations have been shown (15) to be dependent on the concentration of serum in the medium. An inhibitor, para-fluorophenylalanine, of pinocytic vesicle formation was also used with 50% nbcs. In the previous study (14) there was a regular increase in accumulation of I^*-HSA as the serum content was increased. The sample with the inhibitor showed only a fraction of the net uptake compared to the one without it. A polyanionic stimulator of pinocytosis, dextran sulfate, was placed in a culture with 50% nbcs which caused an increase in pinocytosis over the control. Electron micrographs also indicated localization of the ^{125}I-HSA within vesicles.

In a "pulse" experiment (14) the macrophages were exposed to ^{125}I-HSA for 24 hours. The medium was then replaced with non-radioactive

medium for the washout. The amount of ^{125}I-HSA remaining in the cells showed a sharp decline at first which tended to level out with time. The viability was checked by measuring the normal uptake of ^{131}I-HSA as the cells were losing a pulse of ^{125}I-HSA. Therefore the loss of the ^{125}I-label was not caused by cell impairment. Using ^{125}I-bovine gamma globulin in a pulse experiment, similar loss of radioactivity occurred. Using a washout medium of 1% nbcs instead of 50% nbcs made no appreciable change in the loss of the soluble radioactive material from the cells. Therefore digestion and release are not dependent on pinocytosis. Inhibitors of pinocytotic vesicles likewise had no effect on release of material. But a lowering of temperature to 4° did reduce the loss of material to the washout medium. This temperature effect was completely reversible if incubated again at 37°.

The washout medium was also analyzed (14) for TCA soluble radioactive material lost from pulsed cells. The increase in soluble products was about equal to the decrease in the cell radioactivity. Fractionation of the washout medium by gel filtration showed the TCA soluble radioactive material to be of low molecular weight. Using paper chromatography the TCA soluble ^{125}I material corresponded to added ^{131}I-Monoiodotyrosine.

These experiments (14) indicate that a protein was taken up by pinocytosis, digested in the cell and unusable fragments returned to the medium. ^{131}I-Monoiodotyrosine was the only fragment found but small labeled peptides were not excluded. The only factor, of those tested, which affected the digestion and release of the fragments was

a temperature decrease. This was to be expected as many digestive enzymes are temperature sensitive. It was also concluded that regurgitation of the lysosome content into the medium did not occur under these conditions since no fragments of intermediate size were found.

Cohn and Ehrenreich (16) also studied hemoglobin taken up by macrophages in culture. ^{3}H-Leucine labeled hemoglobin was used as the substrate. This avoided the question of a modified amino acid such as ^{125}I-tyrosine acting differently from an unlabeled one. The label made the amino acid indistinguishable from the unlabeled one. To prevent re-utilization of this labeled leucine, cold leucine was added to the culture medium.

The macrophage were tested (16) with ^{125}I-hemoglobin uptake for 24 hours. The accumulation was 10^{-3}µg per µg of cell protein. The loss of a pulse of ^{131}I-Hb was about 50% in 24 hours and was less than the loss of ^{131}I-HSA. The uptake and digestion of ^{3}H-leucine - Hb was the same as that of I^{*}-Hb. The loss from the cells of a ^{3}H leucine - Hb pulse appeared in the medium as leucine. There were no peptides of leucine detected but the possibility of a small number cannot be excluded. Cohn and Ehrenreich showed that the Hb was very similar to HSA in its uptake, digestion, and release of fragments.

These types of studies had also been conducted in other mammalian cells in cultures. Studies of lysosomes from rat embryo fibroblasts were carried out by Tulkens et al. (17). The medium contained 0.1 mg of fluorescein-labeled γ-globulins per ml or 6.3×10^{-7} M. The net uptake of the γ-globulin in a 30-hour period was 8.6µg per mg of cell

protein. This corresponded to 5.4×10^{-8} mmoles per mg of cell protein. Fluorescence microscopy revealed the presence of fluorescence within cytoplasmic bodies. Density gradient centrifugation of fibroblast homogenates exposed to γ-globulin for 30 hours revealed the fluorescence followed the hydrolytic enzymes.

Lysosomal digestion was inhibited by γ-globulin from rabbits which had previously been immunized against proteins of the lysosomal soluble fraction. The in vitro activity of certain lysosomal enzymes decreased when exposed to medium containing lysosomal protein antibodies. Cells were incubated in media with and without antibodies for 4 days. In the control medium there was micromolecular fluorescent material; however, in the antibody treated medium these fragments were absent.

Tulkens (18) cultured rat embryo fibroblasts for 4 days in a 10% calf serum with 9.8 mg fluorescein labeled γ-globulin per 100 ml of medium. Both the cell homogenate and the medium were fractionated by gel filtration. The medium contained 98% of the total fluorescence with 65.5% being macromolecular and 32.5% micromolecular. The cellular content was only 2% of the fluorescence, 1.66% being macromolecular and 0.34% micromolecular. The total macromolecular fluorescence material was 66.16% and the micromolecular was 32.84%. In 4 days 3.3 mg of γ-globulin had been degraded by 12.3 mg of cells.

The work presented in this thesis is a study of the normal behavior of the fibroblast in the rates both of accumulation and digestion of a fluorescein labeled hemoglobin. This study will contribute to improved understanding of the vacuolar apparatus in cells not specialized for a

scavenger role, and will also provide a model for the testing of various nutrients and their effect on the vacuolar apparatus. This study specifically has tested the impact of variation in Mg^{2+} concentration on this system.

EXPERIMENTAL PROCEDURES

Materials - The hemoglobin type I, bovine serum albumin, and fluorescein isothiocyanate (isomer I), were obtained from Sigma Chemical Company, St. Louis, Missouri. The phenol red, N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid, L-glutamine, lactalbumin hydrolysate, sodium deoxycholate, penicillin "G" and streptomycin sulfate were purchased from Nutritional Biochemicals, Cleveland, Ohio. Glucose, NaCl, KCl, $CaCl_2$, $MgSO_4 \cdot 7H_2O$, Na_2SO_4, $NaH_2PO_4 \cdot H_2O$, and Na_2HPO_4 were purchased from J. T. Baker Chemical Co., Phillipsburg, New Jersey. The trypsin (powder), crystal violet, and tissue culture select calf serum were obtained from BBL, Cockeysville, Maryland. The vitamin mixture for basal medium Eagle (100X) came from Microbiological Associates, Bethesda, Maryland. Sephadex G25 and G50 were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. Pregnant Sprague Dawley rats were obtained from Flow Research Lab., Dublin, Virginia.

Tissue Culture Procedures - Standard procedures for obtaining rat embryo fibroblasts were used as recommended by Tulkens (18). Various modifications were made to adapt the procedures to the equipment available in this laboratory.

The primary culture was derived from the fetuses of Sprague Dawley rats, 17 to 20 days pregnant. The rat was killed by decapitation, and the abdomen, sterilized with 70% ethanol, was opened in the sterile atmosphere of a Laminaire hood. The uterus was removed from the rat, and the amniotic sacs containing the fetuses were removed from the uterus. The fetuses were removed from the amniotic sacs and eviscerated.

The residual carcasses were then placed in PBS and minced with scissors.

The tissue was then transferred to the trypsinizing apparatus[2] where it was washed for three to five minutes to remove erthrocytes. The PBS was then drained off and 200 ml of 0.1% trypsin in PBS at 37° was added. The entire mixture was stirred vigorously. The individual cells and small bits of tissue pass through a 40 mesh stainless steel screen to the inner container. After 30 minutes, the cell suspension was withdrawn and strained through an 80 mesh screen into an ice cold centrifuge bottle. One millimeter of calf serum was added to stop the trypsin action. The cell suspension was then centrifuged at 271 x g for 10 minutes. The pellet was washed three times in approximately 50 ml BME and 1 ml calf serum. During the last wash the cells were again strained through 80 mesh screen to remove agglutinated cells. The cell suspension was transferred to a 50 ml graduated centrifuge tube and centrifuged. The resulting pellet contained erythrocytes at the bottom and the colorless fibroblasts in the upper layer. This upper layer was removed and resuspended in 20 ml of BME.

A small portion of the cell suspension was stained with crystal violet and counted in a hemocytometer. The remaining suspension was seeded in Roux bottles at a rate of 15×10^6 cells per bottle. The cells were incubated at 37° for three days.

Subcultures were made in Leighton tubes from the cells grown in the Roux bottles. The medium in the Roux bottles was removed by

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[2]Designed by P. Tulkens, University of Louvain, Belgium.

aspiration and the cells washed once with 50 ml of warm PBS. Ten milliliters of warm trypsin were added to each Roux bottle and then incubated at 37° for 10 to 15 minutes to detach the fibroblasts. To each Roux bottle was added 1 ml of calf serum to stop the trypsin action. Ten ml of BME were added to the bottle and the cell suspension was transferred to a 200 ml centrifuge bottle and centrifuged at 205 x g for 10 minutes. The cells were resuspended and washed once in approximately 50 ml of BME and 1 ml of calf serum. The cell suspension was transferred to a conical centrifuge tube and centrifuged. The volume of cells in the pellet was measured and then resuspended in 50 ml of BME, the seeding rate being 0.08 ml cells per 100 ml of culture medium. The medium was identical to that of the primary culture except for the addition of 2 ml of 0.5 M HEPES buffer, and 2 ml of 5% $NaHCO_3$, to every 100 ml of medium. To each Leighton tube was added 3 ml of the inoculate. The cells were incubated at 37° for two days.

Treatment, Care, and Harvesting the Cells - The seeding rate was chosen so as to produce a monolayer in two days. If this was not achieved, then the experiments were delayed until the cells were nearly confluent. There were 10 tubes in each Leighton tube rack.

During growth and maintenance of the cells, the pH of the medium decreased as a result of organic acids and CO_2 formation. The pH was readjusted to the original value by addition of 5% $NaHCO_3$. The bicarbonate treatment varied from culture to culture but an average was about one to three drops twice daily.

If the experiment involved cells in low Mg^{2+} medium, both control

and test cells were rinsed once in low Mg^{2+} medium to ensure equal treatment. In order to minimize the variation between cells treated with normal or low Mg^{2+} medium, five tubes of each group were placed on the same rack. All work performed on the cells was carried out in the sterile atmosphere provided by the Laminaire hood. Except in certain experiments, the FlHb in the medium was at a concentration of 200µg/ml which amounted to about 3% of the total protein.

Before harvesting, the cells were inspected under tissue culture microscope and all contaminated or unhealthy cultures were discarded. The medium was aspirated from the Leighton tube and each tube washed 3 times with 5 ml of warm PBS. It was important to complete the washing procedure on each tube before proceeding to the next, as the cells would begin to detach after a short period of time. To each tube 3 ml of 0.65% DOC, pH 10.5 were added and then it was mixed for one minute on a vortex mixer. The tubes were then stored frozen until the desired analysis could be made. Upon thawing, the tubes were again briefly mixed.

Medium - The culture medium used for fibroblast was a modified Eagle's basal medium with Earl's balance salt solution supplemented with 10% calf serum (18). The BME was prepared in 5 ℓ lots in the laboratory from its individual constituents (see Table I).

Solution A was prepared separately and was cloudy. In preparation of solution B, the first component was dissolved in one liter of double distilled water. Each additional component was dissolved in approximately 50 ml of water and then added with stirring to solution B.

TABLE I

A. Composition of modified Eagle's basal medium with Earle's BSS for fibroblasts

Solution A	Quantity/10 ℓ	Quantity/5 ℓ	mM
1. Lactalbumin Enzymatic Hydrolysate	50.00g	25.00g	-
2. Phenol red	0.20g	0.10g	5.6×10^{-2}
Solution B			
1. NaCl	64.00g	32.00g	110.0
2. KCl	4.00g	2.00g	5.36
3. $CaCl_2$	2.00g	1.00g	1.82
4. $MgSO_4 \cdot 7H_2O$	4.02g	2.01g	1.64
4a. Na_2SO_4[a]	2.32g	1.16g	1.64
5. NaH_2PO_4	1.25g	0.625g	1.04
5a. $NaH_2PO_4 \cdot H_2O$	1.43g	0.715g	-
5b. $NaH_2PO_4 \cdot 2H_2O$	1.62g	0.810g	-
6. $NaHCO_3$	7.50g	3.75g	8.93
7. Glutamine	0.30g	0.15g	0.205
8. Glucose	45.00g	22.50g	25.0
9. BME vitamin (100X)	100 ml	50 ml	-
10. Penicillin "G"	0.632g[b]	0.316g	-
11. Streptomycin	1.00g	0.50g	-

B. Vitamin concentration in BME

Vitamin	mg/10 ℓ	mg/5 ℓ
1. D-Biotin	2.443	1.222
2. D-Ca-Pantothenate	4.765	2.383
3. Choline Chloride	1.396	0.698
4. Folic acid	4.414	2.207
5. Nicotinamide	1.221	0.611
6. Pyridoxal HCl	2.036	1.018
7. Riboflavin	3.376	0.188
8. Thiamine HCl	3.373	1.687

[a] Used in place of $MgSO_4$ for Low Mg^{2+} medium, molarity of SO_4^{2-}.

[b] 1×10^6 IU and 0.5×10^6 IU, respectively.

When all components had been added, solution A was added to solution B and the volume brought to 4 ℓ. From this solution 800 ml was withdrawn and Na_2SO_4 added. The volume was brought to one liter. This was the low Mg^{2+} medium. To the remainder of the solution $MgSO_4 \cdot 7H_2O$ was added and the volume brought to 4 ℓ. This was designated as the normal or control medium. The medium was then sterilized by filtration with a Millipore 0.22μm filter, and incubated at room temperature for 4 days. If there was no contamination it was stored at refrigerator temperature for up to two months.

The primary cultures were grown in 90% BME and 10% calf serum. The medium for the secondary culture was modified slightly to increase the buffering capacity by the addition of HEPES, 0.5 M, to give a final concentration of 10 mM. The HEPES was in addition to, rather than in place of, phosphate and bicarbonate as proposed by Eagle (19). This helped decrease rapid pH changes in the Leighton tubes due to CO_2 production. In the Leighton tube the medium shifted toward the acidic side much faster than in the Roux bottles. Two percent by volume of $NaHCO_3$, 5%, was also added to the medium. This gave a final molarity of nutrient 86% of the undiluted BME. The Mg^{2+} concentration of calf serum was approximately 0.8 mM. The total Mg^{2+} concentration in the normal culture medium was about 1.8 mM as compared to 0.2 mM in the low Mg^{2+} test medium.

Preparation of Fluorescein Labeled Hemoglobin - The labeling procedure was that of de Lumen and Tappel (20). Hemoglobin (1 g) was dissolved in 20 ml of sodium bicarbonate buffer 0.05 M, pH 8.6, and

allowed to stand for 15 minutes. Fluorescein isothiocyanate was then added and stirred for 30 minutes. The solution was filtered through a Millipore 0.22μm filter to remove undissolved particles.

Purification of the FlHb was accomplished on a Sephadex G-25 medium Column (23 x 0.9cm). The eluant was phosphate buffer 0.05 M, pH 7.6. The unreacted FITC is retained on the column while the FlHb passes through. The FlHb was diluted to 50 ml and dialyzed in the cold for 4 days with 8 buffer changes. The concentration of FlHb was then determined by the Lowry method using Folin-Ciocalteu reagent (21)(22). The FlHb was sterilized with a 0.22μm Millipore filter and stored frozen. Final concentration was approximately 2%.

No attempt was made to determine the fluorescein to hemoglobin ratio since the quantity of FlHb accumulated by the cells can be determined fluorometrically utilizing a standard curve of FlHb of known concentrations.

Fluorometric Measurements - All fluorescence measurements were made with an Aminco-Bowman Spectrophotofluormeter. A standard curve of FlHb was prepared using DOC as a blank. The most concentrated FlHb solution was used as a 100% reference standard. The fluorescence of each sample was then measured and the FlHb content determined from the standard curve.

The excitation beam passed through slits of 4 mm and 2 mm while the emitted light passed through slits of 1 mm and 2 mm.

The measurements of the fractionated medium were made with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

The concentration of the 100% reference standard was approximately 1.4×10^{-7}M FlHb. The cell fluorescence posed a more complicated problem. In order to obtain the sensitivity necessary to detect accumulated FlHb, relatively wide slits were necessary. These wide slits allowed scattered light from the dissolved cells to be measured as fluorescence. To minimize scattering, an excitation wavelength of 460 nm was used while the emission wavelength was 520 nm. There was still some scattering, so the apparent fluorescence of cells not treated with FlHb was measured and subtracted from the fluorescence of the treated cells. The 100% reference standard was approximately 5×10^{-8}M FlHb.

Protein Estimations - Protein determinations were carried out using a standard assay method (21)(22). BSA in distilled water was used as the standard. A wavelength of 620 nm was used to measure the absorption. Absorption measurements were made either on a Bausch and Lomb Spectronic 700 or a Coleman Model 124 Hitachi Double-Beam Grating Spectrophotometer.

Column Studies - Sephadex G-50 was prepared according to the method in the Sephadex manual (23). Columns 0.9 cm in diameter and 12.5 cm in height were packed. Void volumes were about 2.5 ml with band spreading a factor of two for dextran blue. Sample volumes of 0.6 ml of medium were used. A Buchler polystaltic pump was used with 1/32 inch inside diameter tubing for delivering the eluant. The pump was adjusted to deliver 0.5 ml/min. The sample volume, 0.6 ml, was placed on the column by pumping for 72 seconds from the medium. At the same time 0.6 ml of medium was collected, by pumping, and frozen for percent recovery determination. The sample was eluted with NaCl 0.9% and fractions

of 1.5 ml were collected by timing for 3 minutes on the fraction collector. To each fraction 1.5 ml of DOC was added and frozen. The FlHb was determined from a standard curve.

RESULTS

The first series of experiments were devoted to the study of the accumulation of F1Hb in normal and low Mg^{2+} media. An experiment with high Mg^{2+} was also conducted. These experiments were designed to provide information about the metabolism of hemoglobin by fibroblasts in culture. Experiments were performed to determine which substrate concentration was most suitable. In order to determine the loss of accumulated cellular F1Hb by digestion, pulse or spike experiments were carried out. The turnover rate was also studied by analysis of media, utilizing gel filtration.

Magnesium Concentration - The Mg^{2+} concentration of the normal or control medium was 1.73 mM, Table II. Atomic absorption[3] measurements of the Mg^{2+} concentration in BME were close to the calculated value (see Table I). The low Mg^{2+} BME had a Mg^{2+} content of 0.116 mM and mixed with 10% calf serum the Mg^{2+} concentration of the medium was 0.204 mM. Serum Mg^{2+} was determined by atomic absorption to be 0.884 mM. The Mg^{2+} enriched culture medium was prepared by adding 0.1 ml of 1.52 M $MgSO_4$ to 100 ml of normal medium. The concentration of the high Mg^{2+} medium was 3.34 mM.

F1Hb Accumulation - Fibroblasts, grown in normal medium for two days, were treated with F1Hb at a concentration of 200 µg/ml or 3.10×10^{-3} mM. The cells were harvested at 3, 6, 12, approximately 18,

[3]Determined by Mr. Nelson O. Price, Department of Biochemistry and Nutrition, VPI & SU.

TABLE II

Mg^{2+} content of culture medium

A. Mg^{2+} content of BME and calf serum as determined by atomic absorption[a]

Trial	BME Normal	BME Mg^{2+} salt omitted	Serum
1	1.86 mM		0.917 mM
2	-	0.112 mM	-
	-	0.109 mM	-
	-	0.118 mM	-
3	1.60 mM	0.125 mM	0.867 mM
4	-	-	0.808 mM
average	1.73 mM	0.116 mM	0.884 mM

B. Average content of culture medium

	Normal	Low Mg^{2+}	Mg^{2+} enriched
BME	1.73 mM	0.116 mM	1.73 mM
Serum 10%	0.09 mM	0.088 mM	0.09 mM
Added Mg^{2+}	-	-	1.52 mM
Total Mg^{2+}	1.82 mM	0.204 mM	3.34 mM

[a]Determined by Mr. Nelson O. Price, Department of Biochemistry and Nutrition, VPI & SU.

and 24 hours.

The first set of data obtained showed some decrease in the accumulation of FlHb in the cells maintained in low Mg^{2+} medium as compared to the control cells. The data for three experiments are listed in Table III. The procedures were subsequently modified by placing the control and deficient Leighton tubes on the same rack for the same time point. Also a change in media preparation was made at this time. Instead of preparing the low and normal Mg^{2+} media separately, both were prepared together. A quantity of low Mg^{2+} medium was removed before the addition of $MgSO_4$.

Further tests of cellular accumulation in the control and deficient media showed no significant difference. The data in Table IV, although there were occasional differences, showed no trend. Trial 5 was plotted in Figure 1 as a typical accumulation curve.

To eliminate any possibility that omission of SO_4^{2-} in the initial experiments caused the observed differences, an experiment which used a low Mg^{2+} and SO_4^{2-} medium was conducted. Normal and low Mg^{2+} media were also tested at the same time. The accumulation for the first 24 hours was measured as shown in Table V. The results indicated no difference in net uptake among the three media.

One experiment was conducted to see if increasing the Mg^{2+} concentration would affect the accumulation of the prey molecules. The data given in Table VI showed no difference in accumulation by increasing the Mg^{2+} concentration. Also no decrease in accumulation was observed in the deficient test.

TABLE III

Accumulation of FlHb by Fibroblasts in normal and low Mg^{2+} medium

Trial	Time	Normal	Low Mg^{2+}
	hours	FlHb mmoles x 10^8 / CELL PROTEIN mg	
1	0	0.50 ± 0.10	0.19 ± 0.25
	3	6.14 ± 0.16	5.92 ± 0.30
	6	8.09 ± 0.59	6.87 ± 0.55
	12	9.37 ± 0.07	8.27 ± 0.45
	20	10.46 ± 0.18	9.61 ± 0.27
	24	11.11 ± 0.25	10.32 ± 0.26
2	0	0.39 ± 0.26	0.15 ± 0.14
	3	-	5.32 ± 0.55
	6	7.48 ± 0.40	7.18 ± 0.38
	12	8.90 ± 0.74	8.89 ± 0.57
	20	10.86 ± 0.33	9.77 ± 0.46
	24	11.89 ± 0.49	10.88 ± 0.50
3	0	0.16 ± 0.05	0.19 ± 0.09
	3	3.75 ± 0.26	3.91 ± 0.21
	6	5.25 ± 0.35	4.64 ± 0.08
	12	6.40 ± 0.25	5.64 ± 0.32
	21	8.14 ± 0.30	6.78 ± 0.14
	24.5	7.60 ± 0.29	7.40 ± 0.41

TABLE IV

Accumulation of F1Hb by Fibroblasts in normal and low Mg^{2+} medium

Trial	Time	Normal	Low Mg^{2+}
	hours	F1Hb mmoles x 10^8 / CELL PROTEIN mg	
4	0	0.33 ± 0.04	0.17 ± 0.09
	3	3.79 ± 0.19	3.65 ± 0.17
	6	4.77 ± 0.29	4.64 ± 0.16
	12	5.25 ± 0.38	5.41 ± 0.13
	19	6.59 ± 0.17	7.06 ± 0.19
	24	7.16 ± 0.15	6.32 ± 0.45[a]
5	0	1.03 ± 0.24	1.04 ± 0.10
	3	3.84 ± 0.08	5.05 ± 0.31
	6	5.01 ± 0.10	4.92 ± 0.17
	12	7.64[b]	7.73 ± 0.35
	20	9.58 ± 0.29	9.50 ± 0.41
	24.9	8.85 ± 0.35	9.25 ± 0.38
	24.9	10.25 ± 0.11	9.72 ± 0.25
6	0	0	-
	3	5.85 ± 0.73	5.02 ± 1.00
	5.3	6.92 ± 0.65	6.25 ± 0.21
	12	9.17 ± 0.20	9.13 ± 1.08
	20	11.40 ± 0.23	10.91 ± 1.12
	24.2	10.44 ± 0.76	10.54 ± 0.83
	48	16.72 ± 0.99	15.29 ± 0.83

[a]Peeling of the monolayer was evident in these series of tubes.

[b]No standard deviation calculated as this is the average of three values.

Figure 1. F1Hb accumulation by fibroblasts versus time. Closed circles represent accumulation in normal medium and open circles in low Mg^{2+} medium.

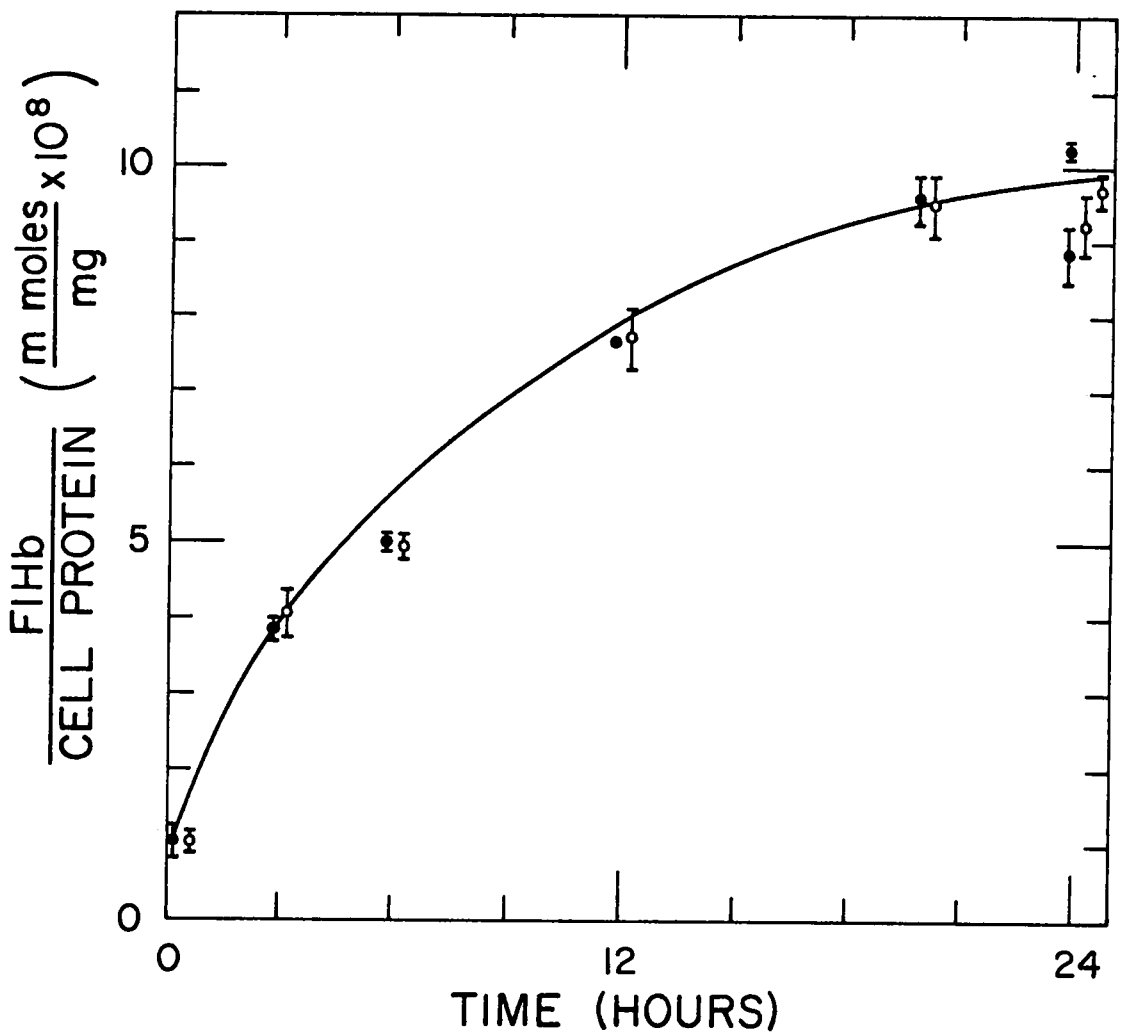

FIHb / CELL PROTEIN (m moles/mg x 10^8)
10
5
0
0
12
24
TIME (HOURS)

TABLE V

Accumulation of F1Hb by Fibroblasts in normal, Low Mg^{2+}, and Low Mg^{2+}, and SO_4^{2-} media

Time	Normal	Low Mg^{2+}	Low Mg^{2+} and SO_2^{2-}
hours	F1Hb mmoles x 10^8 / CELL PROTEIN mg		
0	0.27 ± 0.14	-	-
3	3.40[a]	3.34	3.30 ± 0.11
6	4.30	4.46	4.31 ± 0.24
9	5.36	5.26	5.66 ± 0.44
12	6.15	5.86	6.58 ± 0.18
22	7.90	7.97	7.85 ± 0.16
24	8.64	8.05	9.30 ± 0.62
24.2	8.78	8.51	8.79 ± 0.35

[a]No standard deviation was calculated as these values represent the average of three values.

TABLE VI

Accumulation of FlHb by fibroblasts in normal, Mg^{2+} enriched, and low Mg^{2+} medium 0-24 hours

Time	Normal	Low Mg^{2+}	Mg^{2+} Enriched
hours	FlHb mmoles x 10^8 / CELL PROTEIN mg		
0	0.13 ± 0.14	-	-
3	5.69 ± 0.59	4.34 ± 0.54	5.99 ± 0.64
6	8.43 ± 1.13	7.80 ± 1.41	8.32 ± 1.44
12	10.64 ± 0.87	10.29 ± 1.45	11.36 ± 0.56
20	14.40 ± 1.66	12.67 ± 1.14	12.34 ± 1.66
24	11.72 ± 1.15	11.85 ± 0.76	12.51 ± 0.78
24	13.79 ± 1.06	14.18 ± 1.25	12.23 ± 0.76

In order to study any effect which might occur under extended periods of low Mg^{2+}, several experiments of extended duration were conducted. Accumulation at 24, 48, 72, and 96 hours were measured and are listed in Table VII. Trial 3 in Table IV has an accumulation value at 48 hours. Although the net uptake was not always a smooth curve, there was no significant difference between the control and deficient at any given time.

The accumulation of FlHb was extremely rapid during the first 3 hours with a flattening by 24 hours. There was a small but continual increase in net uptake after 20 hours as seen in Table VII. These accumulation values represented only a net uptake rather than total uptake. Uptake of FlHb would be opposed by digestion and release of the fluorescent micromolecular material. This will be discussed in more detail later.

The zero time values were obtained by rinsing cells, not previously exposed to FlHb, with medium containing FlHb. This FlHb medium was in contact with the cell for about 30 seconds and was followed by the normal washing procedure. It was a measure of the surface binding and the effectiveness of the washing.

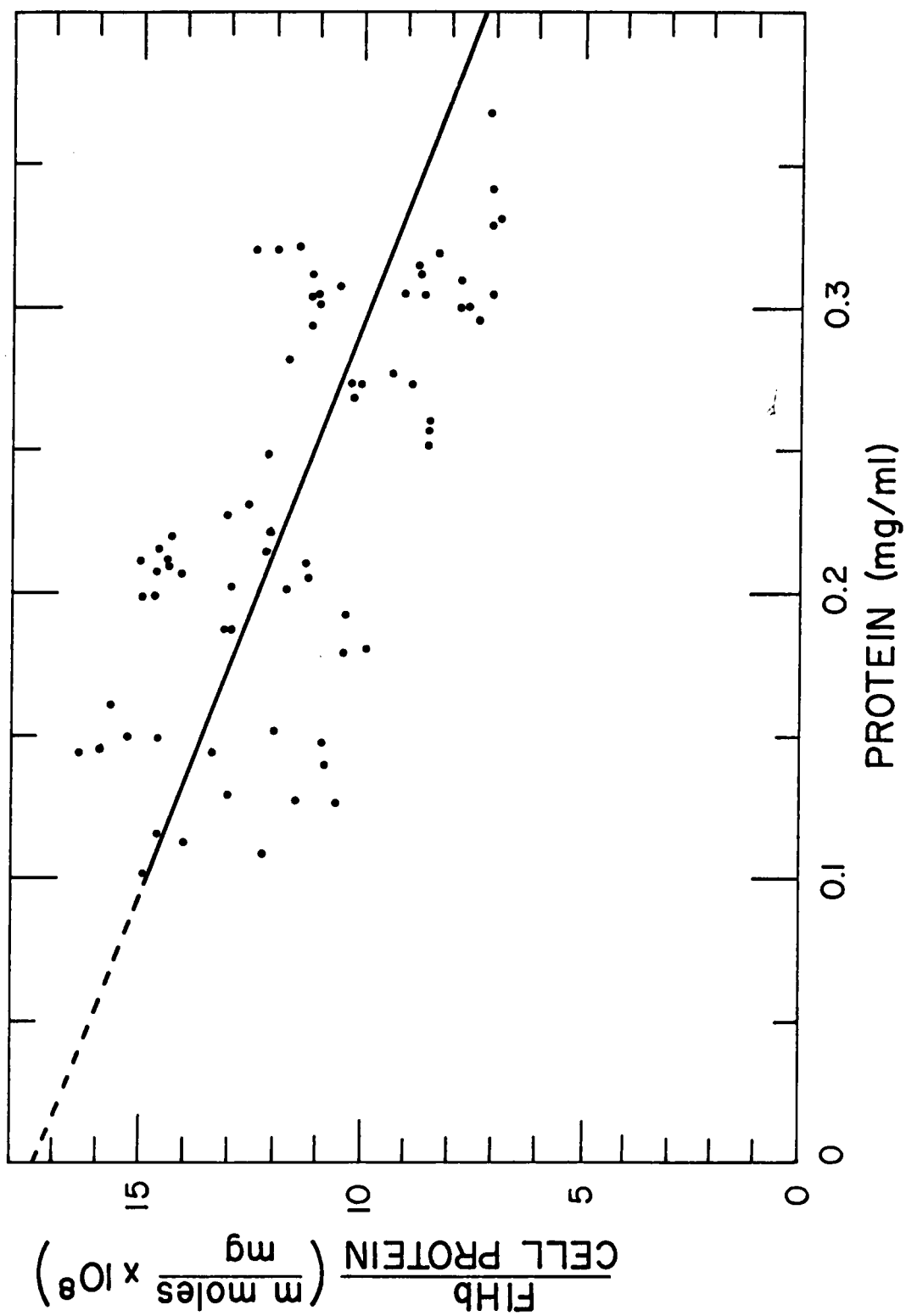
Accumulation versus protein - The net uptake at any given time varied a great deal from one experiment to another. By plotting the accumulation per weight of cell protein versus the cell protein (Figure 2), the differences formed a pattern. As the cell protein decreased the accumulation per weight of cell protein increased. This figure was representative of accumulation for 24 hours of the cells in the

TABLE VII

Accumulation of F1Hb by fibroblasts in normal and low Mg^{2+} medium 24-96 hours

Trial	Time	Normal	Low Mg^{2+}
	hours	F1Hb mmoles x 10^8 / CELL PROTEIN mg	
1	0	1.18 ± 0.09	-
	24	10.50 ± 0.42	10.12 ± 0.32
	48	17.61 ± 0.20	16.33 ± 1.21
	72	14.29 ± 0.66	15.36 ± 0.21
	96	17.98 ± 0.32	18.02 ± 0.14
2	0	0.45 ±	-
	24	12.23 ± 0.83	12.34 ± 0.32
	48	15.88 ± 1.06	14.94 ± 0.43
	72	17.12 ± 0.23	15.67 ± 0.97
	96	17.54 ± 0.73	17.67 ± 1.00

Figure 2. F1Hb accumulation per weight of cell versus cell protein. The fibroblasts were incubated in normal medium for 24 hours. The slope is 25.1 with a vertical intercept of 17.4. This figure includes data from two preparations of F1Hb.


FIHb / CELL PROTEIN (m moles / mg x 10⁸)
0
5
10
15
0
0.1
0.2
0.3
PROTEIN (mg/ml)

control medium and was a combination of data from two preparations of FlHb. This phenomenon occurred at all times after three hours, even though the total uptake was not as great. There was a considerable variation at any given value of protein, the reasons for which are unclear. The method did give a means of evaluating the results of the accumulation experiments, because it enabled a better comparison of net uptakes at high and low protein values.

Figure 2 contains the most comprehensive plot since many more values were available for this particular time. Values much above 0.32 mg/ml of cell protein were rare since much peeling occurred due to overcrowding. The cells in this range often had an "unhealthy" appearance and were more easily detached by washing. At low values of cell protein, less than about 0.08 mg/ml, there often was not a complete monolayer in the center of the Leighton tube.

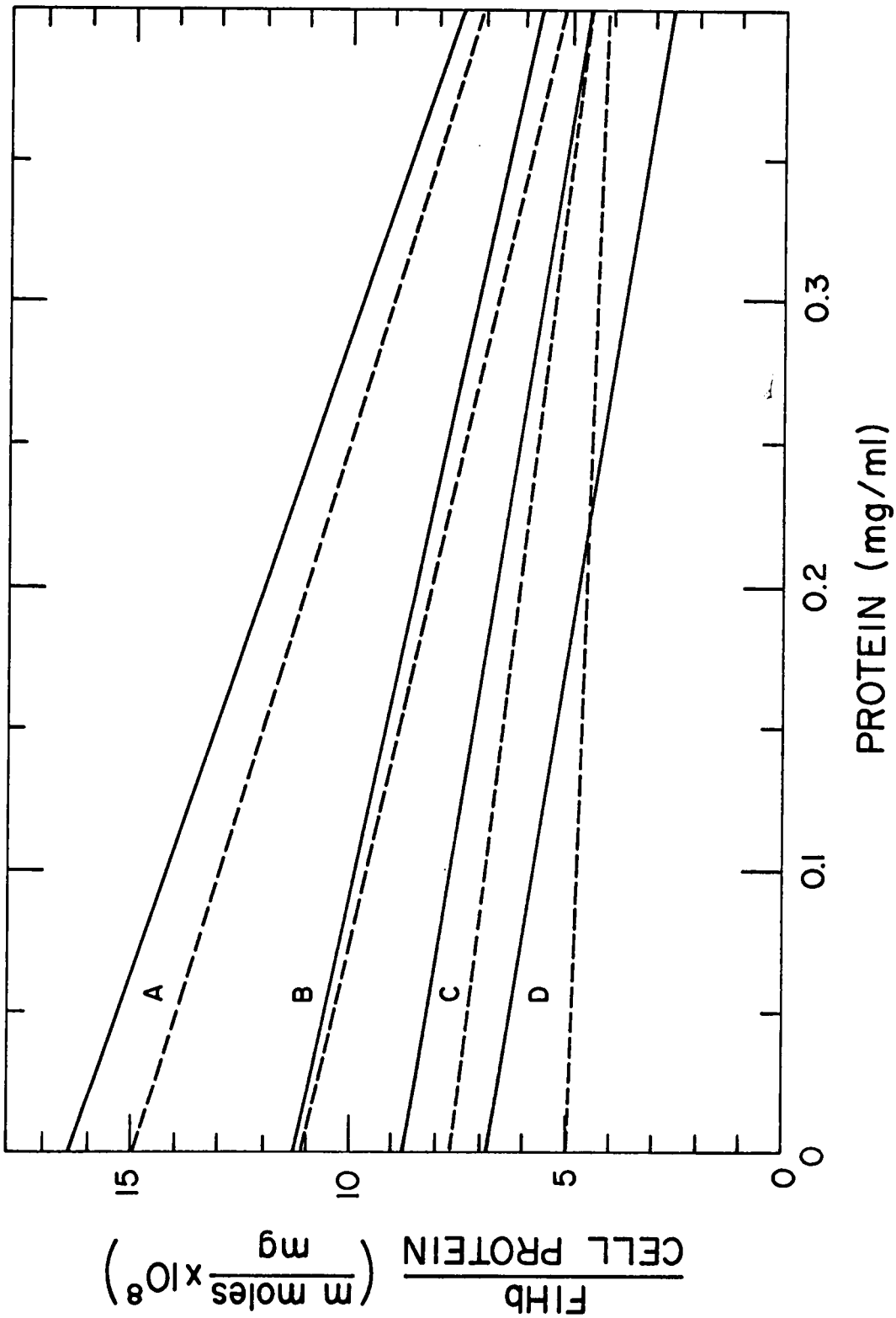
The comparison of FlHb accumulation by fibroblasts in control and low Mg^{2+} medium could be evaluated more exactly by utilizing the accumulation per weight of cell protein versus cellular protein plot. In Table VIII and Figure 3, the comparison was made between the accumulation in normal and low Mg^{2+} medium. The accumulation in normal medium was represented by a solid line while that in low Mg^{2+} medium was a dashed line. The letters A, B, C, and D represented the various times 24, 12, 6, and 3 hours, respectively. The data points, in Figure 3, have been omitted for clarity. All these values were obtained using a single preparation of FlHb in order to eliminate any differences due to slight substrate variation. The lines were determined by the least squares

TABLE VIII

Accumulation of FlHb per weight of cell protein versus cell protein

Media type	Time	Slope	Vertical intercept	Data points
	hours		$\frac{\text{FlHb mmoles} \times 10^8}{\text{CELL PROTEIN mg}}$	Number
Normal	3	10.0 ± 2.9	6.8 ± 0.6	30
Low Mg^{2+}		2.1 ± 2.5	5.1 ± 0.5	34
Normal	6	10.5 ± 3.7	8.8 ± 0.8	34
Low Mg^{2+}		7.9 ± 3.3	7.7 ± 0.7	35
Normal	12	14.0 ± 3.4	11.3 ± 0.8	32
Low Mg^{2+}		14.8 ± 2.9	11.1 ± 0.6	39
Normal	24	22.4 ± 3.2	16.4 ± 0.8	58
Low Mg^{2+}		19.6 ± 3.4	14.9 ± 0.8	38

Figure 3. FlHb accumulation per weight of cell versus cell protein. The fibroblasts were incubated in media for various periods of time: A for 24 hours, B for 12 hours, C for 6 hours, and D for 3 hours. The solid lines represent accumulation in normal media and the dashed lines represent accumulation in low Mg^{2+} media.


A
B
C
D
0
5
10
15
0
0.1
0.2
0.3
PROTEIN (mg/ml)
FIHb / CELL PROTEIN (m moles/mg x 10^8)

method. There appeared to be no significant difference between accumulations in either of the media. The slope of the three hour low Mg^{2+} line was quite different from all the others but this could be attributed to an insufficient number of values in the low protein region.

Dose dependency - Two experiments were performed to determine the most desirable quantity of FlHb to be used in the accumulation experiments. To the control medium, FlHb was added to give final concentrations of 50µg/ml to 300µg/ml. The cells were incubated for 24 hours. A break in the curve appeared between the 50 and 100µg/ml concentration. The data is listed in Table IX and plotted in Figure 4.

The experiment was repeated using Mg^{2+} enriched and low Mg^{2+} media. The accumulation period was 12 hours with the FlHb concentrations ranging from 50µg/ml to 500µg/ml. The results are given in Table X. As in the previous experiment, there was a break in the curve about 100µg/ml. The results of both experiments indicate that 200µg/ml was an acceptable concentration to use, as a small variation in added FlHb would not change the accumulation appreciably. There was no significant difference between accumulation in the Mg^{2+} enriched and the low Mg^{2+} throughout this concentration range.

Spike experiment - In order to determine if a Mg^{2+} deficit would affect the digestion and/or release of digestive products, pulse, or spike experiments were performed. The fibroblasts were grown for two days in Leighton tubes in control medium containing FlHb. After 24 hours the medium was removed, all the cells washed once in low Mg^{2+} medium, and placed in control or low Mg^{2+} medium containing no FlHb.

TABLE IX

Dose dependency of accumulated F1Hb, by fibroblasts, on the medium concentration of F1Hb. Accumulation period was 24 hours in normal medium

Dose	Accumulation
F1Hb (µg/ml)	$\frac{\text{F1Hb mmoles} \times 10^{8}}{\text{CELL PROTEIN mg}}$
50	7.37 ± 0.40
100	10.79 ± 0.40
150	12.87 ± 0.77
200	14.65 ± 0.30
250	15.62 ± 1.04
300	18.16 ± 0.89

Figure 4. Accumulation of F1Hb versus the media concentration of F1Hb. The time period was 24 hours.

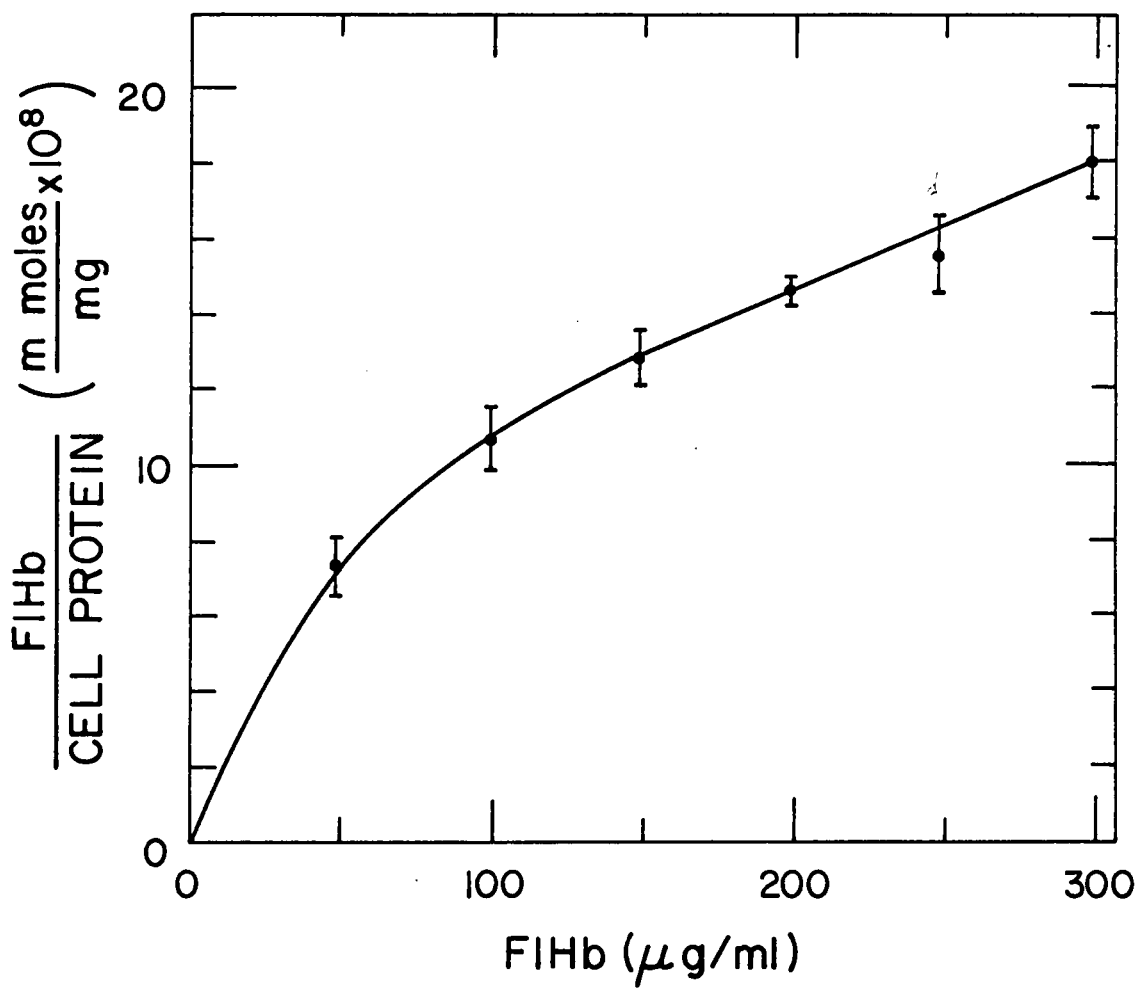

FIHb / CELL PROTEIN (m moles x 10^8 / mg)
20
10
0
0
100
200
300
FIHb (μg/ml)

TABLE X

Dose dependency of accumulated FlHb by fibroblasts, on the medium concentration of FlHb. Mg^{2+} enriched and low Mg^{2+} mediums accumulation period was 12 hours

Dose	Accumulation	
FlHb (µg/ml)	$\frac{\text{FlHb mmoles} \times 10^{8}}{\text{CELL PROTEIN mg}}$	
	Mg^{2+} enriched	Low Mg^{2+}
50	5.84 ± 0.20	5.43 ± 0.58
100	9.15 ± 0.28	7.23 ± 0.22
200	9.44 ± 0.46	8.30 ± 0.42
300	12.62 ± 0.89	11.84 ± 0.50
400	14.72 ± 0.85	14.37 ± 2.30
500	15.49 ± 1.97	14.15 ± 1.00

The cells were harvested at various time intervals from 0 hour to 24 hours.

The amounts of F1Hb in the cells decreased rapidly at first but tended to flatten out after about 9 to 12 hours. The results are given in Table XI and plotted in Figure 5. The closed circles represent the amount remaining in cells cultured in normal medium and the open circles were corresponding values for cells cultured in low Mg^{2+} medium. There did not appear to be enough difference between the normal and low Mg^{2+} to warrant drawing two lines per experiment. Trial 1 was represented by line A and trial 2 by line B.

The log of F1Hb remaining in the fibroblasts in trials 1 and 2 was plotted versus time. The line showed a break about 9 hours (Figure 6). The lines were obtained by the least squares method of points plotted from 0 to 12 hours and from 12 to 24 hours. The closed circles and solid lines represent the amount remaining in cells cultured in normal medium. The open circles and dashed lines represent the values in low Mg^{2+} medium.

Magnesium depletion - An accumulation experiment was conducted under conditions of Mg^{2+} depletion. Cells were cultured for 24 hours after seeding in normal medium. The medium in the control cells was replaced with fresh normal medium after 24 hours while the test cells were given low Mg^{2+} medium. Twenty-four hours later, the medium was again changed, this time to include F1Hb, 200µg/ml. The control cells remained in normal medium and the test cells in low Mg^{2+} medium. The cells were harvested at specific intervals. There appeared to be no

TABLE XI

F1Hb remaining in fibroblasts after pulse or spike of F1Hb 200µg/ml

Trial	Time	Normal	Low Mg^{2+}
	hours	F1Hb mmoles x 10^8 / CELL PROTEIN mg	
1	0	15.72 ± 0.68	-
	3	13.19 ± 0.74	12.25 ± 0.67
	6	11.46 ± 0.27	11.45 ± 0.61
	9	9.62 ± 1.06	10.44 ± 0.71
	12	9.83 ± 0.28	9.80[a]
	24	8.12 ± 0.39	7.96 ± 0.27
2	0	12.43 ± 0.36	-
	3	9.86 ± 0.37	10.39 ± 0.38
	6	9.52 ± 0.24	9.50 ± 0.14
	9	-	-
	12	8.25 ± 0.23	8.31 ± 0.37
	24	6.33 ± 0.51	6.49 ± 0.26

[a]Average of two values.

Figure 5. F1Hb remaining in the cells after a 24 hour spike or pulse. The closed circles are for retention in normal medium while the open circles are for retention in low Mg^{2+} medium. Trial 1 is line A and trial 2 is line B.

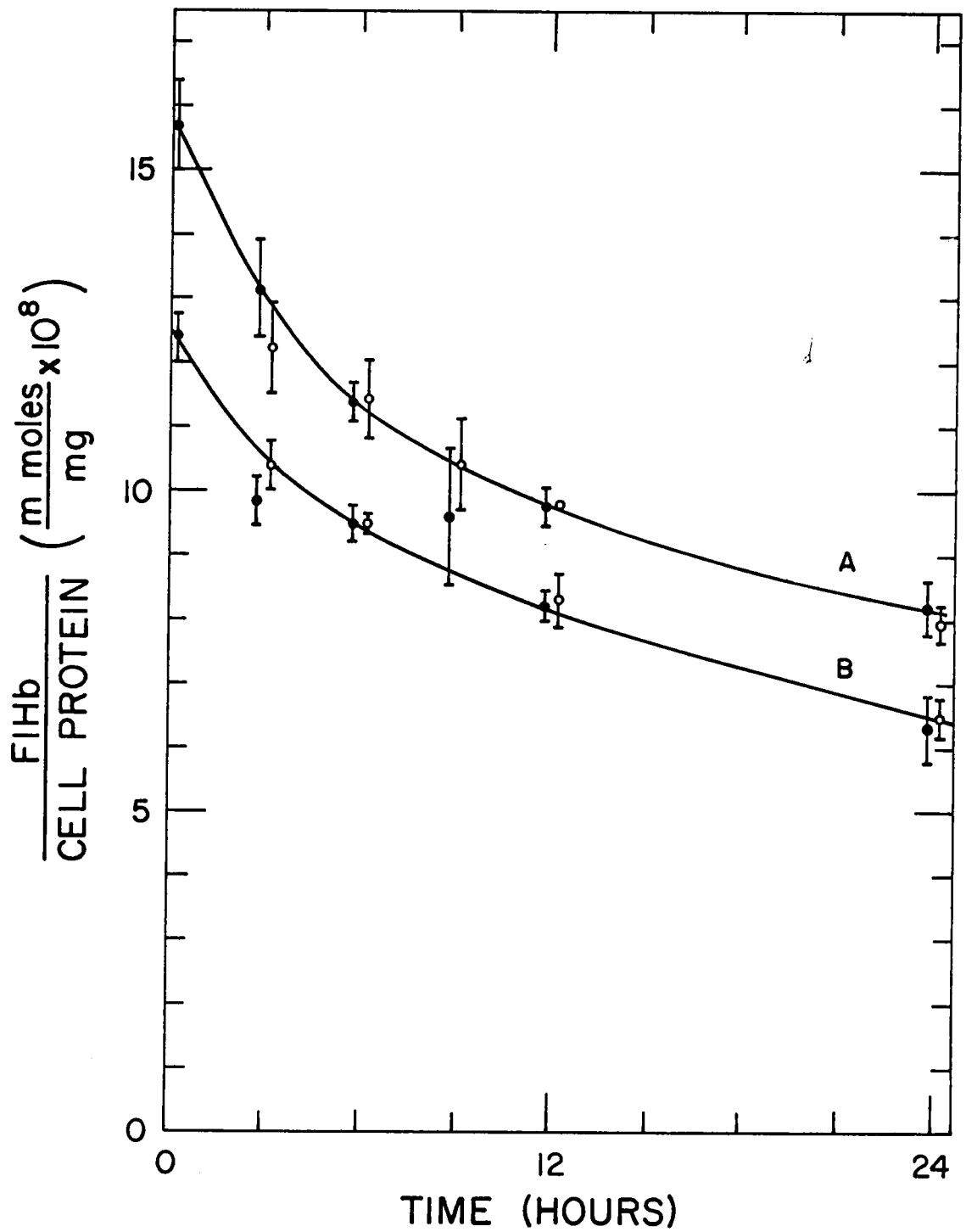
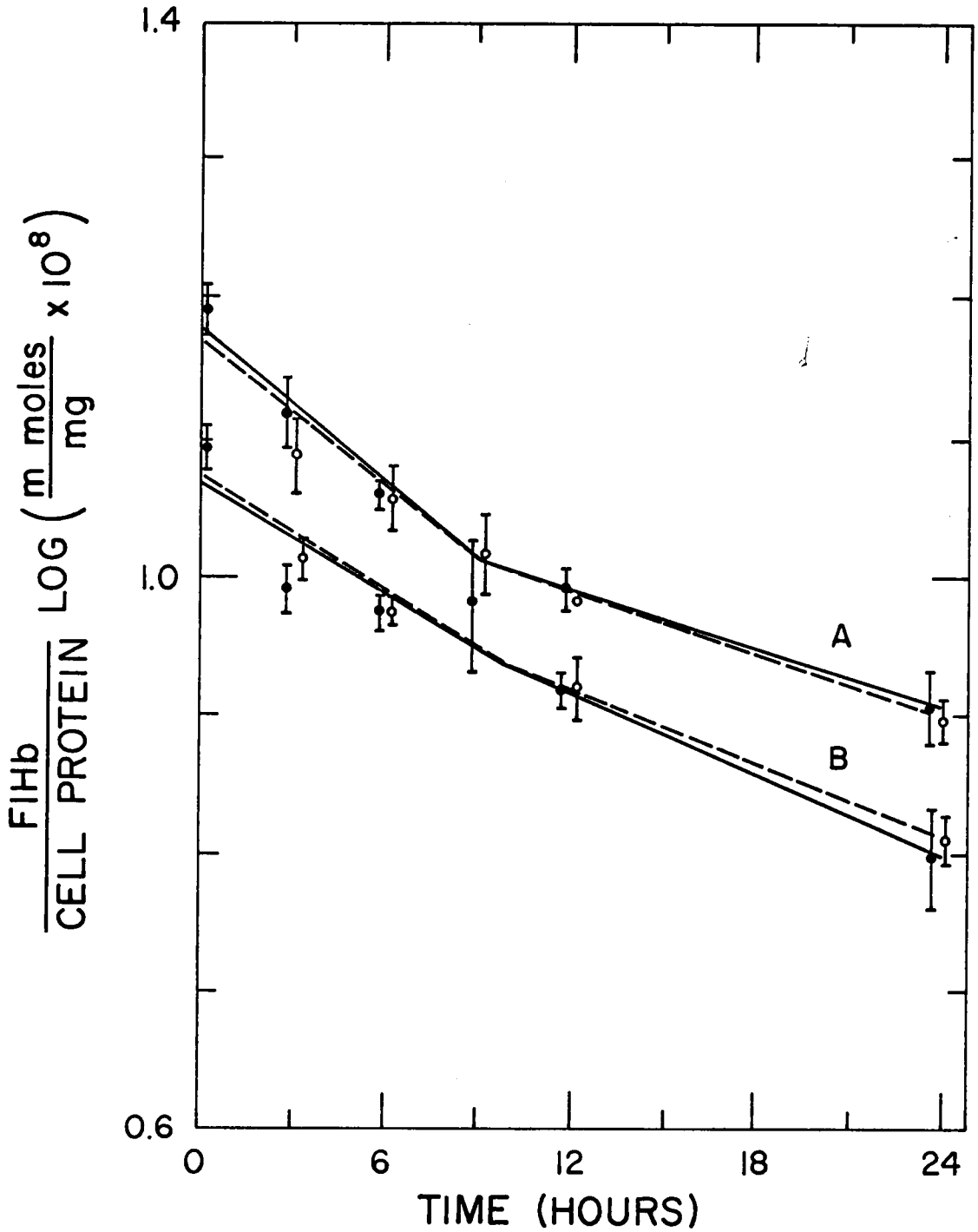

FIHb / CELL PROTEIN (m moles/mg x 10^8)
15
10
5
0
0
12
24
TIME (HOURS)
A
B

Figure 6. A semilog plot of FlHb retained in cells versus time. The closed circles are retention in normal media and the open circles are retention in low Mg^{2+} media. Trial 1 is line A and trial 2 is line B.


1.4
1.0
0.6
0
6
12
18
24
TIME (HOURS)
A
B
FIHb / CELL PROTEIN LOG (m moles / mg x 10^8)

significant difference in FlHb accumulation between the normal and low Mg^{2+} treated cells as shown in Table XII.

A pulse or spike experiment was conducted under similar conditions as the previous experiment. This time both the control and test cells were grown in the low Mg^{2+} medium for 24 hours without FlHb and were maintained under the same conditions in the presence of FlHb for an additional 24 hours. At the end of this period the control cells were given fresh normal medium without FlHb, but the test cells were continued under low Mg^{2+} conditions without FlHb. As in the other spike experiments, there was no difference in the release of FlHb or its products between cells as a function of the Mg^{2+} concentration of the two media (Table XIII).

Column analysis of media - In order to obtain data on digestion and turnover rates, analysis of the fluorescence material in the culture media containing FlHb was conducted. Gel filtration was used to separate the micromolecular components from the macromolecular components.

The normal and deficient media were incubated for 0, 24, 48, 72, and 96 hours without cells. A 0.6 ml sample of medium was applied to the Sephadex G-50 column and eluted with NaCl 0.9%. To each of the 1.5 ml fraction was added 1.5 ml of DOC. On analysis most of the macromolecular species came out in the first five fractions. The amount of fluorescent species past the sixth fraction was very small in zero time trials. As the incubation time increased, a very small shoulder appeared in the fifth and subsequent fractions. The data in

TABLE XII

Accumulation of F1Hb by fibroblasts in normal medium by fibroblasts which have been depleted of Mg^{2+} for 24 hours prior to F1Hb exposure

Time	Normal	Low Mg^{2+}
hours	F1Hb mmoles x 10^8 / CELL PROTEIN mg	
0	0.23 ± 0.24	-
3	2.48 ± 0.16	2.55 ± 0.31
6	4.35 ± 0.31	4.16 ± 0.27
12	5.82 ± 0.37	5.40 ± 0.24
21.6	7.73 ± 0.52	7.50 ± 0.26
24	7.71 ± 0.74	7.31 ± 0.41

TABLE XIII

F1Hb remaining in fibroblasts after pulse or spike of F1Hb 200 µg/ml in low Mg^{2+} medium

Time	Normal	Low Mg^{2+}
hours	F1Hb mmoles x 10^8 / CELL PROTEIN mg	
0	-	7.31 ± 0.41
3	6.80 ± 0.61	7.03 ± 0.43
6	6.43 ± 0.39	6.64 ± 0.26
9	5.22 ± 0.30	5.60 ± 0.29
12	4.84 ± 0.30	5.06 ± 0.28
24	3.88 ± 0.17	4.10 ± 0.11

Tables XIV-XVIII and Figures 7 and 8 showed the increase in this shoulder with time. The graphs are expressed in percentage of total FlHb recovered in order to facilitate comparisons. The fractions containing the phenol red are indicated with brackets.

There is no difference between the normal and deficient medium in the height of the shoulder for any given time. Whether this shoulder was micromolecular material or modified macromolecular FlHb is uncertain.

To determine the percent recovery, a sample of medium 0.6 ml was diluted to 10 ml or 25 ml and the quantity of FlHb present determined. By adding the total fluorescence from the column, and comparing it to the amount present in the unfractionated sample, the percent recovery was calculated. A sample of 0.6 ml was measured with a pipet to check the accuracy of the pump delivery. All values compared reasonably well.

Culture medium without FlHb was also incubated without cells for 0 to 96 hours. The fluorescence was negligible.

In a turnover experiment, fibroblasts in Leighton tubes were cultured for 24, 48, 72, and 96 hours. The medium from each set of tubes was collected and pooled. Analysis by gel filtration revealed an increase of fluorescent material in fractions 6-15. Unlike the shoulder on the edge of the large peak in media incubated without cells, there was a definite peak around fractions eight or nine as shown in Tables XIX-XXIII and Figure 9, especially in the 96 hour trials. By the position, the peak was probably made up of

TABLE XIV

F1Hb distribution in fractioned BME incubated at 37° without cells for 0 time

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0	0	0	0
2	0	0	1.29	7.2
3	14.2	73.3	14.0	78.5
4	4.0	20.5	1.7	9.3
5	0.79	4.1	0.48	2.7
6	0.30	1.6	0.24	1.3
7	0.12	0.6	0.11	0.6
8	0.01	0.0	0.02	0.1
9			0.01	0.0
10			0.01	0.1
11			0.02	0.1
12			0.01	0.0
13				0.0
14				
15				
Total	19.4		17.8	
% recovery	87%		97%	

TABLE XV

F1Hb distribution in fractionated BME incubation at 37° without cells for 24 hours

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0	0	0	0
2	0	1.5	0.24	1.3
3	15.6	79.5	14.9	78.2
4	1.79	9.1	2.00	10.5
5	0.52	2.6	0.52	2.7
6	0.32	1.6	0.34	1.8
7	0.25	1.3	0.26	1.4
8	0.13	0.7	0.14	0.8
9	0.07	0.4	0.11	0.6
10	0.10	0.5	0.11	0.6
11	0.18	0.9	0.12	0.6
12	0.13	0.7	0.12	0.6
13	0.08	0.4	0.09	0.5
14	0.03	0.2	0.05	0.3
15	0.10	0.5	0.05	0.3
16	———	0.2	———	
Total	19.7		19.0	
% recovery	101.0%		108.0%	

TABLE XVI

F1Hb distribution in fractionated BME incubated at 37° without cells for 48 hours

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0	0	0	0
2	12.3	64.5	0	0
3	3.85	20.2	13.0	74.5
4	0.82	4.3	2.28	13.1
5	0.49	2.6	0.61	3.5
6	0.42	2.2	0.42	2.4
7	0.28	1.5	0.30	1.7
8	0.18	0.9	0.18	1.1
9	0.17	0.9	0.16	0.9
10	0.17	0.9	0.13	0.8
11	0.17	0.9	0.14	0.8
12	0.13	0.7	0.20	0.1
13	0.08	0.4	0.03	0.2
14	0.03	0.2		
15				
Total	19.1		17.5	
% recovery	99%		103%	

TABLE XVII

F1Hb distribution in fractionated BME incubated at 37° without cells for 72 hours

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0	0	0.01	0.06
2	6.3	34.0	5.4	28.7
3	9.0	48	10.3	54.6
4	0.90	4.9	0.99	5.3
5	0.58	3.1	0.48	2.6
6	0.52	2.8	0.45	2.4
7	0.36	1.9	0.36	1.1
8	0.26	1.4	0.27	1.4
9	0.20	1.1	0.20	1.1
10	0.15	0.8	0.16	0.8
11	0.10	0.5	0.11	0.6
12	0.05	0.3	0.06	0.3
13	0.02	0.1	0.06	0.3
14	0.01			
15	———		———	
Total	18.5		18.9	
% recovery	102%		111%	

TABLE XVIII

F1Hb distribution in fractionated BME incubated at 37° without cells for 96 hours

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0	0	0	0
2	2.01	11.1	4.1	22.1
3	12.3	67.5	10.9	58.3
4	1.11	6.1	1.06	5.7
5	0.58	3.2	0.54	2.9
6	0.58	3.2	0.55	2.9
7	0.51	2.8	0.43	2.3
8	0.33	1.8	0.33	1.8
9	0.27	1.5	0.27	1.5
10	0.19	1.1	0.21	1.1
11	0.15	0.8	0.16	0.9
12	0.09	0.5	0.07	0.4
13	0.03	0.2	0.05	0.3
14	0.06	0.3	0.01	0.1
15				
Total	18.2		18.7	
% recovery	110%		112%	

Figure 7. Distribution of fluorescent material in normal and low Mg^{2+} media containing FlHb. The fractionation was by Sephadex G-50. Graph A is normal medium and Graph B is low Mg^{2+} medium. Brackets indicate phenol red.

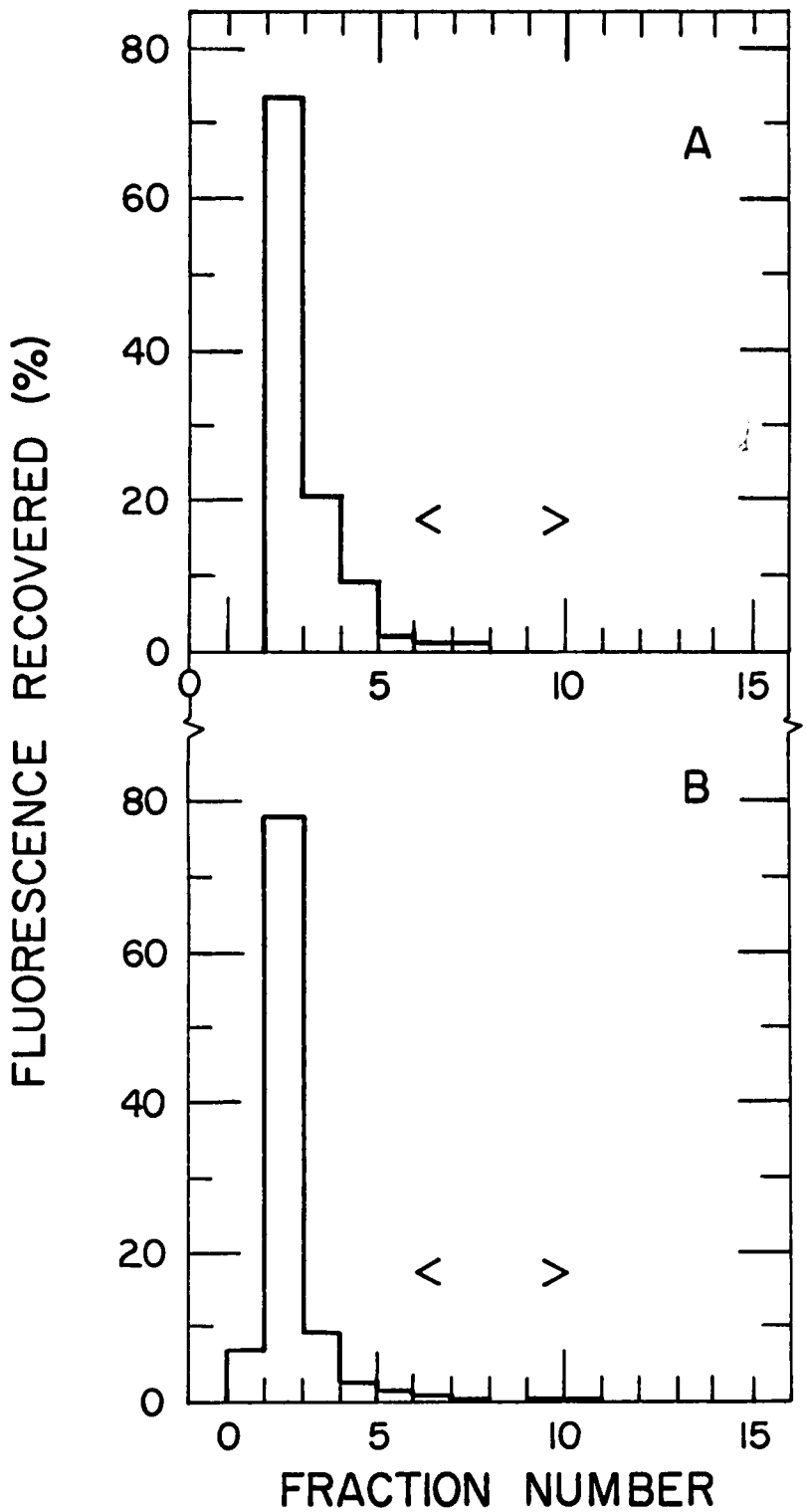

FLUORESCENCE RECOVERED (%)
80
60
40
20
0
A
<
>
0
5
10
15
B
80
60
40
20
0
<
>
0
5
10
15
FRACTION NUMBER

Figure 8. Fluorescent material recovered from media containing F1Hb and incubated without cells. The fractionation was on Sephadex G-50 with fractions 5 through 15 shown here. Sections A, C, E, G, and I were normal media incubated for 0, 24, 48, 72, and 96 hours, respectively. Sections B, D, F, H, and J were low Mg^{2+} media incubated for 0, 24, 48, 72, and 96 hours, respectively. Brackets indicate phenol red.

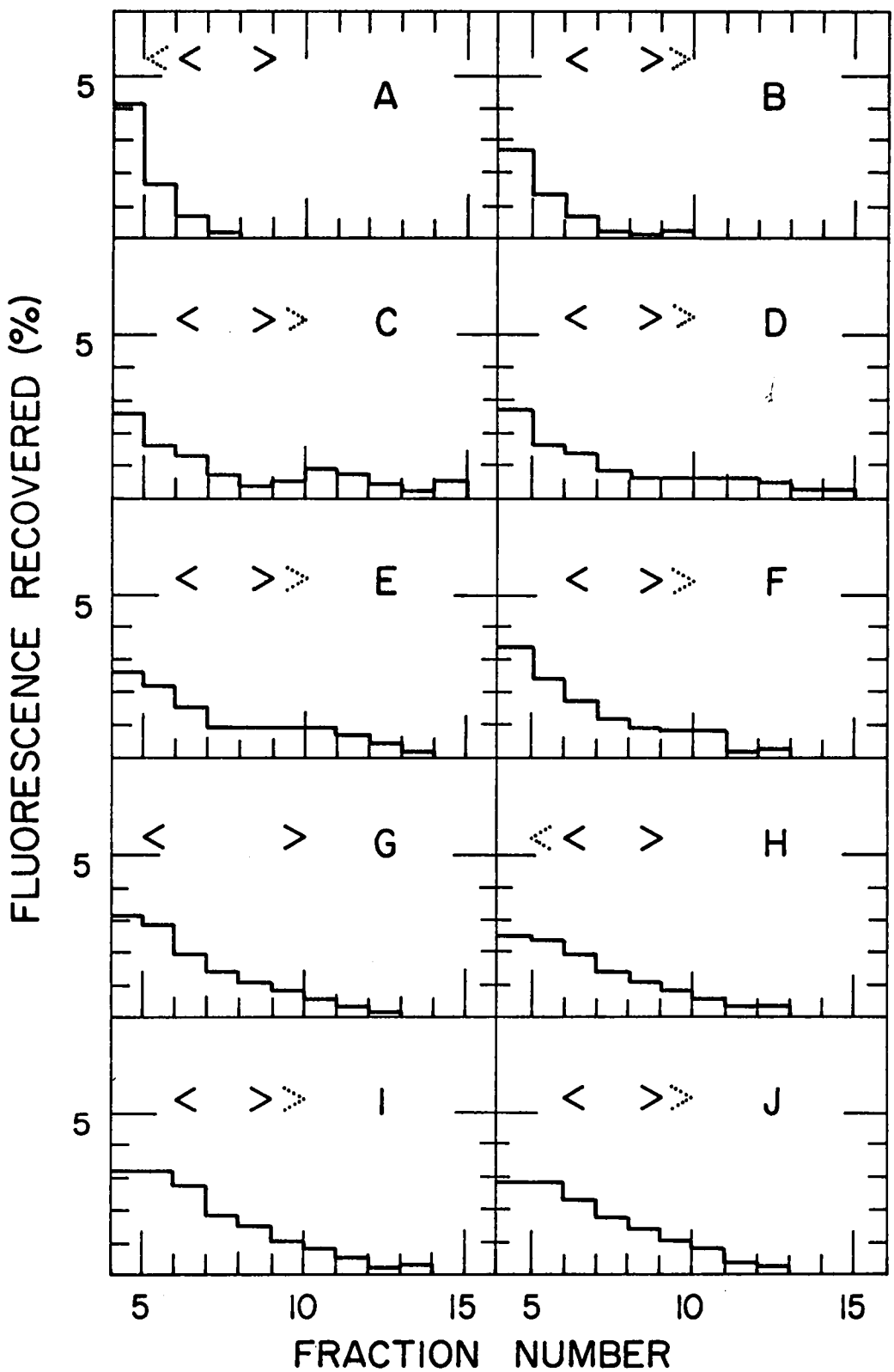

A
B
C
D
E
F
G
H
I
J
5
5
5
5
5
5
10
15
5
10
15
FLUORESCENCE RECOVERED (%)
FRACTION NUMBER

TABLE XIX

F1Hb and its digestive products in fractionated BME incubated with fibroblasts at 37° for 0 time

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0	0	0.06	0.3
2	6.7	31.0	1.95	8.3
3	12.8	59.4	18.6	78.7
4	1.15	1.7	1.92	8.1
5	0.37	0.9	0.46	1.9
6	0.20	0.3	0.22	0.9
7	0.06	0.2	0.08	0.3
8	0.05	0.2	0.06	0.3
9	0.05	0.3	0.06	0.3
10	0.06	0.3	0.06	0.3
11	0.06	0.2	0.06	0.3
12	0.05	0.1	0.06	0.3
13	0.02		0.03	0.1
14			0.02	0.1
15				
16				
Total	21.6		23.6	
% recovery	90%		111%	

TABLE XX

F1Hb and its digestive products in fractionated BME incubated with fibroblasts at 37° for 24 hours

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0.02	0.1	0.02	0.1
2	3.84	19.0	5.83	29.1
3	13.1	65.0	11.2	55.7
4	1.25	6.2	1.17	5.8
5	0.45	2.2	0.42	2.1
6	0.37	1.8	0.33	1.6
7	0.31	1.5	0.26	1.3
8	0.24	1.2	0.23	1.1
9	0.21	1.0	0.16	0.8
10	0.18	0.9	0.14	0.7
11	0.12	0.6	0.12	0.6
12	0.07	0.3	0.11	0.5
13	0.05	0.2	0.06	0.3
14			0.02	0.1
15			0.01	0.1
16				
Total	20.2		20.0	
% recovery	101%		103%	

TABLE XXI

F1Hb and its digestive products in fractionated BME incubated with fibroblasts at 37° for 48 hours

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0.02	0.1	0.03	0.2
2	1.97	10.3	2.25	11.7
3	12.8	67.1	12.6	65.4
4	1.35	7.1	1.50	7.8
5	0.46	2.4	0.51	2.7
6	0.43	2.3	0.41	2.1
7	0.45	2.4	0.41	2.1
8	0.45	2.4	0.41	2.1
9	0.40	2.1	0.35	1.8
10	0.31	1.6	0.29	1.5
11	0.21	1.1	0.22	1.1
12	0.12	0.6	0.15	0.8
13	0.06	0.3	0.07	0.4
14	0.05	0.3	0.04	0.2
15	0.01	0.1	0.01	0.1
16	———		———	
Total	19.1		19.2	
% recovery	102%		113%	

TABLE XXII

F1Hb and its digestive products in fractionated BME incubated with fibroblasts at 37° for 72 hours

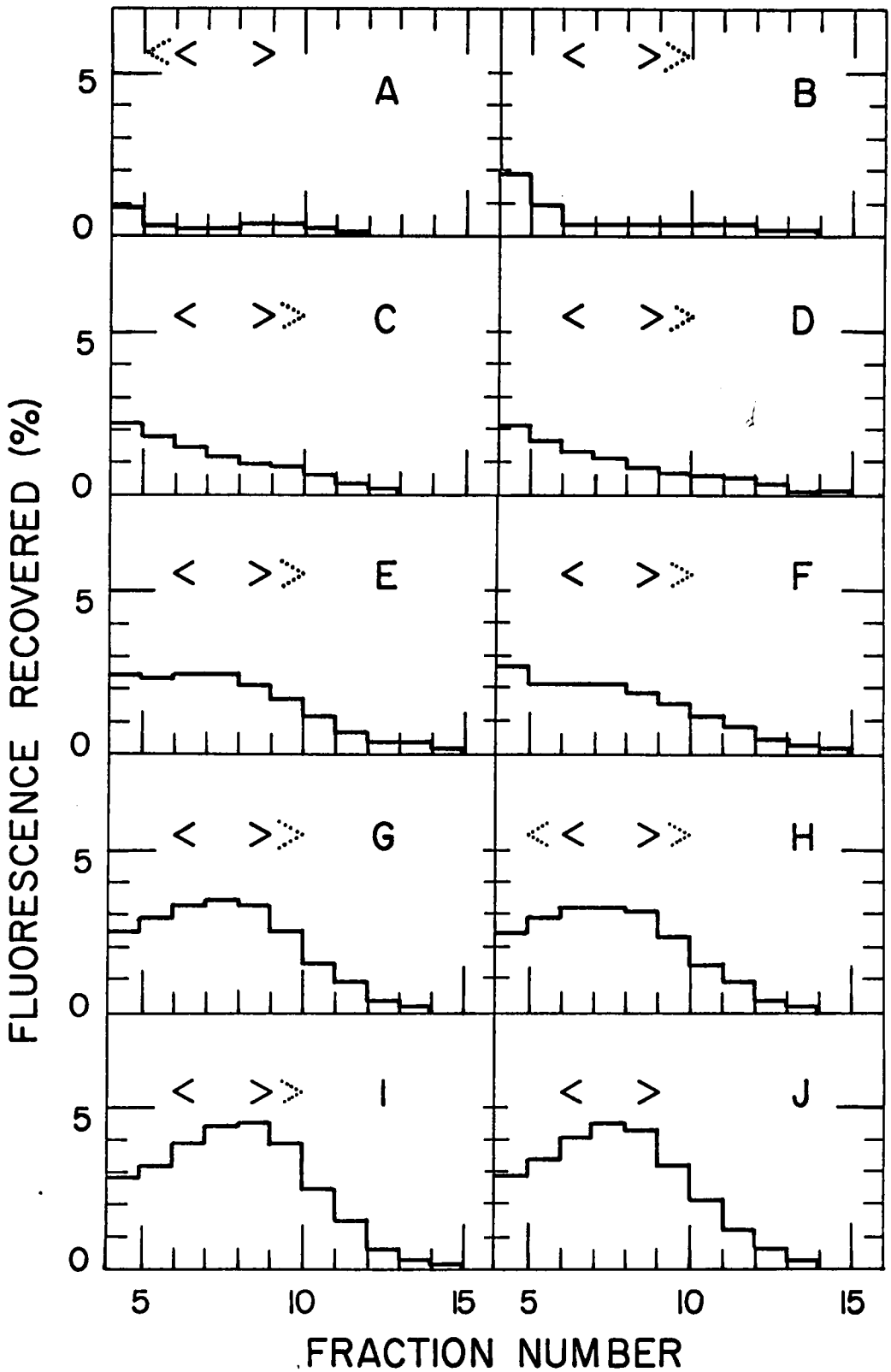
Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0.15	0.7	0.03	0.2
2	1.94	9.5	5.2	25.6
3	12.8	62.5	9.9	48.8
4	1.31	6.4	1.12	5.5
5	0.51	2.5	0.51	2.5
6	0.59	2.9	0.58	2.9
7	0.67	3.3	0.65	3.2
8	0.72	3.5	0.66	3.2
9	0.68	3.3	0.62	3.1
10	0.51	2.5	0.46	2.3
11	0.30	1.5	0.29	1.4
12	0.18	0.9	0.18	0.9
13	0.06	0.3	0.06	0.3
14	0.02	0.1	0.03	0.1
15	0.01	0	0.01	0
16				
Total	20.4		20.3	
% recovery	103%		103%	

TABLE XXIII

F1Hb and its digestive products in fractionated BME incubated with fibroblasts at 37° for 96 hours

Tube	Normal		Low Mg^{2+}	
	mmoles x 10^7	%	mmoles x 10^7	%
1	0.02	0.1	0.02	0.1
2	3.00	12.3	0.70	3.2
3	12.9	52.8	13.7	63.0
4	1.69	6.9	1.62	7.4
5	0.69	2.8	0.63	2.9
6	0.79	3.2	0.74	3.4
7	0.94	3.9	0.89	4.1
8	1.10	4.5	0.99	4.5
9	1.13	4.6	0.93	4.3
10	0.94	3.9	0.69	3.2
11	0.61	2.5	0.46	2.1
12	0.36	1.5	0.26	1.2
13	0.15	0.6	0.12	0.6
14	0.06	0.2	0.02	0.1
15	0.02	0.1		
16				
Total	24.4		21.8	
% recovery	116%		89%	

Figure 9. Fluorescent material recovered from media containing FlHb and incubated with cells. The fractionation was on Sephadex G-50 with fraction 5 through 15 shown here. Sections A, C, E, G, and I were normal media incubated for 0, 24, 48, 72, and 96 hours, respectively. Sections B, D, F, H, and J were low Mg^{2+} media incubated for 0, 24, 48, 72, and 96 hours, respectively. Brackets indicate phenol red.


A
B
C
D
E
F
G
H
I
J
5
0
5
10
15
FLUORESCENCE RECOVERED (%)
FRACTION NUMBER

micromolecular material.

There was no significant difference between the amount of micromolecular fluorescent material, fractions 6-15, present in the control and low Mg^{2+} media at any given day. There was a steady increase in the micromolecular material with time (Table XXIV).

There were two methods to determine the amount of F1Hb digested by the cells. One method was to subtract the amount of fluorescence found in fractions 6-15 of the unincubated F1Hb medium at zero time from the fluorescence of fractions 6-15 of F1Hb medium incubated with cells. The second method was to subtract the fluorescence in fraction 6-15 of media incubated without cells from the corresponding fractions of media incubated with cells for the same period of time.

The results obtained by subtracting the zero time value from the media incubated with cells still showed almost no difference between the normal and low Mg^{2+} in digestion products released. There was a steady rise in the total amount of digestion products with time (Table XXV). If the results were analyzed per 24 hour period (Table XXVI), the results match well. The 96 hour values were higher than the others but not exceptional.

The fluorescent material lost to the medium in the spike experiment was approximated by subtracting the 3 hour average molarity from the 0 hour average molarity of the cell solutions. The mmoles released may then be calculated (Table XXVI). If the 3 hour values were extrapolated to a 24 hour period (Table XXVI), it could be compared to the amount in the medium of the turnover experiment of Table XXIV. For the

TABLE XXIV

Amount of fluorescent material in Sephadex G-50 fractions 6-15 of medium incubated with cells

A. Actual Values

Time	Normal		Low Mg^{2+}	
hours	mmoles x 10^7	%	mmoles x 10^7	%
0	0.55	2.5	0.65	2.9
24	1.55	7.5	1.44	7.1
48	2.49	13.2	2.36	12.2
72	3.73	18.3	3.54	17.4
96	6.10	25.0	5.10	23.5

B. Values corrected for zero time

Time	Normal		Low Mg^{2+}	
hours	mmoles x 10^7	%	mmoles x 10^7	%
24	1.00	5.0	0.79	4.2
48	1.94	10.7	1.71	9.3
72	3.18	15.8	2.89	14.5
96	5.55	22.5	4.45	20.6

TABLE XXV

Amount of fluorescent material corrected for zero time in Sephadex G-50 fractions 6-15 of medium incubated with cells per weight of cell

Time	Normal		Low Mg^{2+}	
	Total	Per 24 hrs	Total	Per 24 hrs
	mmoles x 10^7 / CELL PROTEIN mg		mmoles x 10^7 / CELL PROTEIN mg	
Hour				
24	1.64	1.64	1.37	1.37
48	2.26	1.31	2.78	1.39
72	3.79	1.26	4.01	1.34
96	8.60	2.15	7.77	1.94

TABLE XXVI

Fluorescent material released to the medium in the spike experiment during the first 3 hours

Trial	Normal			Low Mg^{2+}		
	3 hours mmoles x 10^8	Extrapolated to 24 hours mmoles x 10^7	Extrapolated to 24 hours $\frac{\text{mmoles x } 10^7}{\text{CELL PROTEIN mg}}$	3 hours mmoles x 10^8	Extrapolated to 24 hours mmoles x 10^7	Extrapolated to 24 hours $\frac{\text{mmoles x } 10^7}{\text{CELL PROTEIN mg}}$
1	1.94	1.56	2.02	2.04	1.63	2.78
2	2.04	1.63	2.06	1.53	1.22	1.63

24 hour period, 1.00×10^{-7} mmoles were released by the cells in the normal medium in the turnover experiment, as compared to 1.56×10^{-7} mmoles and 1.63×10^{-7} mmoles in the spike experiments. In the low Mg^{2+} turnover experiment only 0.79×10^{-7} mmoles were lost compared to 1.63×10^{-7} mmoles and 1.22×10^{-7} mmoles in the spike experiments. The mmoles per weight of cellular protein of the turnover experiment (Table XXV), if compared to the amount released by the spike (Table XXVI), proved to be in good agreement.

Another way of analysis of the data was to subtract the value of fluorescence in media incubated without cells from the corresponding value of fluorescence incubated with cells. The results of these calculations, listed in Tables XXVII and XXVIII, were much less consistent than the values obtained by subtracting the zero time. There was a steady but small rise in the quantity of material released to the medium. When comparing the amount released by spiked cells (Table XXVI) to that in the media of the turnover experiment (Table XXVIII), there was a large difference. The amount turned over when calculated in this manner was about two to ten times less than the 24 hours extrapolated values of the spike experiments.

TABLE XXVII

Amount of fluorescent material in Sephadex G-50 fractions 6-15 of media incubated with cells less the corresponding value from media incubated with cells

Time	Normal	Low Mg^{2+}
hours	mmoles x 10^7	
24	0.13	0.05
48	0.86	0.79
72	2.1	1.9
96	3.9	3.0

TABLE XXVIII

Amount of fluorescent material in Sephadex G-50 fractions 6-15 of medium, incubated with cells less the corresponding value from media incubated without cells, per weight of cell

	Normal		Low Mg^{2+}	
Time	Total	Per 24 hrs	Total	Per 24 hrs
hours	mmoles x 10^7 / CELL PROTEIN mg		mmoles x 10^7 / CELL PROTEIN mg	
24	0.21	0.21	0.09	0.09
48	1.16	0.58	1.29	0.65
72	2.45	0.81	2.60	0.87
96	6.03	1.51	5.27	1.32

DISCUSSION

The initial step in the metabolism of FlHb is its uptake from the external medium by pinocytosis. There are two possible ways for uptake to occur. The substrate (FlHb) may be soluble in the ingested medium with no membrane binding. This type of uptake occurs in Acanthamoeba casstellanii (8) and mouse macrophages (24) as the uptake is linear within the range of protein concentrations tried. A second type of uptake is of a substrate which is bound to the external membrane. This binding to the membrane tends to concentrate the protein. If the uptake is strictly of membrane bound protein, then a saturating value will cause a leveling off of the uptake curve, even if the medium concentration is increased. An example is the uptake of horseradish peroxidase by rat kidney (25). The saturating effect is not always present. In Ehrlich tumor cells, uptake of albumin is linear over a wide range of concentrations but surface binding is apparent (26). In the results of the dose dependency experiments (Tables IX and X and Figure 4), there is a flattening of the curve but not a complete cessation of increased uptake with increased FlHb concentration. This is interpreted as a combination of substrate binding to the cell surface and soluble substrate in the pinocytotic droplet. The amount binding to the surface approaches saturation at about 100µg/ml as evidenced by the break in the curve of Figure 4. The bound FlHb accounts for the majority of uptake in the lower concentration ranges. The soluble FlHb taken up in the pinocytotic droplet is of lesser importance. As the medium concentration increases, its contribution increases. This could

account for the linear rise past a concentration about 100µg/ml.

As an alternate hypothesis, there may be at least two types of "sites" to which the protein could bind. One type might bind the protein at low solution concentrations and become saturated at about 100µg/ml. The second type might have a lower binding constant and be responsible for the more gentle increase past 100µg/ml in which case the soluble F1Hb in the pinocytotic droplet would play a minimal role.

The fibroblasts when exposed to F1Hb, 200µg/ml, began immediate uptake. The most rapid uptake is from 0 to 6 hours. After this time the slope of the accumulation curve begins to decrease, and by 20 to 24 hours, the curve has almost plateaued. The uptake at 24 hours ranged from about 7 to 15 x 10^{-8} mmoles/mg cell protein. Past 24 hours there is a small but continual rise in the accumulation.

The accumulation is similar to the uptake observed by Tulkens et al. (17) using γ-globulins and rat embryo fibroblasts over a 30 hour period. The γ-globulin uptake is 8.6 x 10^{-3} mg/mg cell protein or 5.4 x 10^{-8} mmoles/mg cell protein. Cohn and Ehrenreich (16) have found an uptake for Hb by macrophage in a 20 hour period to be 1.6 x 10^{-8} mmoles/mg cell protein, a value considerably less than the one reported in this study. In their study of HSA (14)(16) uptake by macrophage, the value is even lower (4.4 x 10^{-9} mmoles/mg cell protein).

The rapid initial rise represents uptake. The decrease in the slope beyond 6 hours could represent digestion and release of the initial input of F1Hb. As time progressed the uptake is almost counterbalanced by release of digested fluorescent products. Since digestive products

of FlHb appears in the media with a constant increase over a four day period, the concept that uptake is counterbalanced by digestion and release would tend to be supported. A second possibility is a burst of pinocytosis which subsides with time. If, however, the release of material is constant as indicated by the turnover experiment, the amount of accumulation by the fibroblasts would be noticably decreased. The "asymptotic" type increase in the accumulation over an extended time period might indicate a lag time in digestion of a small part of the FlHb. There could also be a small accumulation of indigestible residues.

The most striking result in the accumulation of FlHb is the very wide range of values as seen in Tables III and IV. This large difference is difficult to explain until a plot of accumulation per cellular weight versus cell protein is made as shown in Figures 2 and 3. As the protein in the tube decreases, the accumulation per protein increases. The accumulation at lower protein levels has a larger increase with time than at higher protein values. This is shown by the increasing slopes depicted in Figure 3 and Table VIII. The number of points available in any single line, Figure 3, are not necessarily enough to show increased accumulation with decreased protein. However, by evaluating all the plots including Figure 2, this effect appears to be real. The large deviation in the slope of low Mg^{2+} line, as mentioned previously, is probably caused by a lack of sufficient number of data points in the low protein region.

The reasons for the decreased accumulation/mg cell protein with an

increase in total cell protein are unclear. Steinman and Cohn (24) have found no change in accumulation of horseradish peroxidase per weight of cell as the cell protein varies. The time period, only two hours, may not have been long enough to see the effect. A possible consequence of cell crowding may be a decrease in metabolism, which would show as decreased accumulation. There is a correlation between the amount of protein and the size of the cells. At lower protein values the cells have a classical fibroblast shape. At higher protein values, the cells are much more crowded with many more cells per unit area. The cells do not seem to be elongated but rather have a distorted rhombohedral shape which may possibly be referred to as senescence.

The spike experiments, Table XI and Figures 5 and 6, show the loss of labeled material after removal of FlHb from media. The rate of loss in the two experiments appears to be very similar; although, the accumulated protein at 24 hours is different. The released products appear to be micromolecular in nature, based on column studies of the incubated media (Tables XIX-XXIII and Figure 9). This would be in agreement with Tulkens (17)(18) and Ehrenreich and Cohn (14)(16). The released micromolecular labeled material is either an amino acid which cannot be utilized in protein synthesis because of the fluorescein label or a small peptide containing the label. The method of release is unknown. The possibility that a labeled amino acid can diffuse or be transported across the lysosomal membrane is not unreasonable. The labeled amino acid would diffuse or be transported to the external medium from the cytoplasm.

Another possibility is that the material released to the medium by exocytosis is the undigestible fragments containing the label. This appears less likely because a measurable quantity of partially digested Hb would be expected to be found in the medium. Ehrenreich and Cohn (14) also did not find large fragments of the partially digested protein in the washout medium of spiked or pulsed macrophage. Small quantities would not necessarily be found, however.

In work by Mego et al. (27), particles (lysosomes) have been isolated from mouse liver homogenates. The mice have previously been injected with formaldehyde treated ^{131}I-albumin. Following incubation of these particles at 37°, ^{131}I-tyrosine has been found in the incubation solution. This indicates that digestion to the level of amino acids or small peptides occurred.

Based on the limited data of the spike experiments, there appears to be a slight break in the semilog plot of retained fluorescent material versus time. The semilog plot, Figure 6, shows this break to be at 9 hours and indicates that the breakdown of FlHb is not entirely a first order process. One interpretation is that most of the digestion occurs in the lysosome, with small undigested fragments crossing the lysosomal membrane to the cytoplasm where final digestion takes place. This idea, proposed by Coffey (28), suggests that material is broken down in the lysosome with certain undigestible bonds being left unattached. The small peptides, it was postulated, then enter the hyaloplasm by diffusion or transport where the cytoplasmic enzymes finish the degradation. Coffey and de Duve (3) in studies of rat liver

lysosomal enzyme digestion of protein have shown that 70% of the protein bonds are broken by the lysosomal fraction at acidic pH's. The remainder cannot be degraded even at pH 8. The soluble fraction, however, digests all but about 10% of the bonds. This experiment has been performed with fractions of liver homogenates rather than in vivo which can explain why the other 10% is not degraded.

A second interpretation is that most of the protein bonds are easily cleaved by the hydrolytic enzymes. There are a few bonds which are not easily broken, however. Either interpretation helps to explain the slight increase in the accumulation with time.

The half life of the spiked F1Hb appears to be approximately 24 hours in fibroblasts. The half life for ^{125}I-Hb in pulsed or spiked macrophage is also 24 hours (16). Macrophage digests ^{125}I-HSA (14) (16) much faster with a halflife of about 5 to 6 hours. Horseradish peroxidase (24) has a halflife of 20 to 30 hours in macrophage. The loss of the labeled fragments from the fibroblasts is quite similar to that of macrophage. Any differences in rates of digestion of hemoglobin between fibroblasts and macrophage is quite small. This does not rule out the possibility that the release rate from fibroblasts is greater than first order. This digestion rate might well be a function not only of the cell but also of the substrate.

The incubation of media containing F1Hb, at 37° in the absence of cells causes a small shoulder to appear in fractions 5 through 15 of the fractionated media. The largest portion of the shoulder is in fractions 6-10 (Figures 7 and 8). The shoulder becomes slightly

larger as the incubation time increases. The exact nature of the material in the shoulder is unknown, but could be partially denatured FlHb which is retarded slightly by the column. Alternatively this material could be FlHb which has been cleaved into large fragments by the proteases present in the calf serum; the size of which would have been small enough to be fractionated by the Sephadex G-50. It seems unlikely that this shoulder is composed of small peptides or individual amino acids. Ehrenreich and Cohn (14) have shown that incubating ^{125}I-HSA in a 50% nbcs for 48 hours at 37° produces no ^{125}I-monoiodotyrosine. In a test with Hb, Ehrenreich and Cohn (16) have found no increase in TCA soluble Hb fragments, when the medium is incubated without cells. Micromolecular species are released after a period of time, if cells are incubated in media containing FlHb. The data in Tables XIX-XXII and Figure 9 show a steady rise in digested material. One method used to determine the actual amount turned over by the cells is to subtract the amount in the shoulder, fractions 6-15, in media incubated without cells from its corresponding value of released micromolecular material. The values obtained by this method are extremely small (Tables XXVII and XXVIII) and very irregular. If the shoulder in the media incubated without cells is composed of partially cleaved FlHb, then the shoulder is probably present in media incubated with cells, but is masked. Ryser (6) has found that generally the larger the protein the greater its uptake. Therefore, these fragments would not be expected to be taken up as readily as the intact FlHb. The extrapolated 24 hour values for released material in the spike experiment (Table XXVI)

are much greater than the turnover values in Table XXVIII.

The values of the released fluorescent material could have been corrected by subtracting the zero time value. If the fluorescent shoulder in the media incubated without cells is denatured species, its uptake could have been enhanced. Then, as the FlHb is denatured, it would have been preferentially removed from the media. Denaturation enhances digestion (3) but whether it affects uptake is not known. The turnover values (Tables XXIV and XXV) on a 24 hour basis correspond with the amount lost in the spike experiment (Table XXVI). The turnover rate is about 5% per 24 hours. The range of turnover, 1.26 to 2.15 x 10^{-7} mmoles/mg cell protein, is most likely due to biological variation between cultures such as amount of protein in each tube. Tulkens (18) has found that about 32% of the fluorescent γ-globulins are digested to micromolecular species in four days. He used a γ-globulin concentration of 0.29 mg/3 ml of medium for 0.37 mg of cellular protein. The values in this study for FlHb are 0.6 mg/3 ml of medium for an average of 0.7 mg cellular protein.

Release of fluorescent material to the media in the spike experiments is calculated from the 3 hour value extrapolated to 24 hours. It is felt that this short time period is more realistic of release in a continuous turnover situation. The extrapolated 24 hour value averages about 2 x 10^{-7} mmoles/mg cell protein. The 24 hour turnover values, less the zero time, averages 1.5 x 10^{-7} mmoles/mg cell protein. The spike released value might have represented the release of more easily digested portions of FlHb. This could have given a value slightly larger

than the actual value. Any differences could also have been attributed to the differences among cultures.

The comparison of the spike release value to the turnover values, less the corresponding cell free incubation values, is much less satisfactory. The average turnover value in 24 hours is about 0.8 x 10^{-7} mmoles/mg cell protein which is less than half of the average spike release value.

The reduction of the Mg^{2+} concentration in the culture media has no significant effect on any aspect of FlHb metabolism. The accumulation of FlHb cells in low Mg^{2+} media is not depressed from the control; this indicates that pinocytosis in rat embryo fibroblasts is not affected by the lowered medium Mg^{2+} concentration. The observed lowering of accumulation in the initial experiments is attributed to different handling techniques of the cells. The plots of FlHb accumulation per cellular protein versus cell protein (Table VIII and Figure 3) shows no statistical difference between normal and low Mg^{2+} media.

Even extended duration of exposure to the low Mg^{2+} media has little effect on the net uptake. There is a 9 fold increase in Mg^{2+} in the normal medium as compared to the low Mg^{2+} medium. When the Mg^{2+} concentration is elevated to about 17 times greater than the low Mg^{2+} medium, there is still no effect on accumulation. Although the accumulation increases as the FlHb concentration increases, there is no significant difference between accumulation in cells in normal or low media. Depletion of Mg^{2+} prior to treatment with FlHb causes no decrease in accumulation. Low Mg^{2+} media causes no change in rate of release of

digested FlHb. The media containing FlHb in which the cells are grown shows no changes in quantities of digestive products from the normal to the low Mg^{2+} condition. The modification of FlHb in media incubated without cells is also independent of Mg^{2+} concentration within the tested limits. In no aspect of this study is there a significant difference between cells in low Mg^{2+} and normal media. The very small differences in accumulation have been sporadic and could not have been considered to constitute any statistical differences.

The Carlsberg studies by Chapman-Andresen and others (11)(12)(13) demonstrates an increase of pinocytosis with increased Mg^{2+} concentration. This effect occurs at Mg^{2+} concentrations much greater than the ones used in this study. Their organisms of study were free living amoebae whereas this study utilizes cells isolated from a mammalian source. The variations in ionic strength caused by Mg^{2+} concentrations changes in the fibroblast culture media are insignificant compared to the total ionic strength. If the fibroblast is as sensitive to ionic strength as the amoeba is, then it might be possible to produce pinocytosis by drastic alterations in media Na^{+} and Mg^{2+} concentrations.

Bunce and Bloomer (24) have found the Mg^{2+} level in normal rat serum is 0.88 mM while the normal medium used in this study contained 1.8 mM. The Mg^{2+} level in the serum of a rat maintained on Mg^{2+} deficient diet for at least 3 days is 0.44 mM while the low Mg^{2+} medium contained 0.21 mM Mg^{2+}. Both the high and low limits of Mg^{2+} concentration in sera are exceeded in the culture media used in this study.

Magnesium deficiences have produced a wide range of effects from

calcification in the kidney to interference in bacterial cell wall synthesis (30). The effects of a low Mg^{2+} diet upon the rat kidney could have been due to the unique function of the kidney cells. The effects observed might also have been secondary effects caused by alteration of the normal function of other organs and/or cells which in turn caused changes in the kidney cells. These changes could well have been produced in the whole animal and not found in culture of isolated cells. There is, however, no effect observed in the culture of rat embryo fibroblast.

This system could be useful in studying the effect of nutrients and hormones on pinocytosis and other metabolic activities. This cell study would be useful in comparing the reactions to low Mg^{2+} to that of other cells. This system would be excellent for comparison of low Mg^{2+} on the effect of pinocytosis in kidney to that of fibroblasts.

More study is needed on the digestion and release of labeled material in pulse experiments to determine if the release is indeed greater than first order. Brief pulses followed by cell fractionation at various times may help locate the presence of digested fragments in fractions other than the light mitochondral fraction. Comparison studies with similarly labeled HSA might also be of value in tracking the digestive products from the lysosome to the external media.

The study of FlHb incubated in culture media without cells needs more attention. Longer columns or smaller porosity packing material may be useful in determining the nature of the fluorescent material in this shoulder. Dialysis may also be useful in the determination of the

shoulder components. Incubation of F1Hb in the modified BME without calf serum should determine if the serum is responsible for the shoulder.

SUMMARY

Rat embryo fibroblasts in cell culture were used to study FlHb uptake, digestion, and release of products to the medium. The cells were grown in modified BME with 10% calf serum. Both normal media and low Mg^{2+} media were tested to determine any effects of low Mg^{2+} on the metabolism. The substrate, FlHb, was prepared by reacting fluorescein isothiocyanate with Bovine Hb. Accumulation and spike experiments were conducted to obtain base line information on normal activity of fibroblast. The cells, dissolved in DOC, were analyzed for fluorescence and for protein by the Lowry method. Turnover experiments were performed over 96 hours to determine the quantities of FlHb metabolized in normal and low Mg^{2+} conditions. Column fractionation with Sephadex G-50 was used to analyze the media from these turnover experiments.

The accumulation values ranged from 7 to 15 x 10^{-8} mmoles/mg cell protein. Variations in the substrate concentrations demonstrated a change in surface binding at approximately 100µg/ml. In cells which had been pulsed for 24 hours with FlHb, the rate of loss appeared to be slightly greater than first order. The half life of ingested FlHb was about 24 hours. The turnover of FlHb was from 1.26 to 2.15 x 10^{-7} mmoles/mg cell protein in one day. This amounted to about 5% of the total substrate present in the medium.

Low Mg^{2+} media had no effect on the metabolism of FlHb by fibroblasts. The accumulation of FlHb was greater in fibroblasts than I^{*}-HB in macrophages. The rate of loss of digestive products from spiked experiments was about the same. The release of digestive products from

fibroblasts appeared to be slightly greater than first order. This would lend support to Coffey's and de Duve's postulation that digestion occurs in both the lysosome and in the cytoplasm. The turnover rate of FlHb was similar to Tulkens' work with γ-globulin metabolism in fibroblasts.

This study has provided base line information on a relatively undifferentiated cell type which can be compared to studies with other cells.

REFERENCES

1. de Duve, C. (1969). The lysosome in retrospect. In Lysosomes in Biology and Pathology. Vol. 1. (Dingle, F. and Fell, H. B., Eds.), pp 3-40, North Holland Publishing, Amsterdam.

2. de Duve, C. and Wattiaux, R. (1966). Functions of lysosomes. Ann. Rev. Physiol. 28, 435-492.

3. Coffey, J. W. and de Duve, C. (1968). Digestive activity of lysosomes I. The digestion of proteins by extracts of rat liver lysosomes. J. Biol. Chem. 243, 3255-3263.

4. Novikoff, A. B., Essner, E., and Quintani, N. (1964). Golgi apparatus and lysosomes. Fed. Proc. 23, 1010-1022.

5. Cohn, Z. A. (1970). Endocytosis and the vacuolar system. Cell Membranes, Biological and Pathological Aspects. (Richter, G. W. and Scarpelli, D. G., Eds.), pp 129-135, Williams & Wilkins, Baltimore.

6. Ryser, H. J.-P. (1970). Transport of macromolecules, especially proteins, into mammalian cells. In Proceedings of the Fourth International Congress on Pharmacology. (Eigenmann, R., Ed.), pp 96-118, Schwabe, Basel & Stuttgart.

7. Ryser, H. J.-P. (1968). Uptake of protein by mammalian cells: an underdeveloped area. Sci. 159, 390-396.

8. Woessner, J. F. (1965). Acid hydrolases of connective tissue. International Tissue Research. Vol. 3. (Hall, D. A., Ed.), pp 201-260, Academic Press, New York.

9. Shea, S. M. and Karnovsky, M. J. (1970). Transport of macromolecules, especially proteins, into mammalian cells. The point of view of the electron microscopist. In Proceedings of the Fourth International Congress on Pharmacology. (Eigenmann, R., Ed.), pp 118-123, Schwabe, Basel and Stuttgart.

10. Bowers, B. and Olszewshi, T. E. (1972). Pinocytosis in Acanthamoeba casstellanii kinetics and morphology. J. Cell Biol. 53, 681-694.

11. Chapman-Andresen, C. (1965). The induction of pinocytosis in amoebae. Arch. Biol. 76, 189-207.

12. Chapman-Andresen, C. (1958). Pinocytosis of inorganic salts by Amoebae proteus (Choas diffluens. Compt. Rend. Lab. Carlsberg. 31, 77-92.

13. Cooper, B. A. (1968). Quantitative studies of pinocytosis induced in Amoeba proteus by simple cations. Compt. Rend. Lab. Carlsberg. 36, 385-403.

14. Ehrenreich, B. A. and Cohn, Z. A. (1967). The uptake and digestion of iodinated human serum albumin by macrophages in vitro. J. Exp. Med. 126, 941-958.

15. Cohn, Z. A. (1966). The regulation of pinocytosis in mouse macrophages I. metabolic requirements are defined by the use of inhibitors. J. Exp. Med. 124, 557-571.

16. Ehrenreich, B. A. and Cohn, Z. A. (1968). Fate of hemoglobin pinocytosed by macrophages in vitro. J. Cell. Biol. 38, 244-248.

17. Tulkens, P., Trouet, A., and van Hoof, F. (1970). Immunological inhibition of lysosome function. Nature 228, 1282-1285.

18. Tulkens, P. (1970). Les Lysosomes des Fibroblastes de Rat en Culture. unpublished memoire, Université Catholique de Louvain.

19. Eagle, H. (1971). Buffer combinations for mammalian cell culture. Sci. 174, 500-503.

20. de Lumen, B. O. and Tappel, A. L. (1970). Fluorescein hemoglobin as a substrate for cathepsin D and other proteases. Anal. Biochem. 36, 22-29.

21. Lowry, O. H., Rosebrough, N. J., Farr, L. A. and Randall, R. J. (1951). Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

22. Bailey, J. L. (1967). Techniques in Protein Chemistry, 2nd Ed., pp 340-341, Elsevier Publishing Co., New York.

23. Pharmacia Fine Chemicals (1972). Sephadex Gel Filtration in Theory and Practice, pp 31-49, Upsala, Sweden.

24. Steinman, R. M. and Cohn, Z. A. (1972). The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. J. Cell. Biol. 55, 186-204.

25. Straus, W. (1962). Colorimetric investigations of the uptake of an intravenously injected protein (horseradish peroxidase) by rat kidney and effects of competition by egg white. J. Cell. Biol. 12, 231-246.

26. Ryser, H, Aub, J. C., and Caulfield, J. B. (1962). Studies on protein uptake by isolated tumor cells. II. Quantitative data on the adsorption and uptake of I^{131}-serum albumin by Ehrlich ascites tumor cells. J. Cell. Biol. 15, 437-449.

27. Mego, J. L., Bertini, F., and McQueen, J. D. (1967). The use of Formaldehyde treated ^{131}I-albumin in the study of digestive vacuoles and some properties of these particles from mouse liver. J. Cell. Biol. 32, 699-707.

28. Coffey, J. W. (1966). Protein digestion by hepatic lysosomes. J. Cell. Biol. 31, 21A.

29. Bunce, G. E. and Bloomer, J. E. (1972). Effect of magnesium deficiency on serum and urinary ions in rats: studies with ion-selective electrodes. J. Nutri. 102, 863-872.

30. Garrett, A. J. (1969). The effect of magnesium ion deprivation on the synthesis of mucopeptide and its precursors in Bacillus subtilis. Biochem. J. 115, 419-430.

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STUDIES ON THE UPTAKE AND FATE OF HEMOBLOBIN PINOCYTOSED BY RAT EMBRYO FIBROBLASTS CULTURED IN NORMAL AND LOW MAGNESIUM LEVEL

by

William Wills Bradford

(ABSTRACT)

Rat embryo fibroblasts were used to study the metabolism of F1Hb in normal and low Mg^{2+} media. The fibroblasts were cultured in a modified Eagle's basal medium with a 10% calf serum supplement. The test substrate was in a concentration of 200µg/ml. Accumulation and spike experiments were conducted to measure uptake and digestion. Turnover experiments measured rates of digestion of a pinocytosed substrate.

The accumulation was between 7 and 15 x 10^{-8} mmoles/mg cell protein. The half life for ingested F1Hb was about 24 hours. There was considerable variation in accumulation per weight of cell protein when compared to the quantity of protein present. The lower the protein, the higher the accumulation. From dose dependent experiments there appeared to be surface binding saturation. The turnover rate was approximately 5% of the F1Hb per day. There was no difference between the metabolism of F1Hb by fibroblasts in the low Mg^{2+} or normal media.