THE ACCUMULATION AND DISTRIBUTION OF EVANS BLUE IN THE KIDNEY OF RATS FED NORMAL OR LOW MAGNESIUM DIETS

by

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LIST OF ABBREVIATIONS

RSA-^{125}I	^{125}I-labeled Rabbit Serum Albumin
N	Nuclear Fraction
M	Heavy Mitochondrial Fraction
L	Light Mitochondrial Fraction
P	Microsomal Fraction
S	Supernatant
NBC	Nutritional Biochemical Company
S.E.M.	Standard Error Mean
p	probability
n.s.	not significant

INTRODUCTION

Background Information - One of the earliest studies of magnesium deficiency in the rat was that of Greenberg et al. in 1936 (1). Histological examinations of various soft tissues revealed evidence of marked degenerative changes which increased with time, particularly in the kidneys, from animals which had been fed a magnesium deficient diet over a period of 18 to 120 days. Control animals showed no evidence of gross or histological changes in the kidneys.

More recent studies have been performed in an attempt to determine the sequence of changes which lead to the profound degenerative state. The earliest alteration observed in the kidney due to magnesium deficiency was mild to moderate swelling of the proximal convoluted tubules (2, 3). Using the glyoxyl reaction and micro-incineration Battifora et al. (2) have demonstrated increased amounts of ionic intracellular calcium coincident with the cellular swelling. Within one week, both intracellular and intraluminal calcium deposits were observed near the cortico-medullary junction in the proximal and distal convoluted tubules and the loop of Henle. The intracellular calcium deposits were described as either lying free in the cytoplasm or associated with lysosome-like bodies.

The intraluminal calcium deposits seem to be the primary sites of extensive calcium accumulation (3). Oliver et al. (3) found that at earliest detection (second week of magnesium depletion) these intraluminal calcium deposits were spherical laminated microliths which gave a strong positive reaction to periodic acid Schiff (PAS) stain and a weak positive reaction for calcium and phosphate. As the period of deficiency was extended, the intensity of the PAS stain diminished and the calcium and phosphate reaction became dominant. They concluded that these microliths contained a matrix of mucopolysaccharide (PAS positive) substances which serve as a point of precipitation for calcium and phosphate. The continued deposition of calcium and phosphate onto this matrix is in a laminer manner.

The mechanism of formation of these intraluminal calcium deposits is not known. There is reason to believe, however, that the induction of kidney stones by means of restriction of magnesium intake may serve as a model for calculus disease in the kidney. Boyce and King (4) have proposed an "active matrix" theory of kidney stone genesis. They have noted that the induction of renal calculi by administration of either thiacetazone, excess vitamin D, or parathyroid hormone was always preceded by the appearance in the tubular lumen of laminated PAS positive bodies which subsequently calcified. They suggested that these lithogenic agents shared in common the ability to inflict a specific disturbance upon renal tubular metabolism such that an initiator of calcification, "active matrix," was expelled into the tubular space. They proposed that the initiation of renal calculus formation requires disruption of specific enzyme systems within the proximal tubular cell. This derangement of kidney metabolism gives rise to a PAS positive matrix which serves as the nucleus for subsequent mineral accretion.

The theory of Boyce and King fails to explain the nature and origin of the PAS positive laminated matrix. Bunce and Bloomer (5) in 1972 have presented an hypothesis which attempts to explain the identity of the matrix and the method of appearance in the tubular lumen. They have suggested that the PAS-positive matrix may originate in the vacuolar apparatus and that intracellular imbalances in calcium and magnesium ion concentrations may stimulate its egress into the tubular lumen. The following sequence of events has been hypothesized. Pinocytosis is known to occur in the renal proximal tubular cells where it serves as the means of recovery of protein from the glomerular filtrate. Protein is first adsorbed onto the glycocalyx (cell coat) within the tubular lumen. The pinocytotic vesicles subsequently formed move toward the interior of the cell where they may merge either with one another, primary lysosomes, or secondary lysosomes. Under normal conditions, the ingested prey molecules are first denatured and then hydrolyzed by acid hydrolases to their monomeric units which may then return to the cellular pool by diffusion into the cytoplasm. Under conditions of magnesium deficiency, this chain of events may be interrupted. Regurgitation, that is, exocytotic return of vacuolar material into the compartment of its origin, may take place before digestion is complete. This regurgitation may be stimulated by an increased calcium ion concentration within the cytoplasm, or distortions in the normal Ca^{++}/Mg^{++} ratio. This process would deliver partially digested and denatured proteins and the glycoproteins of the glycocalyx into the tubular space. Crystals of calcium phosphate, formed within the vacuole from the calcium ion present in the original vacuolar fluid and inorganic phosphate released by the action of acid phosphatase, would be attached to this proteinaceous mass. This proteinaceous PAS positive mass studded with clusters of calcium phosphate would serve as a site for further precipitation within the tubular lumen.

Use of Evans Blue - In order to study the possible role of the vacuolar apparatus in calculus disease initiated by the consumption of a low magnesium diet, it would be useful to have a harmless, non-allergenic, persistent, and unique label which could be employed to detect deviations of the normal flow of macromolecules. The acid bi-sazo dye Evans blue, T-1824, was thought to be suitable as such a marker. The use of Evans blue offers several advantages over other methods of labeling. When administered intravenously it binds preferentially with serum albumin and passes into the glomerular filtrate. The albumin-dye complex is then reabsorbed into vesicles by the renal proximal tubular cells (6, 7, 8, 9). Since Evans blue enters the pinocytotic vesicles as a complex with circulating albumin, there is no introduction of a foreign protein with its attendant foreign protein reaction. The dye can be detected by both histochemical and spectrophotometric techniques and persists in the tissues in detectable amounts for several days.

The research to be reported in this thesis has been devoted to the study of the normal behavior of Evans blue in the vacuolar apparatus of rat liver and kidney and to the determination of changes which might be imposed by the feeding of a low magnesium diet.

LITERATURE REVIEW

Review of Vacuolar Apparatus - Extracellular macromolecules are taken into cells by a process called endocytosis, either pinocytosis or phagocytosis (10, 11, 12, 13). Pinocytosis is the uptake of fluids, soluble material, and materials too small to be seen with a microscope; whereas, phagocytosis is uptake of larger extracellular material that can be seen under a microscope. The endocytosed materials may be digested, stored, or even ejected unaltered by a regurgitation type of exocytosis. In most cases the material becomes digested by lysosomal enzymes. The process proceeds by an invagination of the cell membrane resulting in the formation of a phagosome or vacuole within the cell enclosing the extracellular materials. Since these substances are in complex molecular form, they must be broken down or digested by the cell to a simpler form which can then be utilized for new synthesis. The newly formed phagosome migrates across the cell. During this migration it may become fragmented by budding into smaller vacuoles or associated by fusion with other phagosomes. Its ultimate fate is to either fuse with a vacuolar body containing hydrolytic enzymes or with the membrane of the cell wall. The vacuolar body may be either a "primary lysosome," a newly synthesized granule which has not yet reacted with the substrates, or a "secondary lysosome," a digestive vacuole which has already been the site of hydrolytic events. In either case the membranes of the two fuse to form a digestive vacuole. In this way macromolecular substances from the medium surrounding the cell are exposed to lysosomal hydrolases and are degraded to products which diffuse through the vacuolar membrane into the cytoplasm, where they may be used in various synthetic processes. In cases where there are indigestible substances a residual body may occur which may be retained or ejected from the cell into the external environment by a process similar to phagocytosis in reverse known as exocytosis. It has been proposed that some phagosomes do not fuse with vacuoles containing hydrolytic enzymes but migrate to the cell membrane, fuse, and discharge their contents into the extracellular compartment. This process is known as diacytosis if the phagosome moves completely across the cell before it is ejected and regurgitation if the phagosome is ejected into its original excellular compartment (10, 11, 12, 13).

It should be emphasized that the processes described above are performed in a closed space made of vacuoles isolated from the cytoplasm and surrounded by a membrane similar to the plasma membrane. Phagosomes and secondary lysosomes can be considered as portions of the extracellular medium enclosed in the cell, into which primary lysosomes disgorge their content of digestive enzymes. The term vacuolar apparatus has been given to this set of vacuoles (12, 13).

Uptake of Albumin by the Kidneys - All body cells except the red blood cells are capable of endocytosis to some degree; however, the role of endocytosis is particularly important in the renal proximal tubular cells of the kidney where it serves as the means of recovery of protein from the glomerular filtrate. Several workers have shown that protein, including serum albumin, is reabsorbed by the renal proximal tubular cells by means of pinocytosis and then degraded intracellularly within lysosomes (14, 15, 16, 17, 18).

The first evidence that absorbed foreign proteins became located in particles having lysosomal properties came from tissue fractionation experiments by Straus (14) and Straus and Oliver (15), who studied the uptake of egg white and horseradish peroxidase following intravenous injections. They demonstrated that peroxidase was first taken up into apical vacuoles, referred to as phagosomes, and subsequently transferred to lysosomes, identified histochemically by their acid phosphatase content.

In 1962 Straus (16) studied the changes in the concentration of horseradish peroxidase in kidney homogenates, kidney subcellular fractions, blood serum, and urine over a period of several days following administration of the protein. As mentioned before, his earlier cytochemical studies indicated that injected horseradish peroxidase first appeared in smaller phagosomes which subsequently merge to form larger phagosomes (14, 15). He attempted to show a differential distribution of the foreign protein based upon the size of the vacuolar particles.

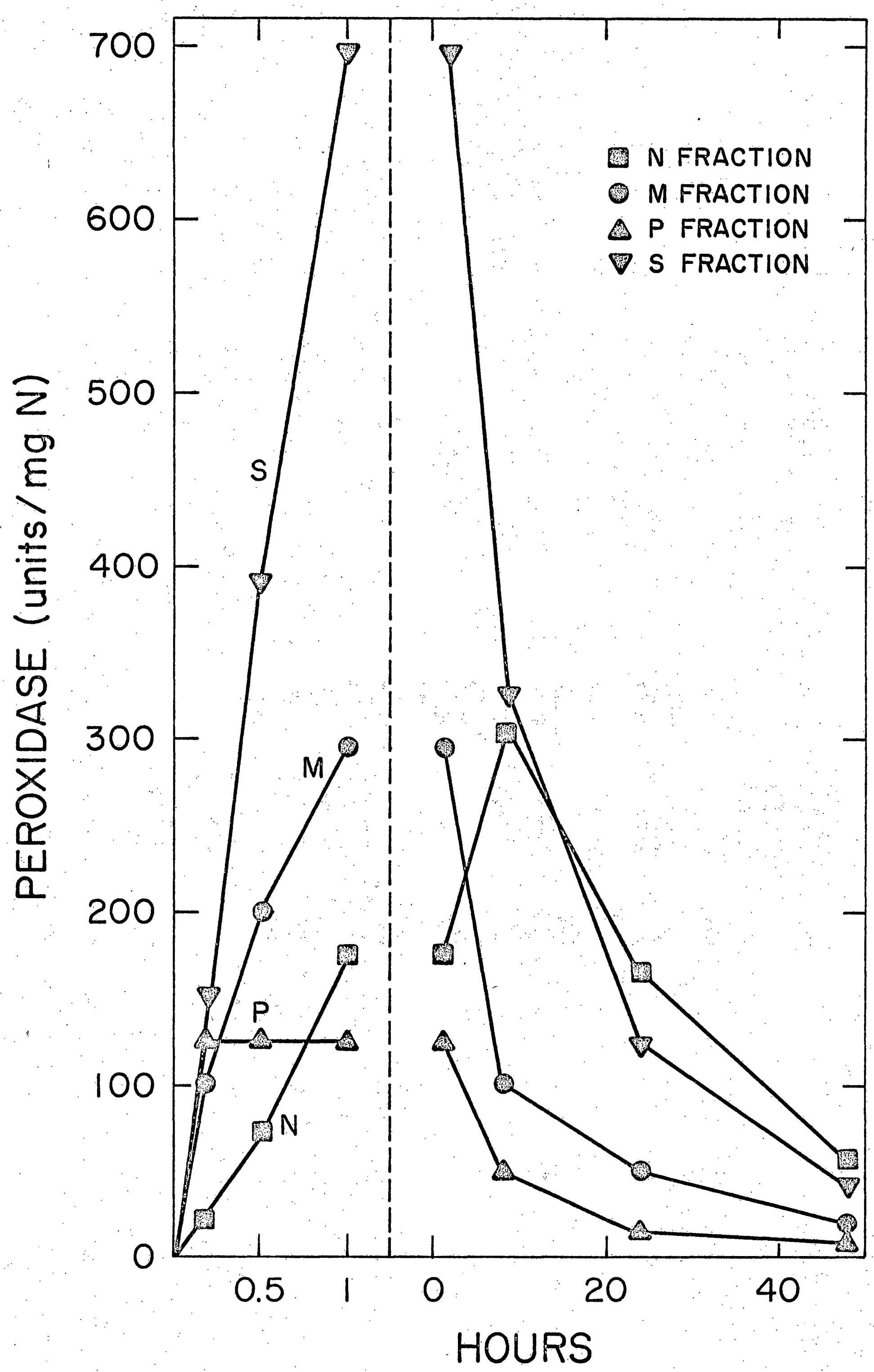
Rats were injected intravenously with horseradish peroxidase and then sacrificed at times varying from a few minutes to several days post-injection. The kidneys were homogenized and divided by fractional centrifugation into four subcellular fractions: nuclear (N) containing nuclei, large phagosomes, and large mitochondria; mitochondrial (M) containing mitochondria and intermediate sized phagosomes; microsomal (P) containing microsomes and small phagosomes; and a final supernatant (S). The fractions as well as an aliquot of the original homogenate, were analyzed for peroxidase activity at each time interval.

As is seen in Figure 1, the concentration of peroxidase at various periods following injection was quite different for the fractions. The microsomal fraction (small phagosomes) was saturated with peroxidase soon after administration, and its peroxidase content decreased quickly after one hour. In the nuclear fraction (large phagosomes) the concentration of peroxidase increased from low to very high levels during the first few hours and then decreased slowly over a period of several days. The concentration of peroxidase in the mitochondrial fraction (intermediate phagosomes) showed behavior intermediate between the nuclear and microsomal fractions. Straus claimed that these results supported his hypothesis that small phagosomes take part in the formation of large phagosomes. Two possibilities were offered for the large amount of peroxidase found in the supernatant (S). Cytochemical observations have shown that a relatively large portion of the peroxidase in kidney cells was localized in "canaliculi" (intercellular spaces), and in membranes, and cytoplasmic strands adjacent to the canaliculi (19). Straus suggested that a portion of this peroxidase was released during the experimental procedures and then appeared in the supernatant. He also suggested that peroxidase may have been released *in vivo* from the phagosomes due to possible changes in the permeability of their membranes.

Changes in the peroxidase content of total homogenate at different intervals after administration were also observed. It was found that peroxidase activity increased to its highest point in the first few hours and then decreased rapidly in the following hours.

Figure 1 - Uptake of Horseradish Peroxidase with Time by Rat Kidney Subcellular Fractions

Data for this figure came from the work of Straus (16).


N FRACTION
M FRACTION
P FRACTION
S FRACTION
S
M
P
N
PEROXIDASE (units/mg N)
0
100
200
300
400
500
600
700
0.5
1
0
20
40
HOURS

Straus showed that the concentration of peroxidase in blood serum and urine decreased exponentially. Peroxidase was cleared from the blood in the first five to six hours; however, small amounts of peroxidase were excreted in the urine for several days.

Straus later investigated the relationship between lysosomes and phagosomes by combining a cytochemical procedure for acid phosphatase, the azo dye method, with a cytochemical reaction for peroxidase with benzidine (20). By applying these reactions successively to the same tissue sections, the lysosomes were stained red, and the phagosomes were stained blue. The procedure for injection of horseradish peroxidase and execution of the rats was the same as before, but this time kidneys were fixed, sectioned, and stained by the two reactions mentioned above for histochemical analysis.

Three different stages were distinguished by microscopic observation in the cells of the convoluted tubules after entry of peroxidase. In the first stage, the first 30 minutes following the injection, phagosomes (peroxidase-positive granules) were seen at the base of the brush border or close to the plasma membrane. They were separate from preexisting lysosomes (acid phosphatase-positive granules) which were located in the apical and intermediate zones of the cells. In the second stage, 30 minutes to three days after administration, the color reactions for acid phosphatase and peroxidase were observed in the same granules. Straus termed these "phago-lysosomes." A third stage was distinguished when no peroxidase was detected in the lysosomes. This occurred three days after injection; however, the reaction for acid phosphatase remained positive. Since the lysosomes contain cathepsin, it was assumed that the peroxidase was digested gradually within the phago-lysosomes.

Straus's work demonstrates that a foreign protein, horseradish peroxidase, is taken up by kidney convoluted tubular cells. Direct evidence has been presented which suggests that serum albumin is normally filtered in the glomerulus and reabsorbed by the kidney proximal tubular cells from the glomerular filtrate (17).

Maunsbach (17) has shown that radioactive labeled albumin enters the proximal tubular cells. In his studies ^{125}I-labeled homologous albumin was injected directly into single proximal tubules with micropipettes. After different time intervals the albumin-perfused tubules were analyzed by electron microscopic autoradiography.

The results from this experiment using labeled albumin were similar to the results obtained by Straus (20) using horseradish peroxidase. Five to ten minutes after its injection albumin was located in both large and small apical vacuoles and apical cell membrane invaginations. Maunsbach suggested that the albumin was taken into apical cell membrane invaginations, which then pinched off to form small apical vacuoles. These then either fused with one another to form large apical vacuoles or fused with preexisting large apical vacuoles. After 30 minutes from the start of absorption the albumin was located in large cytoplasmic bodies which were acid phosphatase-positive and, therefore, identified as heterolysosomes by Maunsbach.

It is noteworthy that ultrastructural changes did not occur in the tubular cells during the absorption of albumin. This strongly suggests that the labeled protein entered, but did not interrupt, a normally occurring pathway for protein absorption and digestion by tubular lumen cells.

Maunsbach further concluded that albumin is reabsorbed and concentrated at discrete sites in kidney cells by a fractionation experiment (17). Animals were injected intravenously with ^{125}I-labeled homologous albumin. Following fractional centrifugation of a kidney homogenate he found high albumin concentration in a purified lysosomal fraction.

In addition Maunsbach conducted basic studies on the capacity of the kidney to catabolize albumin (17). He isolated different sub-lysosomal components and incubated them _in vitro_ with labeled albumin. The results suggested that the lysosomal matrix contained one or more albumin-degrading enzymes.

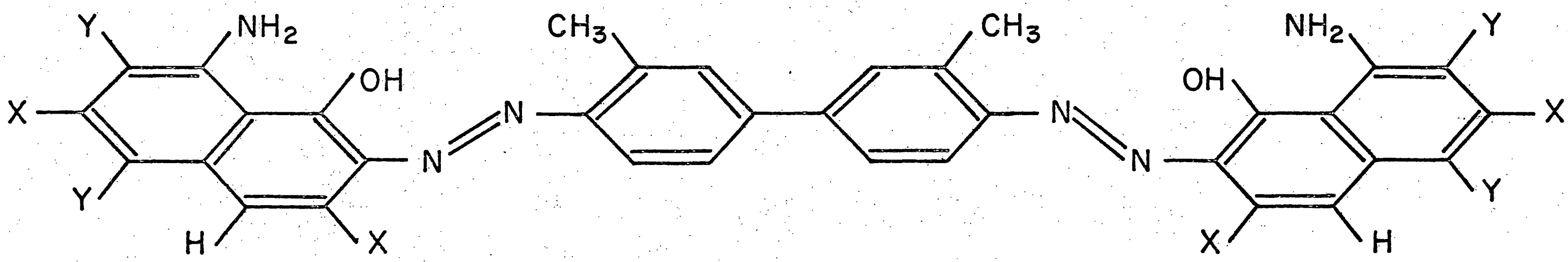
A more recent study of albumin uptake and digestion by kidney renal tubular cells was undertaken by Bourdeau _et al._ (18). Isolated single nephrons from rabbit kidney were divided into proximal convoluted, proximal straight, and cortical collecting tubular segments. These were perfused for varying lengths of time with ^{125}I-labeled rabbit serum albumin (RSA). After perfusion the segments were either embedded in Epon, where they were counted with a gamma spectrometer to quantitate protein accumulation, or fixed and analyzed by electron microscopic autoradiography to determine sequential localization of radioactivity. The protein accumulation data show that proximal convoluted and proximal straight segments accumulated RSA-^{125}I in an almost linear fashion as a function of time; whereas, cortical collecting segments did not accumulate measurable amounts of protein. The rate of accumulation of RSA-^{125}I in the proximal convoluted tubule is 2.6 times as great as that in the proximal straight tubule. Therefore, the accumulation rate is greatest in the convoluted segment, and there appears to be a progressive decrease along the nephron.

The electron microscopic autoradiography data showed the uptake and concentration of RSA-^{125}I in proximal tubular cells over a period of time. After 10 minutes of perfusion radioactivity was located in the base of the brush border, in apical tubular invaginations, and small and medium sized apical vacuoles. By 45 minutes cytoplasmic vacuoles contained the radioactivity, and these could now be detected in the middle third of the cell. After 90 minutes the number of cytoplasmic structures in which radioactivity had been concentrated was increased. Combined electron microscopic autoradiography and histochemistry showed that some radioactivity was concentrated in acid phosphatase-positive-bodies, presumably secondary lysosomes. From these data one can see the movement of RSA-^{125}I from tubular invaginations to larger cytoplasmic vacuoles and finally to concentrated membrane-bound structures, some of which are acid phosphatase positive.

The results from Maunsbach (17) and Bourdeau et al. (18) clearly demonstrate that serum albumin is reabsorbed from the glomerular filtrate by kidney proximal tubular cells. Their data strongly suggest that the albumin is reabsorbed by pinocytosis and that the resulting phagosomes fuse with vacuolar bodies containing hydrolytic enzymes (either primary or secondary lysosomes) where digestion of the albumin may take place.

Evans Blue - Evans blue is a member of a group of acid bisazo dyes. It has a molecular weight of 960 and a maximum absorption at λ 605. Its structure and that of trypan blue, a structural analogue, are seen on the following page.

Rawson (6) in 1943 was the first to indicate that Evans blue, trypan blue, and other bisazo dyes bind strongly to the albumin fraction of serum. She ascertained by several different methods (electrophoresis, ultracentrifugation, effects of plasma proteins upon spectral absorption of dye, cellophane-staining) that at pH 7.4 in solutions of human albumin up to 14 molecules of Evans blue are linked to one molecule of plasma albumin. The greatest stability of the dye-protein complex is attained up to ratios of 8 molecules of Evans blue per molecule of plasma albumin. At such concentrations the dye is wholly and preferentially bound by the albumin fraction. If the dye concentration is increased sufficiently the dye may also be bound by the globulin fraction, preferentially the α globulins. She also showed that in the equilibrium system between the protein-dye complex and the free dye and plasma protein, the concentration of free Evans blue is infinitesimal. This was demonstrated by ultracentrifugation of a solution of dye-protein complex in which a blue-stained layer sedimented leaving an unstained supernatant. Therefore, at a ratio of 8 molecules of Evans blue to 1 molecule of plasma protein, all the dye is bound to the plasma protein.



NAME	SUBSTITUENTS X	SUBSTITUENTS Y
EVANS BLUE	H	SO_3Na
TRYPAN BLUE	SO_3Na	H

Trypan blue also binds to plasma protein, but this complex is more unstable than the Evans blue-protein complex. When the trypan blue-protein complex is dialyzed in a cellophane bag some of the trypan blue is dissociated and stains the cellophane; whereas, the Evans blue-protein complex under same circumstances leaves the dialyzing membrane colorless (6).

As a consequence of the binding of Evans blue to serum albumin, its complete elimination from the bloodstream takes several days. Only negligible quantities of Evans blue appear in the urine; therefore, the primary rate for clearance of the dye from circulation is presumably by uptake into the tissues. Evans blue remains within an animal's tissue for many weeks after administration. It is taken up by the cells of the reticulo-endothelial system as well as by certain epithelial cells, such as those of the kidney proximal convoluted tubules and the liver parenchyma. Once in the cells Evans blue appears as distinct blue granules, which is evidence of both active concentration from more dilute solutions in the blood plasma and separation from the remainder of the cytoplasm (21).

Gilson (7) in 1949 was one of the first workers to employ the use of Evans blue in studying the renal reabsorption of plasma proteins. He labeled certain of the plasma proteins _in vivo_ by intracardiac injections of Evans blue and found the dye concentrated in the cells of the proximal convoluted tubules. Rats were injected with 0.5 ml of a 5% Evans blue solution (12.5 mg/100 g body wt) and killed at 10 minutes, 3 hours, and 24 hours after injection. It was calculated that in the dose administered all the dye combined with the plasma protein. On macroscopic examination of injected animals all tissues were to a greater or lesser degree blue. Unstained frozen kidney sections were studied from each of the three time periods. In animals killed 10 minutes after injection blue granules were observed all superficially distributed in the cytoplasm of proximal tubular cells toward the tubular lumen. In the 3 hour animals blue granules were observed more deeply throughout the cytoplasm. The tissues of the 24 hour animals showed the most dense aggregations of blue particles in the cytoplasm of proximal tubular cells. Gilson interpreted these findings as evidence of glomerular filtration and tubular reabsorption of plasma protein and protein-Evans blue complexes (7).

In a more recent study Wilde *et al*. (9) has followed the path of reabsorption into tubular cells. By electron micrographs he has shown the uptake or reabsorption of an albumin-Evans blue complex by proximal tubular cells. He injected rats with a 3.5% solution of Evans blue in saline (12.6 mg/100 g body wt.) and followed the uptake for six days.
First hour. After 5 minutes of mixing in the circulation system no visible dye entered the proximal epithelium. At 1 hour small dark blue granules were scattered evenly just under the brush border.
First day. After 1 day blue granules were larger and migrated completely away from the brush border, mostly toward the middle of the cells.
Third day. Blue granules were clumped into special clusters located above and around the nucleus near the Golgi after 3 days. These granules had achieved their maximum size at this time, and the zone just below the brush border was now clear.

Sixth day. Clusters of granules seemed smaller, disrupted into strands and faded. The faded appearance may indicate that the Evans blue is no longer bound to the albumin which had accompanied it into the cells.

Wilde et al. concluded that reabsorption of filtered blood albumin-Evans blue across proximal tubular cells takes place via the membranous vacuoles rather than as free molecules through the cytoplasm. Any free albumin-Evans blue would appear as a blue screen, and all cellular blue was seen in fine or coarse dots which represent protein-dye bound or contained within membranous vacuoles (9).

The works cited above demonstrate that Evans blue binds to plasma albumin, and this plasma albumin-Evans blue complex is filtered out of the blood stream by the glomerulus and then reabsorbed as membranous vacuoles by the cells of the proximal tubules.

Work with Trypan Blue - Lloyd et al. (21) have employed trypan blue, a structural analogue of Evans blue, in a different fashion than the authors cited above. In attempting to explain teratogenesis, they were interested in the possibility of inhibition of lysosomal enzymes by the dye. Trypan blue was localized in subcellular fractions of liver from dye-treated rats, and each fraction was analyzed for acid phosphatase activity. Rats were injected subcutaneously with trypan blue and their livers were removed 16 hours later. Successive centrifugation yielded nuclear (N), heavy mitochondrial (M), light mitochondrial (L), and microsomal (P) pellets and a final supernatant (S). Figure 2 illustrates the distribution of trypan blue and acid phosphatase in liver fractions from an injected rat and the distribution of

Figure 2 - Distribution of Acid Phosphatase in Rat Liver of an Uninjected Rat and Distribution of Acid Phosphatase and Trypan Blue in Rat Liver of a 16 Hour Injected Rat

Data are expressed in bar graphs in which subcellular fractions going from left to right are:

N - Nuclear

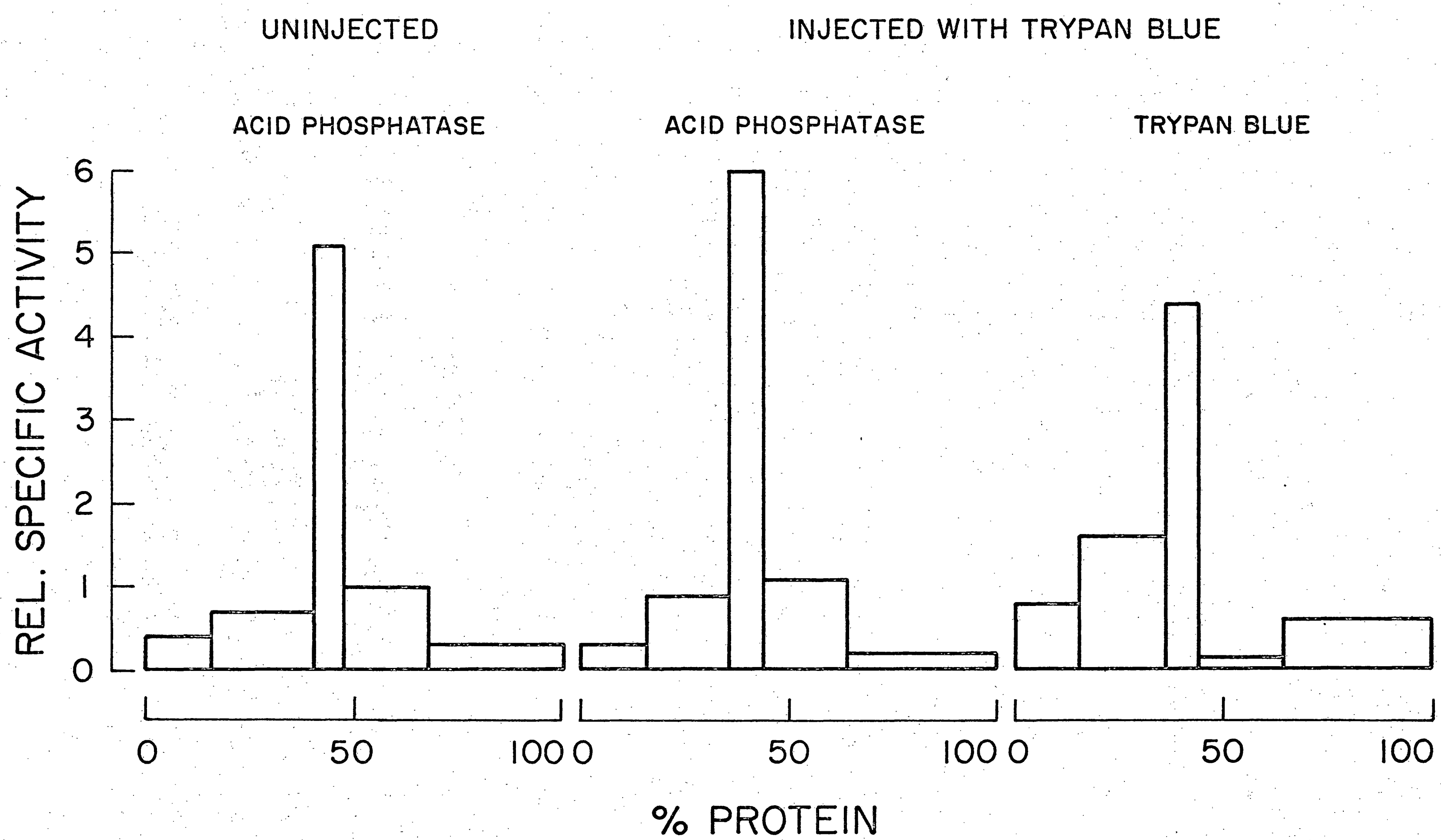
M - Heavy Mitochondrial

L - Light Mitochondrial

P - Microsomal

S - Supernatant

Data for this figure came from the work of Lloyd _et al_. (21).


UNINJECTED
INJECTED WITH TRYPAN BLUE
ACID PHOSPHATASE
ACID PHOSPHATASE
TRYPAN BLUE
REL. SPECIFIC ACTIVITY
0
1
2
3
4
5
6
0
50
100
0
50
100
0
50
100
% PROTEIN

acid phosphatase in liver fractions from an uninjected rat. As seen, the trypan blue does not significantly alter the distribution pattern of acid phosphatase, which indicates that the lysosomal enzyme was not inhibited by trypan blue. The distribution of the dye itself is similar to that of acid phosphatase, with the highest specific activity being in the light mitochondrial fraction.

Having demonstrated the effect of trypan blue in liver, Lloyd et al. (21) turned to the organ of primary interest, rat visceral yolk-sac (strongly phagocytic), and investigated the intracellular distribution of trypan blue in this tissue after injection. Rats were injected subcutaneously with the dye and killed at different time periods afterwards. Within 8 hours both light and electron microscopy revealed accumulation of trypan blue in large membrane-bound vacuoles in the supranuclear region of the cells. It was suggested that the dye first appeared in pinocytotic vesicles which migrated deeper into the cell where they fused to form larger vacuoles. It was clearly demonstrated that these trypan blue containing vacuoles were acid phosphatase positive, which would indicate that they were heterolysosomes.

The yolk-sacs from injected animals were also homogenized and fractionated by differential centrifugation as previously done with the liver with the exception that no microsomal fraction was isolated (21). Assays for trypan blue, acid phosphatase, and protein were performed on animals killed 24, 48, and 72 hours after injection. As seen in Figure 3, the distribution of trypan blue is unequal, and the highest specific activity is found in the nuclear fraction. Coupled with the microscopic

Figure 3 - Distribution of Trypan Blue in Rat Visceral Yolk-Sac at Various Times Following Injection

Data are expressed in bar graphs in which subcellular fractions going from left to right are:

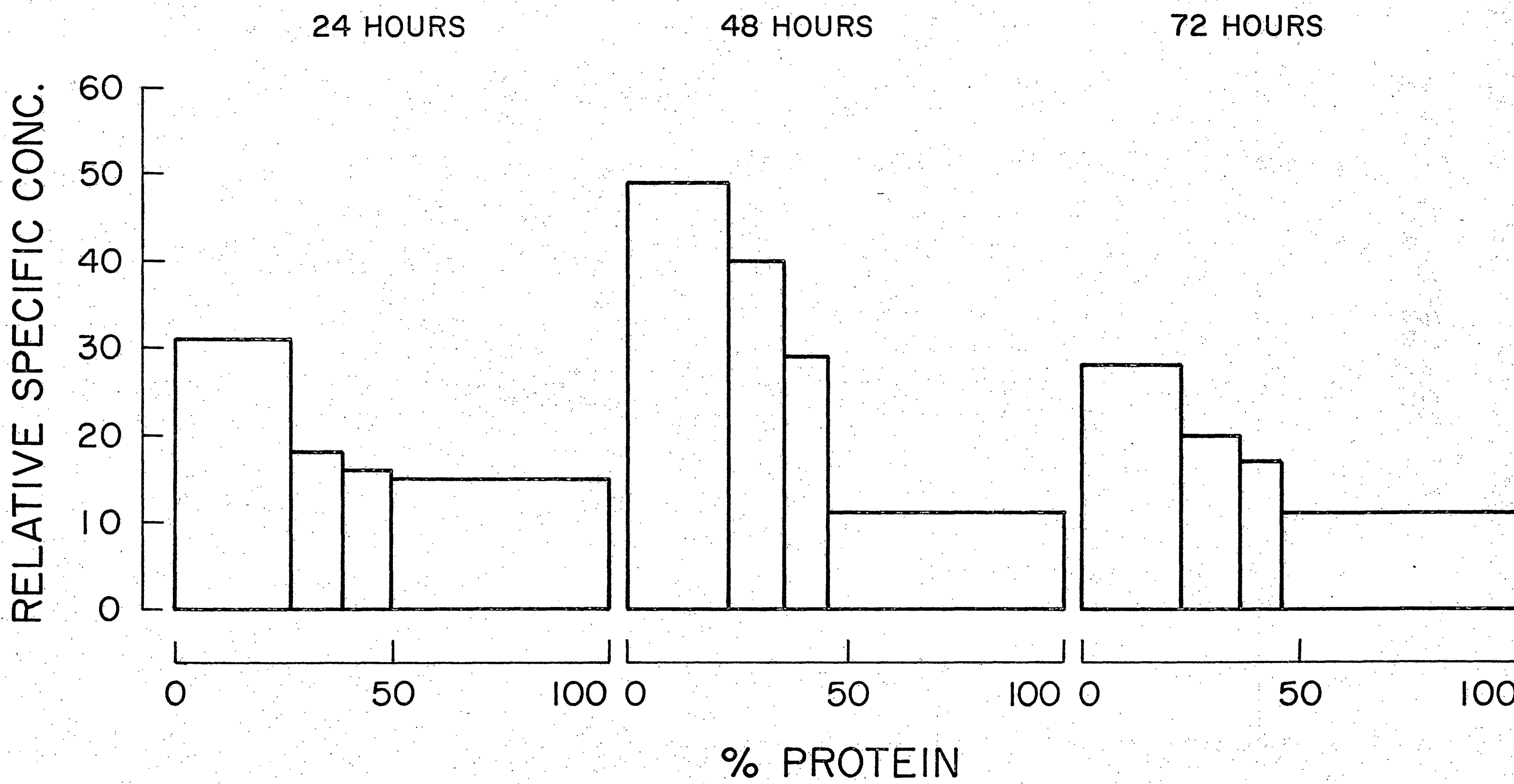
N - Nuclear

M - Heavy Mitochondrial

L - Light Mitochondrial

S - Supernatant

Data for this figure came from the work of Lloyd *et al*. (21).


24 HOURS
48 HOURS
72 HOURS
RELATIVE SPECIFIC CONC.
0
10
20
30
40
50
60
0
50
100
% PROTEIN

observations this implies that large heterolysosomes containing trypan blue sediment in the nuclear fraction. The distribution of acid phosphatase also shows large quantities in the nuclear and heavy mitochondrial fractions. Comparison of these results with those obtained for liver homogenates shows a diversity of size among the elements of the vacuolar system (apparatus) in the liver as compared to the yolk-sac.

Lloyd _et al_. (21) concluded from these and other observations that when trypan blue was bound to protein it did not significantly inhibit lysosomal enzymes. However, when large amounts of trypan blue were incubated _in vitro_ with lysosomal enzymes significant inhibition of these enzymes was seen.

From the literature survey just concluded, it appears that the dye Evans blue when injected into the circulatory system of the rat will bind to serum albumin and enter renal proximal tubular cells by pinocytosis. The rate of entry and size distribution of the dye containing vacuoles can be estimated by analysis for Evans blue following separation by fractional centrifugation.

EXPERIMENTAL PROCEDURES

In the experiments to be discussed in this thesis rats were injected intravenously with Evans blue and then sacrificed at designated time periods afterwards. The tissue of interest (either liver or kidney) was removed, homogenized, and divided by fractional centrifugation into five subcellular fractions: nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and supernatant (S). Each fraction was analyzed for Evans blue, protein, and acid phosphatase activity.

Animal Management - Male Sprague-Dawley rats, individually housed in single, stainless steel rat cages, were used in all experiments. Rats used for both liver and control kidney studies were allowed to consume *ad libitum* a normal rat pellet ration for at least five days prior to injection with Evans blue. The rats used for magnesium deficient kidney studies were meal fed (5:00-9:00 p.m.) a magnesium supplemented basal diet (700 ppm of magnesium) for seven days and then offered a basal low magnesium diet (20 ppm of magnesium), Table I, for three days before injection with Evans blue.

One rat weighing approximately 275 g was used for the liver study. It was injected intravenously with 6.5 mg Evans blue per 100 g body wt. of a 3.5% Evans blue solution in 0.9% saline. Either three or four approximately 220 g rats (200-240 g) were used for each kidney experiment. They were injected intravenously with 12.6 mg Evans blue per 100 g body wt. of a 3.5% Evans blue solution in saline. Wilde *et al.* (9) injected this same amount into their rats and claimed that all the Evans

Table I

Diet Composition

Ingredients	Percent of Diet by Weight
Casein (vitamin-free NBC*)	20
Sucrose	26.6
Corn starch	33.2
Alphacel	5
Vitamin mix (complete NBC*)	2.2
Wesson oil	8
Mineral mix** (Mg omitted)	5

*Nutritional Biochemical Company

**For contents see reference 5

blue was bound to serum albumin. It was calculated that 12.6 mg Evans blue/100 g body wt. injected intravenously would give approximately a 8:1 ratio of Evans blue to serum albumin; therefore, it was assumed that all the Evans blue injected in both the liver study and the kidney experiments was bound to serum albumin.

A specially made rat restraining device which exposes the rat's tail was used for all injections. By the use of a 26 gauge disposable needle and a 1 ml disposable syringe, Evans blue was injected directly into one of the tail veins. Immediately the eyes, ears, and nose of the animal turned blue. This was an indication that Evans blue entered the animal's circulatory system immediately. Animals that did not turn blue within seconds were not used. At the designated time period after injection the animals were sacrificed by decapitation.

Fractionation Scheme - After sacrifice, the tissue of interest (either liver or kidney) was removed, perfused thoroughly with 0.25 M sucrose, and homogenized. The homogenization for liver was accomplished in a Potter-Elvehjem homogenizer with 0.25 M sucrose. The tissue was homogenized three separate times, each time being one up and down pass of the homogenizer, and the homogenate was filtered once through glass wool to remove large cell debris. Kidneys, on the other hand, were homogenized by hand with a Dounce homogenizer and 0.25 M sucrose. They too were homogenized three separate times; once with five up and down passes with a loose pestle, and twice with two up and down passes with a tight pestle. Both liver and kidney homogenates were then subjected to fractional centrifugation.

The procedure for fractional centrifugation was taken from FEBS Summer Course, 1965, University of Louvain (22). This procedure was originally perfected for liver but has been employed also for kidney. Tissue was separated into five subcellular fractions by weight; namely, nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and soluble or supernatant (S) (Table II). Each fraction was washed with 0.25 M sucrose. Since the fractionation was quantitative, the washing fluids were always combined with the previous supernatants before proceeding with the sedimentation of the next fraction. The volumes of the fractions and the combined supernatants were carefully measured or adjusted in such a way that the concentration of each fraction was always known. Centrifugations to remove nuclear and cell debris were carried out in a Sorvall centrifuge (SS-34 rotor) at 121 x g. The remaining centrifugations were performed in a Beckman-Spinco ultracentrifuge, model L, (rotor 40) at the designated speeds. The whole fractionation was performed near 0° C, using ice-cold solutions and equipment.

Analytical Techniques - Sellers et al. (8) in his work developed a procedure for extraction of Evans blue from tissues. This procedure was adapted for extraction of Evans blue from the subcellular fractions.

To an aliquot of each fraction was added 2% aerosol OT (dioctyl sulfosuccinate sodium salt). This mixture was allowed to stand with frequent mixing for 30 minutes. Aerosol OT releases Evans blue from the tissue. Pure acetone was added to precipitate the proteins, and this mixture was allowed to stand for 15 minutes with frequent agitations.

Table II

Procedure for Fractional Centrifugation

Homogenized tissue in 0.25 M sucrose

Centrifuged at 121 x g for 10 minutes. Resuspended pellet in 0.25 M sucrose, rehomogenized, and recentrifuged at 121 x g for 8 minutes. Repeated once.

N

Nuclear fraction

E

Cytoplasmic fraction

Centrifuged at 11,000 x g for 3 minutes. Washed twice.

Supernatant

Centrifuged at 42,500 x g for 6 minutes, 45 seconds. Washed twice.

M

Heavy Mitochondrial fraction

Supernatant

Centrifuged at 90,000 x g for 33 minutes. Washed once.

L

Light Mitochondrial fraction

S

Supernatant

P

Microsomal fraction

The mixture was then centrifuged at 30,900 x g for 30 minutes in a Sorvall centrifuge (SS-34 rotor). The supernatant which contained all the Evans blue originally in the fraction was taken for spectrophotometric analysis at 605 mμ. Using an Evans blue standard curve the mg of Evans blue per g of kidney were calculated for each subcellular fraction.

The widely used Lowry method (23) with the Folin-Ciocalteu phenol reagent was used for protein estimation. The mg of protein per g of kidney were estimated for each fraction.

Acid phosphatase activity was determined by the amount of inorganic phosphate hydrolyzed from β-glycerophosphate in a 10 minute period. The assay procedure was taken from FEBS Summer Course, 1965, University of Louvain (22). An aliquot of each subcellular fraction was incubated for 10 minutes at 37° C in the presence of sodium β-glycerophosphate (0.5 M pH 5), sodium acetate-acetic acid buffer pH 5 (1 M), sucrose (1 M), triton X-100 (0.2%), and water. The triton X-100 was added to release enzyme from vacuoles. The reaction was stopped at 10 minutes by adding ice-cold trichloro-acetic acid, TCA (8%). The denatured proteins were eliminated by filtration, and inorganic phosphate was determined in each filtrate. Blanks were run by incubating an aliquot with all the components of the assay mixture except the substrate, sodium β-glycerophosphate, which was added after the TCA.

Inorganic phosphate was determined by the Fiske and Subbarow method (24). To an aliquot of filtrate was added ammonium molybdate solution (in 5 N H_2SO_4), Elon solution (1% Elon and 3% $NaHSO_3$), and water. The contents of each test tube were mixed thoroughly and allowed to turn blue for approximately two hours, after which the O. D. was read by a spectrophotometer at 660 mμ. A standard containing 1 mM phosphate instead of filtrate was included in each series of determinations.

The units of acid phosphatase activity per g of kidney were calculated for each fraction. One unit of acid phosphatase activity corresponds to a rate of hydrolysis of 1 μmole sodium β-glycerophosphate per minute.

RESULTS

General Introduction - The data to be presented in this thesis are divided into three phases: liver studies, kidney studies, and low magnesium studies.

Rat liver was used to standardize the techniques of fractional centrifugation, Evans blue administration, and Evans blue recovery from the different fractions. Also it was of interest to obtain a distribution pattern for Evans blue in rat liver subcellular fractions and to compare this pattern to the distribution pattern for trypan blue in rat liver subcellular fractions obtained by Lloyd et al. (21), discussed in the LITERATURE REVIEW.

Only one complete experiment was performed with rat liver. The animal was executed 24 hours after injection of Evans blue.

It was the intent of the kidney experiments to ascertain the dynamic behavior in kidney after injection of Evans blue. The accumulation of Evans blue by the total kidney and the distribution of Evans blue in the subcellular fractions were obtained at different time periods after injection. Experiments were performed at 1/2 hour, 1 hour, 3 hours, and 12 hours post-injection.

Serum was also collected at each time interval, and the Evans blue present was extracted. The 12 hour injected animals were placed in metabolism cages immediately after administration of Evans blue, and urine was collected for the first 6 hours after injection and the last 6 hours before sacrifice. Evans blue was extracted from the urine collected.

Once the patterns for accumulation of Evans blue by total kidney and distribution of Evans blue in subcellular kidney fractions had been established, the same experiments were repeated with three day magnesium deficient rats to determine the influence of a low magnesium intake on the rate of accumulation and distribution of Evans blue in rat kidney. Bunce and Bloomer (5) found significant drops in serum and urine magnesium and urine calcium within 24 to 48 hours after offering of first deficient diet and increases in kidney calcium within three days. They suggested that calcification was underway by day three. From these observations it was decided that if changes did occur in kidney cells as a result of magnesium deficiency, they would be initiated by day three.

Liver Studies - The distribution of Evans blue obtained for rat liver subcellular fractions 24 hours post-injection is presented in Figure 4. The plot expresses the amount of Evans blue in each fraction relative to the amount of protein in each fraction. The largest amount of Evans blue was found in the supernatant, and the next largest was in the heavy mitochondrial fraction. However, the highest relative specific concentration for Evans blue was found in the light mitochondrial fraction, which has also been shown to contain the highest relative specific activity of acid phosphatase. No measurable amount of Evans blue was found in the nuclear fraction.

Kidney Studies - As stated earlier the accumulation of Evans blue by rat kidney was studied at 1/2 hour, 1 hour, 3 hours, and 12 hours after injection of Evans blue. Table III shows the means of the total Evans blue extracted at each time period, as well as the amount

Figure 4 - Distribution of Evans Blue in Rat Liver Subcellular Fractions 24 Hours Post-injection

Data are expressed in a bar graph in which subcellular fractions going from left to right are:

N - Nuclear

M - Heavy Mitochondrial

L - Light Mitochondrial

P - Microsomal

S - Supernatant

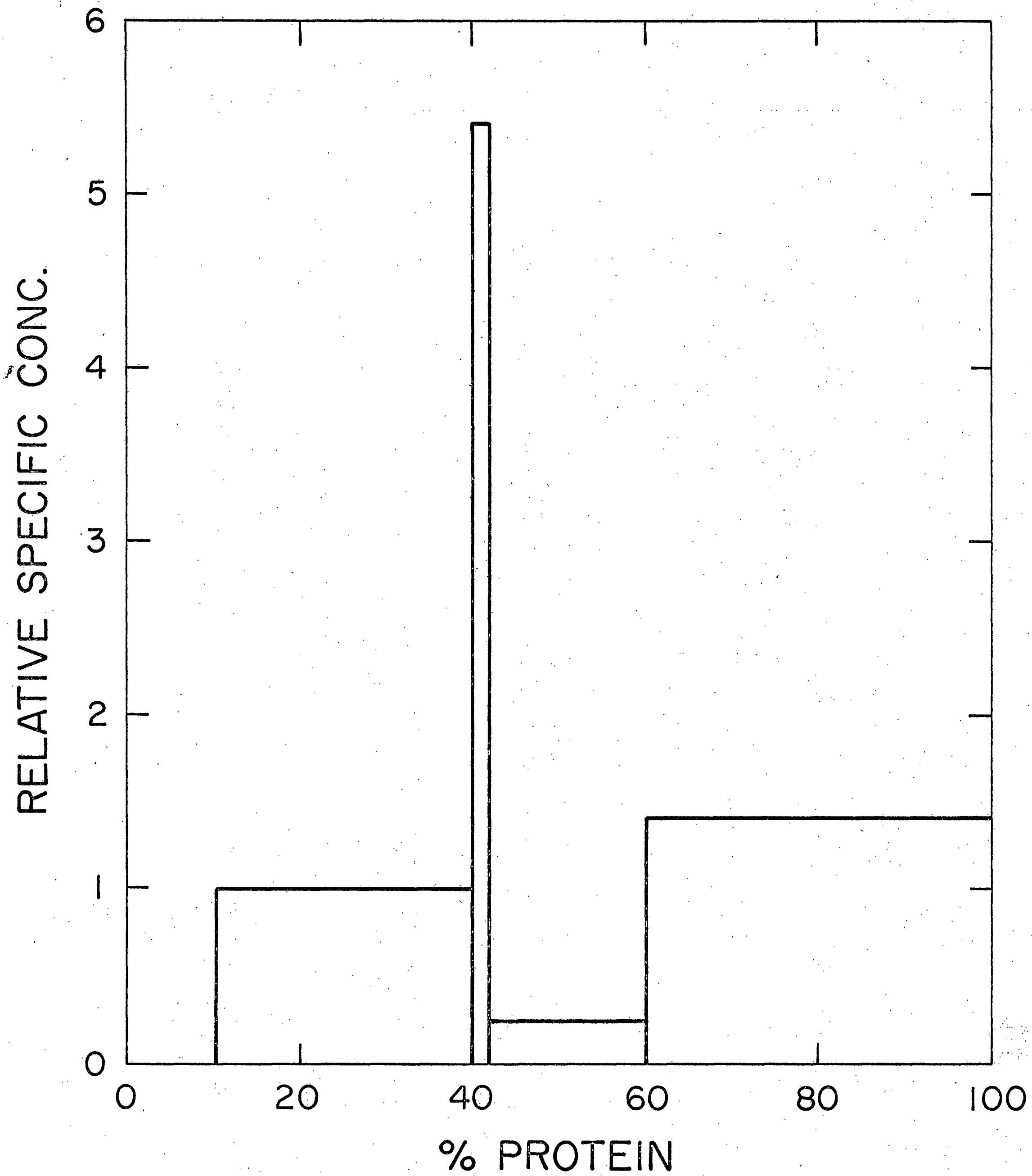

RELATIVE SPECIFIC CONC.
6
5
4
3
2
1
0
0
20
40
60
80
100
% PROTEIN

Table III

Extraction of Evans Blue from

Rat Kidney Fractions and Serum at each Time Interval

	mg Evans blue/g Kidney			
Fraction	1/2 Hour	1 Hour	3 Hours	12 Hours
total	0.265	0.288	0.348	0.568
N	0.086	0.120	0.168	0.255
M	0.043	0.052	0.054	0.083
L	0.010	0.008	0.009	0.017
P	0.014	0.013	0.013	0.026
S	0.112	0.095	0.104	0.187
Serum*	1.904	1.655	1.290	0.690

*mg Evans blue/ml serum

extracted from each subcellular fraction at each time period. All values are the mean of at least two experimental determinations and are expressed in mg Evans blue/g kidney. The individual values are seen in Table IV and Table V. Also appearing in Table III are the mg Evans blue/ml serum extracted at each time interval.

As seen, the total amount of Evans blue extracted increased with time. This can be expressed as the net uptake of Evans blue by whole rat kidney with time and can be seen graphically in Figure 5. Table III also shows an increase in Evans blue in the nuclear fraction with time. The other fractions remained rather constant for the first 3 hours and increased at different degrees in the next 9 hours. The light mitochondrial and microsomal fractions show only a slight increase, and the heavy mitochondrial fraction shows a little greater increase; however, the supernatant shows a very substantial increase over these 9 hours. The net uptake of Evans blue with time by the subcellular fractions is seen graphically in Figure 6.

The percentage of the total Evans blue in each subcellular fraction at each time interval was calculated and is presented in Table VI. The data show that in the first 3 hours after injection the percentage of Evans blue in the nuclear fraction increased while the percentage in the supernatant decreased. It is interesting that in the next 9 hours the percentage of Evans blue in the nuclear fraction decreased while the percentage in the supernatant increased. The percentage of Evans blue in the other three fractions was virtually unchanged throughout the 12 hours.

Table IV

Extraction of Evans Blue from

Rat Kidney Fractions and Serum

	mg Evans blue/g Kidney							
	1/2 Hour Trials*					1 Hour Trials*		
Fraction	1	2	3	4	Mean	1	2	Mean
total	0.304	0.222	0.291	0.242	0.265	0.283	0.293	0.288
N	0.098	0.078	0.087	0.083	0.086	0.120	0.120	0.120
M	0.049	0.029	0.054	0.040	0.043	0.050	0.054	0.052
L	0.009	0.008	0.011	0.010	0.010	0.009	0.007	0.008
P	0.016	0.013	0.014	0.011	0.014	0.014	0.012	0.013
S	0.132	0.094	0.125	0.098	0.112	0.090	0.100	0.095
Serum**	1.880	1.730	2.185	1.820	1.904	1.670	1.640	1.655

*either three or four rats were used for each trial

**mg Evans blue/ml serum

Table V

<u>Extraction of Evans Blue from</u>

<u>Rat Kidney Fractions and Serum</u>

	mg Evans blue/g Kidney						
	3 Hour Trials*				12 Hour Trials*		
Fraction	1	2	3	Mean	1	2	Mean
total	0.337	0.334	0.372	0.348	0.563	0.573	0.568
N	0.173	0.160	0.170	0.168	0.255	0.256	0.255
M	0.053	0.040	0.068	0.054	0.071	0.095	0.083
L	0.006	0.009	0.011	0.009	0.019	0.014	0.017
P	0.009	0.017	0.013	0.013	0.028	0.023	0.026
S	0.096	0.108	0.110	0.104	0.190	0.185	0.187
Serum**	---	1.250	1.330	1.290	0.640	0.740	0.690

*either three or four rats were used for each trial

**mg Evans blue/ml serum

Figure 5 - Net Uptake of Evans Blue by Rat Kidney

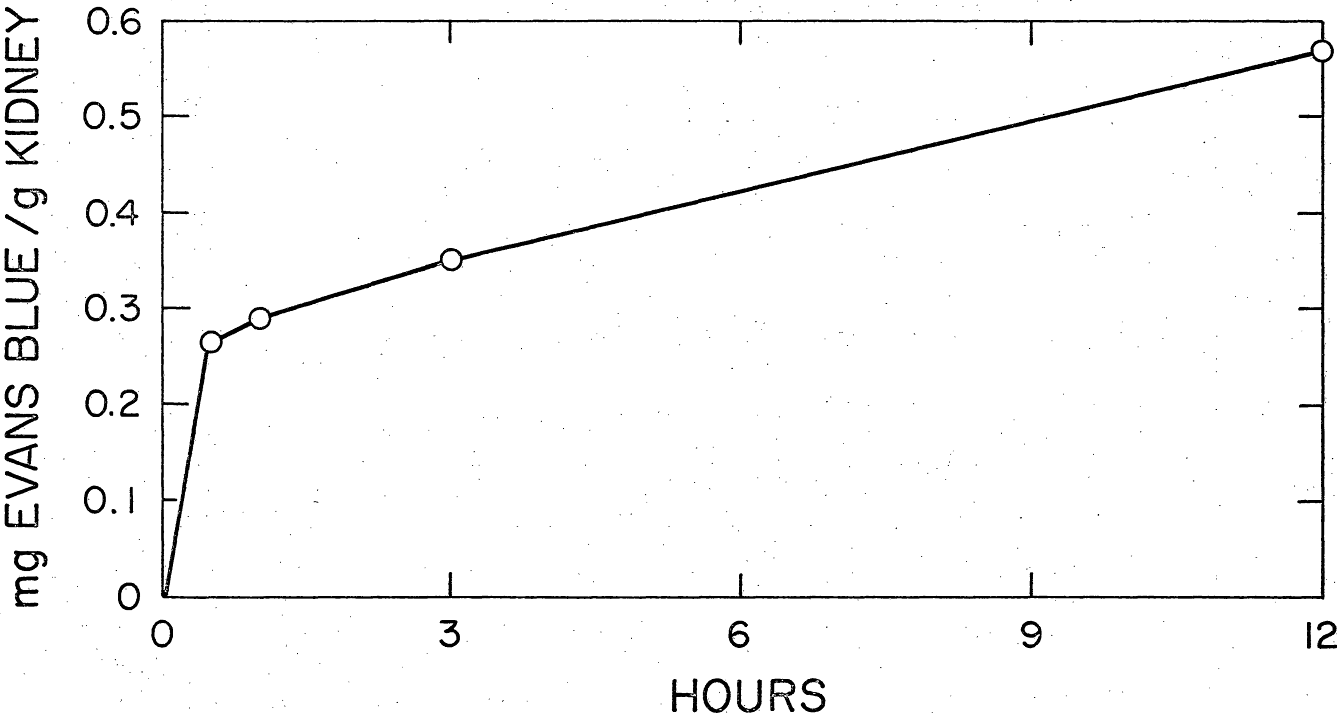

mg EVANS BLUE /g KIDNEY
0.6
0.5
0.4
0.3
0.2
0.1
0
0
3
6
9
12
HOURS

Figure 6 - Net Uptake of Evans Blue by Rat Kidney Subcellular Fractions

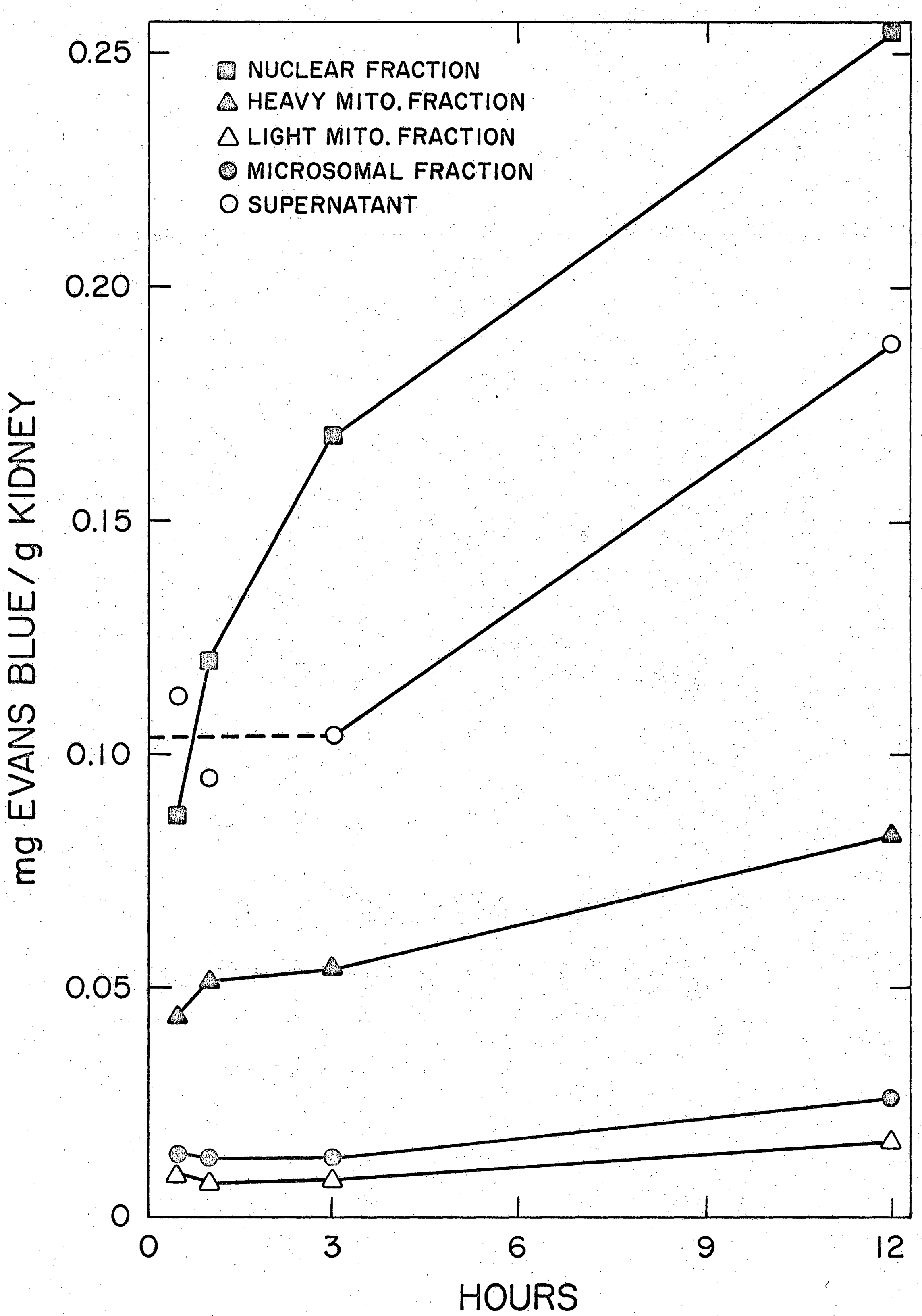

NUCLEAR FRACTION
HEAVY MITO. FRACTION
LIGHT MITO. FRACTION
MICROSOMAL FRACTION
SUPERNATANT
mg EVANS BLUE / g KIDNEY
0.25
0.20
0.15
0.10
0.05
0
0
3
6
9
12
HOURS

Table VI

Percent of Total Evans Blue in each Subcellular Fraction at each Time Interval

	% Evans blue			
Fraction	1/2 Hour	1 Hour	3 Hours	12 Hours
N	33	42	48	45
M	16	18	15	15
L	4	3	3	3
P	5	4	4	4
S	42	33	30	33

In Table VII is presented the mg Evans blue/g protein for total rat kidney and for the subcellular fractions at each time period after injection. These data are very similar to the extraction data presented in Table III. The total amount, as well as the amount in the nuclear fraction increased with time. The other fractions remained rather stable during the first 3 hours and then increased over the next 9 hours.

The recovery of Evans blue from serum is presented in Table III and Figure 7. As seen, the mg Evans blue/ml serum decrease with time.

Table VIII shows the % acid phosphatase activity, relative specific activity of acid phosphatase (% acid phosphatase/% protein), and the % protein in the subcellular fractions at each time interval after injection of Evans blue. The slight variability between values for the same fraction at different time periods is not significant since as much as a 5% difference has been observed for the supernatant with uninjected rats.

Gilson (7) and Wilde et al. (9) have claimed that Evans blue accumulated in vacuoles; however, the appearance of Evans blue in the various fractions does not of itself prove that Evans blue was present within membrane bound vacuoles. Superficial binding to surface sites or protein aggregates was also possible. Control experiments were designed to determine the distribution of the dye in a homogenate from a non-injected rat and to test the latency of the dye in the nuclear fraction from an injected animal.

Table VII

mg Evans Blue/g Protein for Rat Kidney

Subcellular Fractions at each Time Period

	mg Evans blue/g Protein			
Fraction	1/2 Hour	1 Hour	3 Hours	12 Hours
total	1.88	1.88	2.30	3.61
N	1.73	2.19	3.21	4.91
M	1.63	1.70	1.80	2.34
L	2.38	2.07	2.26	3.88
P	0.86	0.72	0.73	1.38
S	2.53	2.05	2.20	4.01

Figure 7 - Evans Blue in Rat Serum at Various Times Following Injection

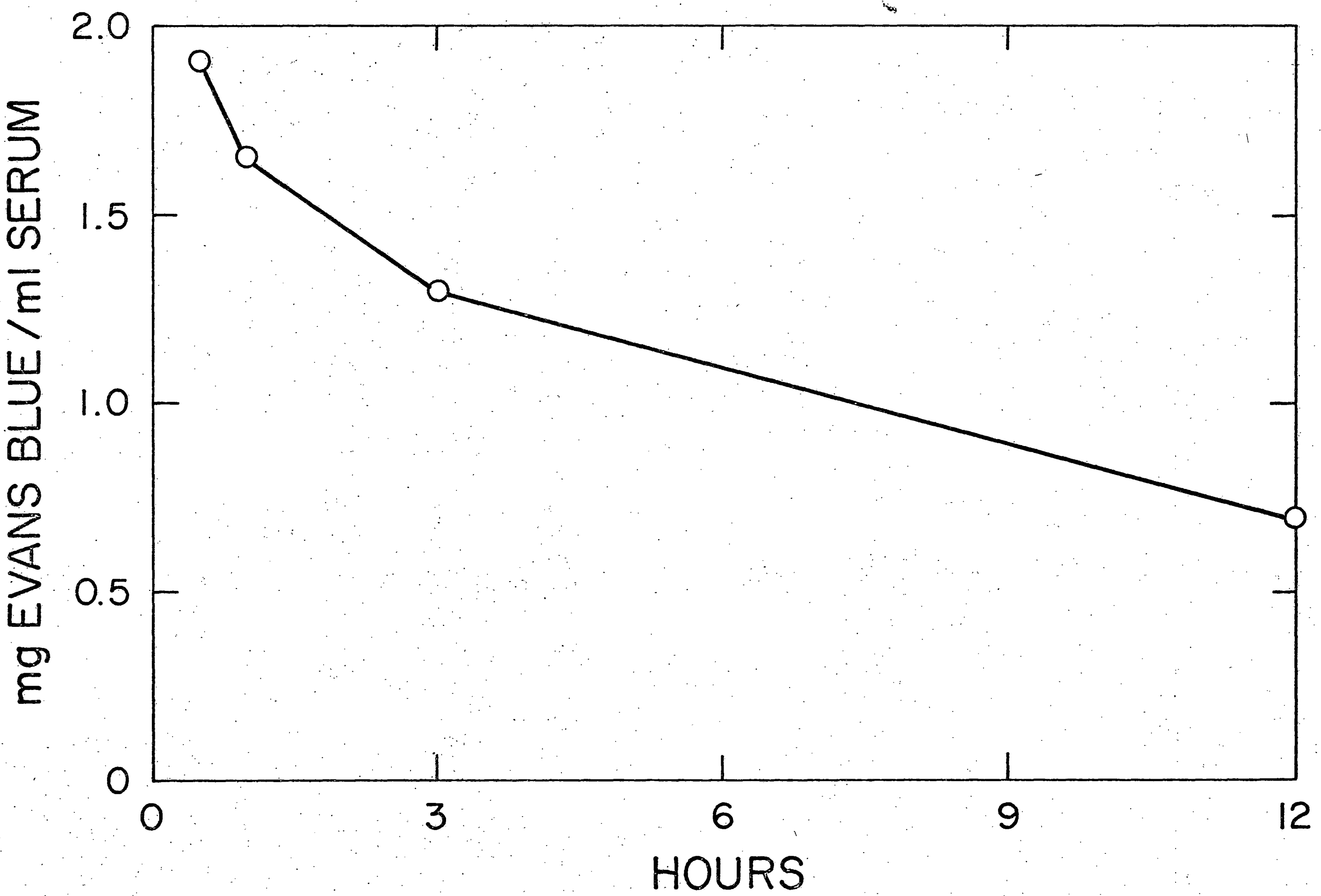

2.0
1.5
1.0
0.5
0
mg EVANS BLUE / ml SERUM
0
3
6
9
12
HOURS

Table VIII

Percent Acid Phosphatase Activity, Relative Specific Activity, and Percent Protein in Rat Kidney Subcellular Fractions at Various Times after Evans Blue Injection

	1/2 Hour		1 Hour		3 Hours		12 Hours	
Fraction	% Ac. P.	% Prot.	% Ac. P.	% Prot.	% Ac. P.	% Prot.	% Ac. P.	% Prot.
N	40.62 [1.15]	35.30	39.88 [1.12]	35.69	42.73 [1.24]	34.58	37.88 [1.15]	33.02
M	24.53 [1.31]	18.74	21.86 [1.09]	19.97	19.69 [0.99]	19.85	19.64 [0.87]	22.54
L	8.06 [2.70]	2.98	7.63 [3.03]	2.52	6.67 [2.53]	2.64	6.43 [2.30]	2.79
P	13.81 [1.19]	11.56	14.18 [1.21]	11.70	14.58 [1.24]	11.72	17.62 [1.47]	11.98
S	12.99 [0.41]	31.42	16.46 [0.55]	30.13	16.32 [0.52]	31.21	18.44 [0.62]	29.68

[ ] = Relative Specific Activity, % Acid Phosphatase/% Protein

To 10 g of kidney from a non-injected rat was added 2.5 mg of Evans blue in a 0.25 M sucrose solution (0.25 mg Evans blue/g kidney). The tissue was homogenized and fractionated by the procedures previously used, and Evans blue was extracted from each subcellular fraction. Table IX shows the mg Evans blue/g kidney recovered in each fraction and the % Evans blue and relative specific concentration in each fraction. Values are from one experimental determination. As seen, the largest amount of Evans blue was recovered in the supernatant; only slight amounts were found in the other fractions. Figure 8 shows this distribution in bar graph form, and Figure 9 shows the distribution of Evans blue in a 12 hour injected rat.

The latency of Evans blue in the nuclear fraction was determined by adding 2% triton X-100, which is known to disrupt vacuolar membranes and release the substances inside, to an aliquot of kidney nuclear fraction from 3 hour injected rats. This solution was mixed thoroughly and allowed to stand in ice for 30 minutes. It was then centrifuged at 121 x g for 10 minutes in a Sorvall centrifuge (SS-34 rotor) to yield a pellet and a soluble supernatant. Evans blue was extracted from both pellet and supernatant, and acid phosphatase activity was analyzed in both. The mg Evans blue/g kidney extracted from each fraction, as well as the % Evans blue, % acid phosphatase, and % protein in each fraction are presented in Table X. Two experiments were performed in this study. The data show that after treatment with triton X-100 almost 84% of the Evans blue originally located in the nuclear fraction was found in the soluble fraction. The total Evans blue extracted (pellet + supernatant)

Table IX

Distribution of Evans Blue after Addition of Free Evans Blue to Homogenate of a Non-injected Rat

Fraction	mg Evans blue/g Kidney	% Evans blue
total	0.238	95.20
N	0.022	9.25 [0.24]
M	0.008	3.36 [0.22]
L	0.001	0.42 [0.25]
P	0.009	3.78 [0.42]
S	0.198	83.19 [2.34]

[ ] = Relative Specific Concentration, % Evans blue/% Protein

Figure 8 - Distribution of Evans Blue in Rat Kidney Subcellular Fractions after Addition of Free Evans Blue to Homogenate of a Non-injected Rat

Data are expressed in a bar graph in which subcellular fractions going from left to right are:

N - Nuclear

M - Heavy Mitochondrial

L - Light Mitochondrial

P - Microsomal

S - Supernatant

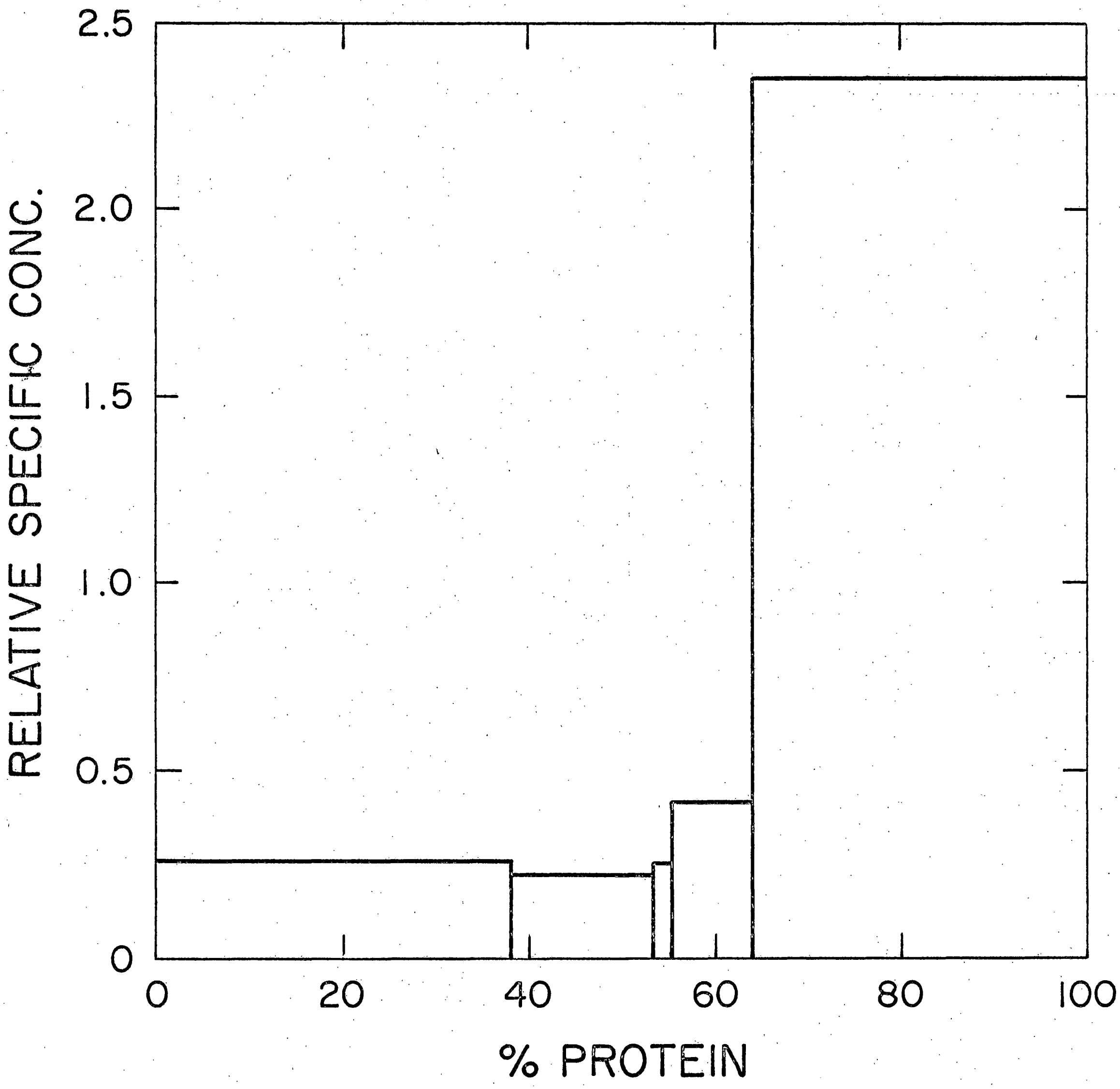

2.5
2.0
1.5
1.0
0.5
0
RELATIVE SPECIFIC CONC.
0
20
40
60
80
100
% PROTEIN

Figure 9 - Distribution of Evans Blue in Rat Kidney Subcellular Fractions at 12 Hours after Injection

Data are expressed in a bar graph in which subcellular fractions going from left to right are:

N - Nuclear

M - Heavy Mitochondrial

L - Light Mitochondrial

P - Microsomal

S - Supernatant

5

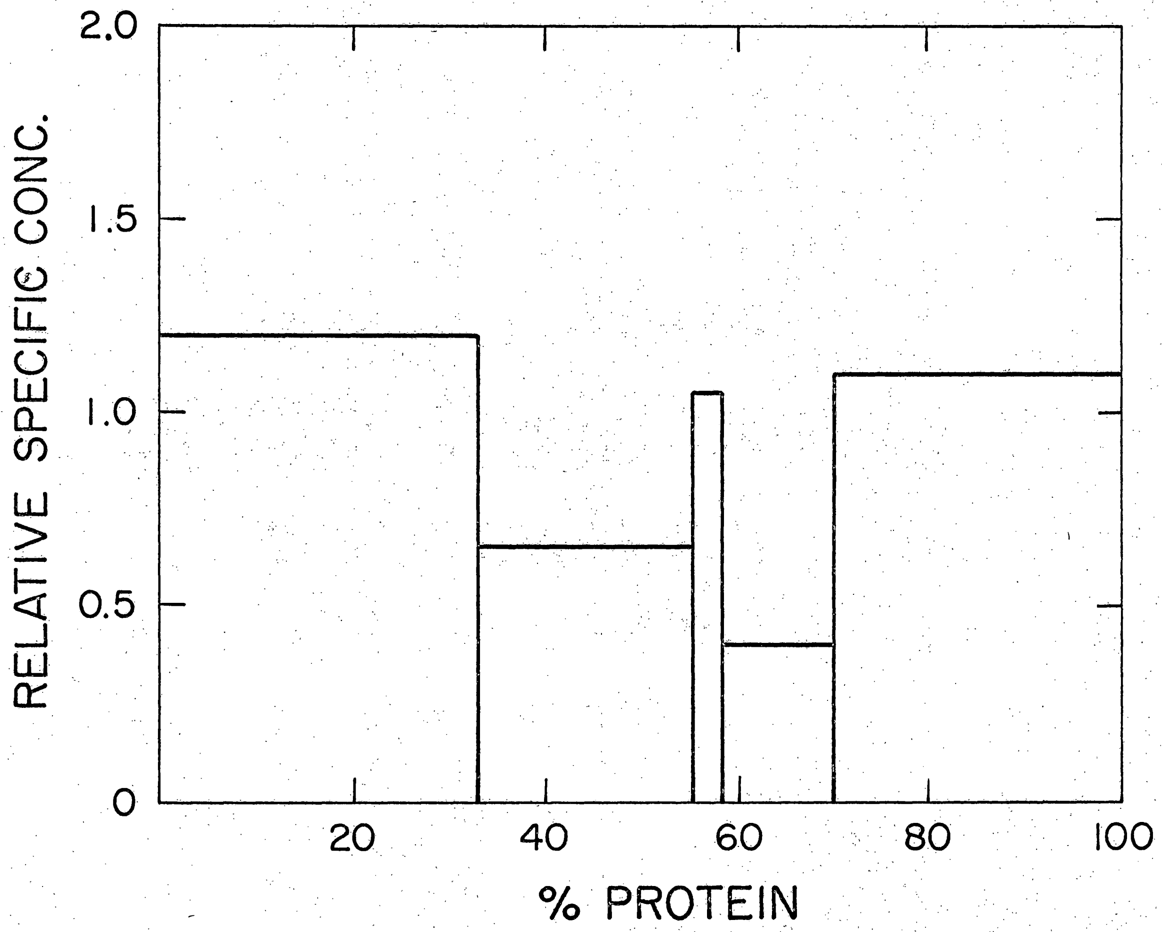


Table X

Distribution of Evans Blue, Acid Phosphatase, and Protein in Rat Kidney Nuclear Fraction after Treatment with Triton X-100

	Fractions			
	Original N	Pellet	Supernatant	P + S
mg Evans blue/g Kidney	0.168			
Trial 1		0.028	0.137	0.165
Trial 2		0.025	0.135	0.160
Mean		0.027	0.136	0.163
% Evans Blue		16.30	83.70	97.02
Units Ac. Pase./g Kidney	1.707	0.386	1.586	1.977
% Acid Phosphatase		19.57	80.42	115.50
mg Protein/g Kidney	42.09	1.40	35.13	36.53
% Protein		3.82	96.18	86.80

was 97.02% of that originally found in the nuclear fraction. Also 80% of the acid phosphatase activity and 96% of the protein was found in the soluble fraction.

The percentage of Evans blue taken up by rat kidney in relation to the other body tissues was determined from balance calculations. The circulating blood volume of a 200 g rat is about 15 ml, and the hematocrit value is about 60% (8). From this information it was calculated that the total volume of serum in a 220 g rat was 6.6 ml. By the use of Figure 7 (decrease of Evans blue in rat serum) the amount of Evans blue in the blood at 1/2 hour after injection was determined to be 12.54 mg, and the amount at 6 hours was found to be 7.26 mg. Therefore, in this 5 1/2 hour period 5.28 mg Evans blue were eliminated from the blood stream. It was estimated that the two kidneys of a 220 g rat weigh approximately 1.6 g. This information and the data in Figure 5 (uptake of Evans blue by rat kidney) were used to calculate the mg of Evans blue taken up by two kidneys at 1/2 hour (0.424 mg) and 6 hours (0.675 mg) after injection. The net amount of Evans blue taken up by two kidneys in this 5 1/2 hour period was 0.251 mg. Table XVI (to be discussed later) shows that 0.138 mg of Evans blue were lost through the urine in the first 6 hours. No fecal determinations were made. In the 5 1/2 hour period 5.280 mg of Evans blue were eliminated from the blood stream, and only 0.138 mg were lost through the urine; therefore, 5.142 mg of Evans blue were taken up by the different body tissues. Knowing that two kidneys took up 0.251 mg of Evans blue in this time period, it was calculated that two kidneys accounted for 4.75% of the total uptake of Evans blue by rat tissues.

Low Magnesium Studies - The total amount of Evans blue extracted from three day magnesium deficient rats at 1/2 hour, 1 hour, 3 hours, and 12 hours after injection is presented in Table XI. Also, the amount of Evans blue extracted from each subcellular fraction at each time interval is stated in this table. For comparative purposes the Evans blue extracted from the control rats, Table III, was added to Table XI. All values are the mean of at least two experimental determinations and are expressed in mg Evans blue/g kidney. Table XII and Table XIII show the individual values for Evans blue extracted in each magnesium deficient experiment. The mg Evans blue/ml serum extracted at each time interval for both magnesium deficient rats and control rats also appear in Table XI.

The accumulation of Evans blue by three day magnesium deficient rat kidney increased with time. The net uptake appears to be similar for control and deficient rats for the first hour; however, there is a very distinct difference at 3 hours with the deficient kidneys containing more Evans blue. The net uptake at 12 hours appears to be similar again. This difference in uptake of Evans blue at 3 hours can be seen graphically in Figure 10, a plot of the net uptake of Evans blue with time for both control and deficient rat kidney. Table XI also shows an increase in Evans blue with time in the nuclear fraction of deficient rat kidney. This uptake seems to be very similar to the uptake found in the control rats. The light mitochondrial and microsomal fractions for the deficient rats remained rather constant for the first 3 hours and then rose slightly in the next 9 hours, which is similar to the

Table XI

Extraction of Evans Blue from Control and Three Day Magnesium Deficient Rat Kidney Fractions and Serum at each Time Interval

	mg Evans blue/g Kidney										
	1/2 Hour		1 Hour		3 Hours					12 Hours	
Fraction	Control	Def.	Control	Def.	Control	S.E.M.	Def.	S.E.M.	p*	Control	Def.
total	0.265	0.234	0.288	0.296	0.348	$\pm$0.012	0.463	$\pm$0.003	0.001	0.568	0.574
N	0.086	0.068	0.120	0.115	0.168	$\pm$0.004	0.172	$\pm$0.007	n.s.	0.255	0.261
M	0.043	0.044	0.052	0.056	0.054	$\pm$0.008	0.106	$\pm$0.019	0.100	0.083	0.079
L	0.010	0.012	0.008	0.008	0.009	$\pm$0.001	0.013	$\pm$0.001	n.s.	0.017	0.020
P	0.014	0.014	0.013	0.012	0.013	$\pm$0.002	0.015	$\pm$0.001	n.s.	0.026	0.026
S	0.112	0.096	0.095	0.105	0.104	$\pm$0.004	0.157	$\pm$0.015	0.050	0.187	0.188
Serum**	1.904	2.300	1.655	1.990	1.290		1.530			0.690	0.795

*p value for control compared to deficient. A p value of 0.05 or less was considered significant; n.s. = not significant

**mg Evans blue/ml serum

Table XII

Extraction of Evans Blue from Three Day Magnesium Deficient

Rat Kidney Fractions and Serum

	mg Evans blue/g Kidney					
	1/2 Hour Trials*			1 Hour Trials*		
Fraction	1	2	Mean	1	2	Mean
total	0.237	0.230	0.234	0.290	0.302	0.296
N	0.068	0.068	0.068	0.118	0.112	0.115
M	0.044	0.043	0.044	0.053	0.059	0.056
L	0.013	0.010	0.012	0.009	0.008	0.008
P	0.016	0.013	0.014	0.010	0.013	0.012
S	0.096	0.096	0.096	0.100	0.110	0.105
Serum**	2.340	2.260	2.300	1.880	2.100	1.990

*either three or four rats were used for each trial

**mg Evans blue/g Kidney

Table XIII

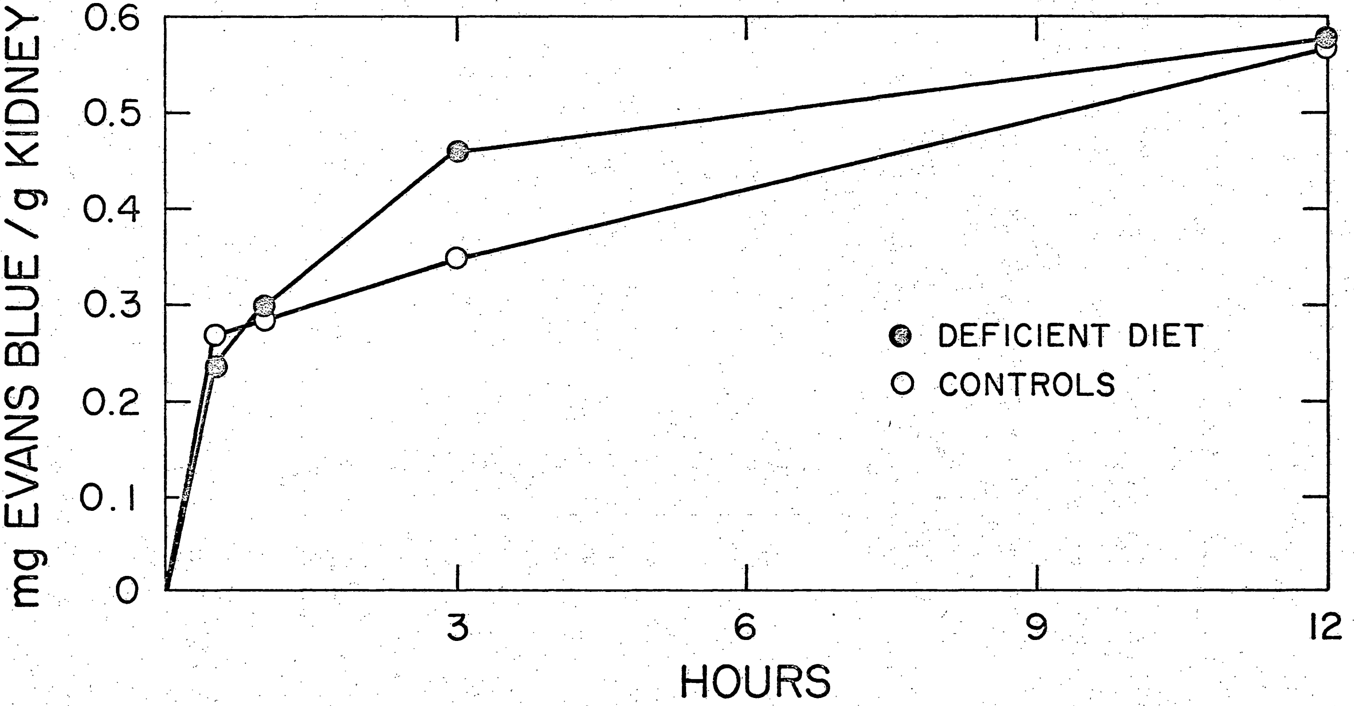
Extraction of Evans Blue from Three Day Magnesium Deficient Rat Kidney Fractions and Serum

	mg Evans blue/g Kidney						
	3 Hour Trials*				12 Hour Trials*		
Fraction	1	2	3	Mean	1	2	Mean
total	0.468	0.458	0.461	0.463	0.554	0.594	0.574
N	0.185	0.160	0.170	0.172	0.272	0.250	0.261
M	0.071	0.135	0.111	0.106	0.066	0.092	0.079
L	0.012	0.014	0.012	0.013	0.018	0.023	0.020
P	0.016	0.015	0.014	0.015	0.022	0.029	0.026
S	0.184	0.134	0.154	0.157	0.176	0.200	0.188
Serum**	1.500	1.525	1.560	1.530	0.815	0.775	0.795

*either three or four rats were used for each trial

**mg Evans blue/g Kidney

Figure 10 - Net Uptake of Evans Blue by Three Day Magnesium Deficient Rat Kidney and Control Rat Kidney



mg EVANS BLUE / g KIDNEY
0.6
0.5
0.4
0.3
0.2
0.1
0
3
6
9
12
HOURS
DEFICIENT DIET
CONTROLS

control data for these fractions. The heavy mitochondrial fraction was the most variable of the deficient rat kidney subcellular fractions. The amount of Evans blue extracted at 1 hour was slightly higher than at 1/2 hour; however, the amount extracted at 3 hours was substantially higher. This large increase in Evans blue in the heavy mitochondrial fraction of deficient rats at 3 hours after injection was not seen with the control rats. At 12 hours the amount dropped to approximately the same as was found in the heavy mitochondrial fraction of control rats at this time. The Evans blue extracted from the supernatant of deficient rat kidney was similar to that of the control for the first hour; however, it too increased markedly at 3 hours while the amount of Evans blue extracted from the control at 3 hours remained the same. The amount of Evans blue found at 12 hours was the same for both the deficient and control supernatants. The net uptake of Evans blue with time by the subcellular fractions of magnesium deficient rats can be seen graphically in Figure 11. If this figure is compared to Figure 6, the net uptake of Evans blue by control rat kidney subcellular fractions, one can compare the uptake of Evans blue by deficient rat kidney subcellular fractions to the uptake obtained by control rat kidney subcellular fractions. The greatest difference seems to be in the heavy mitochondrial fraction and the supernatant.

The percentage of the total Evans blue in each subcellular fraction at each time interval for the deficient rats was calculated and is presented in Table XIV along with the percentages from the control rats. Differences are again seen at 3 hours after injection. The percentage

Figure 11 - Net Uptake of Evans Blue by Three Day Magnesium Deficient Rat Kidney Subcellular Fractions

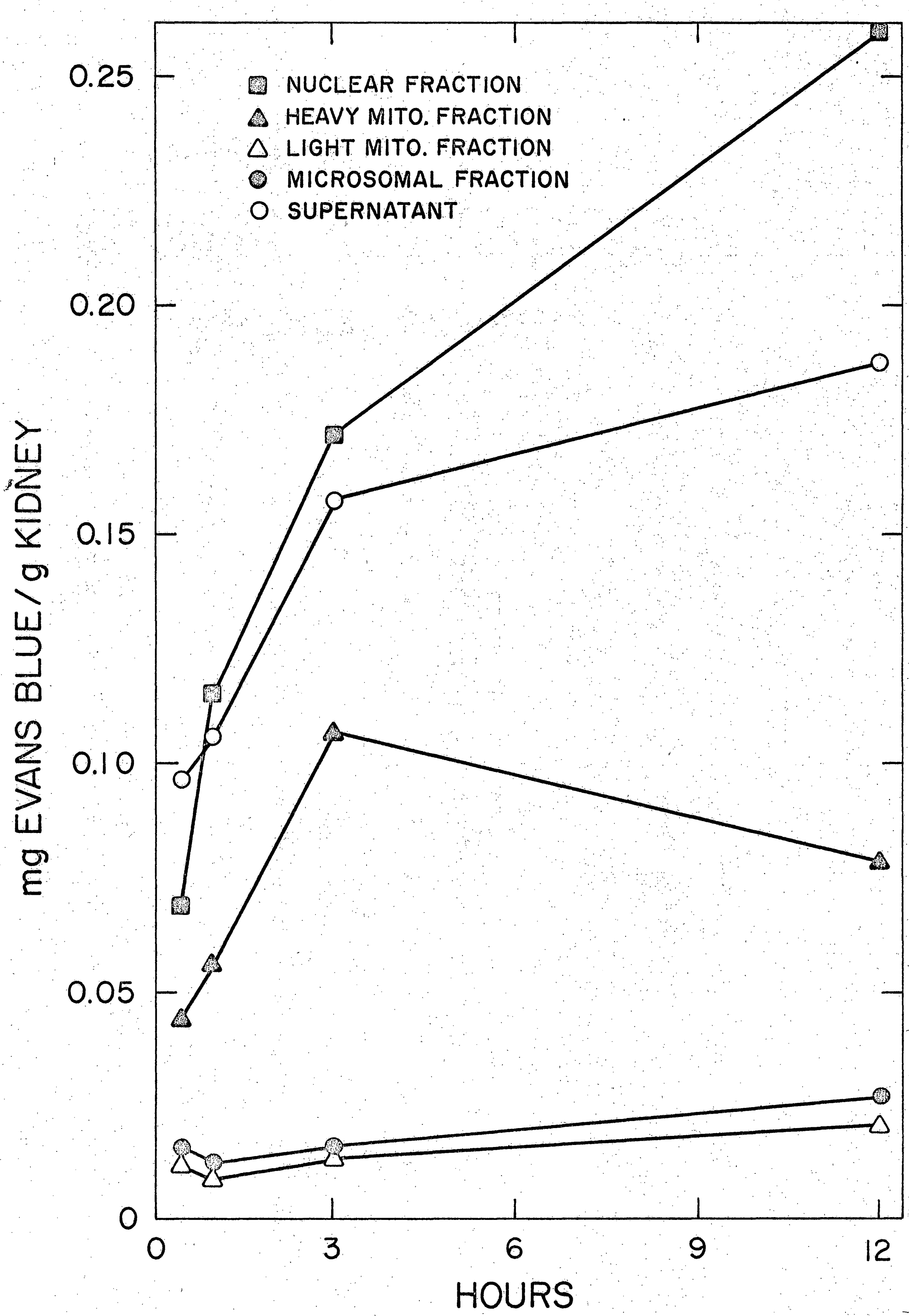

NUCLEAR FRACTION
HEAVY MITO. FRACTION
LIGHT MITO. FRACTION
MICROSOMAL FRACTION
SUPERNATANT
mg EVANS BLUE / g KIDNEY
0
0.05
0.10
0.15
0.20
0.25
0
3
6
9
12
HOURS

Table XIV

Percent of Evans Blue in each Subcellular Fraction at each Time Interval for both Control and Three Day Magnesium Deficient Rats

	% Evans blue							
	1/2 Hour		1 Hour		3 Hours		12 Hours	
Fraction	Control	Def.	Control	Def.	Control	Def.	Control	Def.
N	33	29	42	39	48	37	45	46
M	16	19	18	19	15	23	15	14
L	4	5	3	3	3	3	3	3
P	5	6	4	4	4	3	4	4
S	42	41	33	35	30	34	33	33

of Evans blue in the nuclear fraction from deficient rats was much lower than that of the control rats. However, the percentages of Evans blue found in the heavy mitochondrial fraction and supernatant were higher for the deficient rats. After 12 hours the distribution was almost identical.

Table XI and Figure 12 show a decrease in Evans blue with time in serum of magnesium deficient rats. The amount of Evans blue extracted at each time period was higher for the deficient rats than for the control rats.

The % acid phosphatase activity, the relative specific activity of acid phosphatase, and the % protein found in each subcellular fraction from deficient rats at each time interval after injection of Evans blue are seen in Table XV. The variability between values for the same fraction is not significant.

Urine was collected from both control and deficient rats at the end of the first 6 hours after injection of Evans blue and at the end of 12 hours after injection. Table XVI shows the mg Evans blue extracted at each time period for both control and deficient rats. As seen, the deficient rats excreted almost three times as much Evans blue in the first 6 hours as did the control rats. However, in the next 6 hour period the amount of Evans blue excreted by the deficient rats is similar to the amount excreted by the control rats.

Figure 12 - Evans Blue in Three Day Magnesium Deficient Rat Serum and Control Rat Serum at Various Times Following Injection

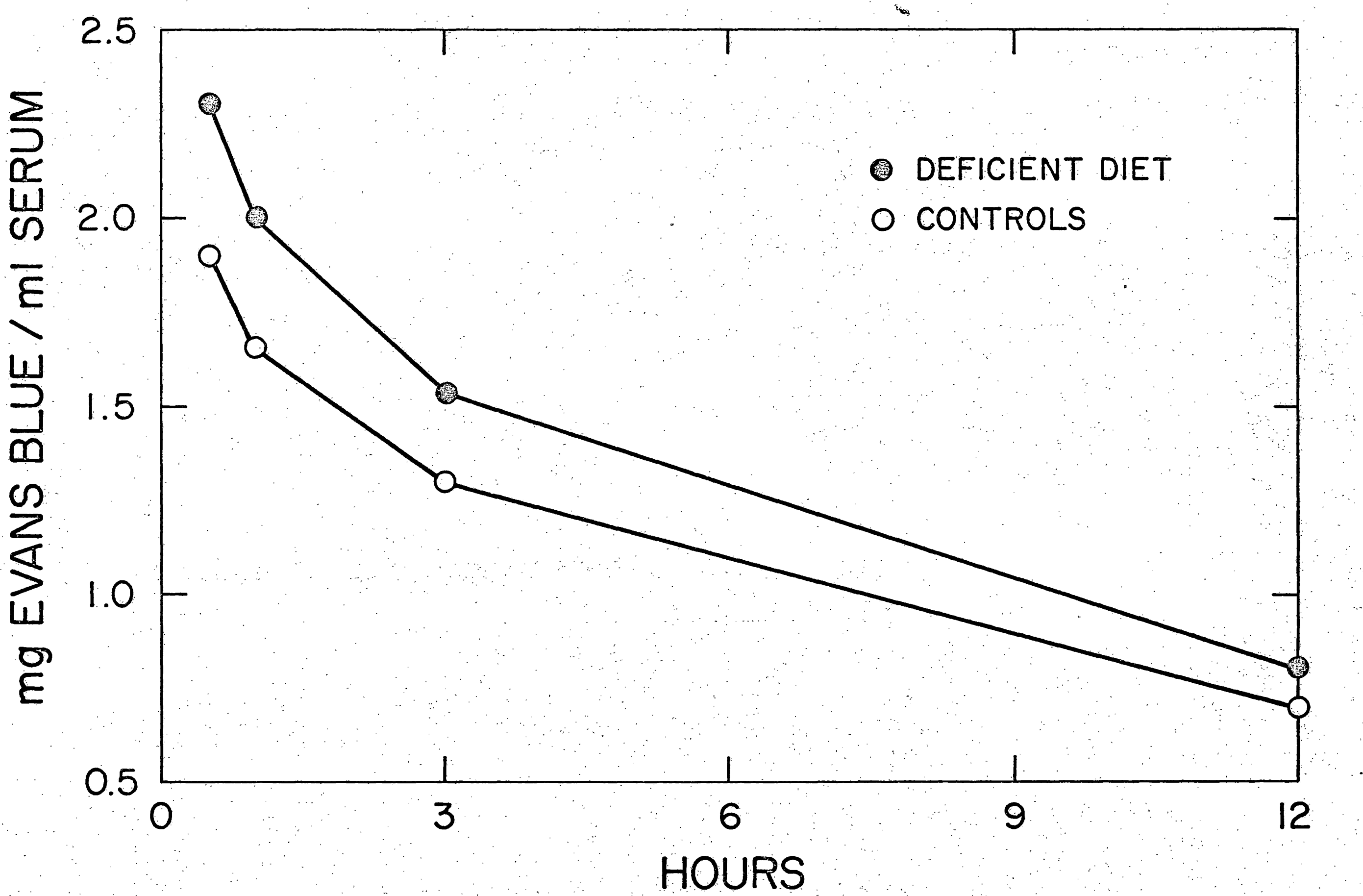

mg EVANS BLUE / ml SERUM
2.5
2.0
1.5
1.0
0.5
0
3
6
9
12
HOURS
DEFICIENT DIET
CONTROLS

Table XV

Percent Acid Phosphatase Activity, Relative Specific Activity, and Percent Protein in Three Day Magnesium Deficient Rat Kidney Subcellular Fractions at Various Times after Evans Blue Injection

	1/2 Hour		1 Hour		3 Hours		12 Hours	
Fraction	% Ac. P.	% Prot.	% Ac. P.	% Prot.	% Ac. P.	% Prot.	% Ac. P.	% Prot.
N	35.87 [1.23]	29.25	39.94 [1.23]	32.52	37.85 [1.25]	30.20	38.00 [1.21]	31.39
M	21.99 [1.02]	21.48	23.19 [1.15]	20.18	21.03 [1.00]	21.03	19.40 [1.00]	19.35
L	9.10 [2.82]	3.23	6.48 [2.47]	2.62	6.44 [2.29]	2.81	6.99 [2.28]	3.06
P	15.34 [1.08]	14.22	14.00 [1.11]	12.65	15.49 [1.15]	13.45	18.46 [1.46]	12.65
S	17.71 [0.56]	31.83	16.40 [0.51]	32.03	19.19 [0.59]	32.51	17.15 [0.51]	33.55

[ ] = Relative Specific Activity, % Acid Phosphatase/% Protein

Table XVI

Evans Blue Extracted from Control and Three

Day Magnesium Deficient Rat Urine

	mg Evans blue Extracted	
Hours of Collection	Control	Deficient
1-6	0.138	0.396
6-12	0.043	0.054

DISCUSSION

Differences and Similarities in Liver, Kidney, and Yolk-Sac Using Bisazo Dyes - The distribution pattern obtained for Evans blue in rat liver subcellular fractions (Figure 4) shows the highest relative specific concentration of Evans blue in the light mitochondrial fraction. In comparison, the distribution pattern for trypan blue in liver fractions obtained by Lloyd et al. (21), Figure 2, shows the highest relative specific concentration of trypan blue in the light mitochondrial fraction also. These distribution patterns probably reflect the accumulation of bisazo dye in small vacuoles which sediment in the light mitochondrial fraction. In contrast, the distribution pattern obtained for Evans blue in rat kidney subcellular fractions (Figure 9) shows the highest relative specific concentration of the dye no longer in the light mitochondrial fraction (as with liver) but in the nuclear fraction. This pattern for kidney indicates an accumulation of Evans blue by larger vacuoles which sediment more rapidly in a centrifugal field. It was concluded from these data that differences exist between these two tissues in the various steps involved in the handling of macromolecules. The data suggest a greater diversity of size among the vacuoles in the kidney than in the liver. The difference may be a function of the quantity of material presented to the cells and/or engulfed by the cells. More Evans blue may have been presented to the kidney cells than the liver cells which resulted in the uptake of greater quantities of Evans blue into large vacuoles. It is also possible that the rate of pinocytosis was more rapid in the kidney cells than in the liver cells. Lloyd _et al_. (21) using visceral yolk-sac which is known to be a strongly pinocytic tissue found a different distribution pattern for uptake of trypan blue (Figure 3) than that observed in liver (Figure 2). The highest relative specific concentration for trypan blue was found in the nuclear fraction as was found for Evans blue in kidney. They also suggested a greater diversity of size among the vacuoles in the yolk-sac than in the liver.

A comparison can also be made of the uptake of horseradish peroxidase in liver and kidney. Jacques (25) in studying the uptake of horseradish peroxidase by rat liver has obtained a distribution pattern for peroxidase similar to the distribution pattern obtained for trypan blue in liver fractions by Lloyd _et al_. (21), Figure 2. The highest relative specific concentration of peroxidase activity was in the light mitochondrial fraction just as with trypan blue and Evans blue. Straus (16), however, in studies of uptake by rat kidney, has found an accumulation of horseradish peroxidase in the nuclear and heavy mitochondrial fractions (Figure 1) just as was found with Evans blue in kidney and trypan blue in yolk-sac.

From these observations it was concluded that the vacuolar apparatus in rat kidney behaves differently than in liver. The uptake of Evans blue in kidney cells is by large vacuoles which sediment in the nuclear and heavy mitochondrial fractions; whereas, the uptake in liver cells is by smaller vacuoles which sediment in the light mitochondrial fraction.

Uptake of Evans Blue by Rat Kidney - In Table III is presented the uptake of Evans blue by rat kidney in mg Evans blue/g kidney, and in Table VII is presented the uptake in mg Evans blue/g protein. Both sets of data are very similar in relation to accumulation of Evans blue in total kidney and in the subcellular fractions. They both indicate a gradual accumulation of Evans blue in total kidney and nuclear fraction. Figure 5 and Figure 6 present these findings graphically for mg Evans blue/g kidney. In comparison, Straus (16) has shown an increasing accumulation of horseradish peroxidase activity in total kidney and nuclear fraction during the first few hours following injection (Figure 1). These results are similar to the accumulation obtained for Evans blue in that they both reveal an accumulation of injected material in the nuclear fraction of rat kidney. It is suggested that the same sequence of events which resulted in the accumulation of horseradish peroxidase in the nuclear fraction of rat kidney took place for Evans blue in rat kidney.

The uptake of Evans blue in rat kidney has been studied microscopically by Gilson (7) and Wilde *et al*. (9); however, no one has described the sequence of events following injection of Evans blue. For this reason the following hypothesis is suggested. Evans blue-albumin complexes enter kidney cells by pinocytosis which results in the formation of small Evans blue containing phagosomes. These small phagosomes either merge with other phagosomes to form intermediate sized phagosomes which then merge to form large phagosomes or vacuoles, or fuse directly with preexisting large vacuoles. These large phagosomes or vacuoles are of such size that they sediment in the nuclear fraction. Approximately 40% of the total acid phosphatase activity was found in the nuclear fraction (Table VIII); therefore, these large phagosomes or vacuoles probably contained lysosomal enzymes and should be termed large heterolysosomes. Lloyd et al. (21) in their work with yolk-sac have claimed that trypan blue accumulated in large heterolysosomes which sedimented in the nuclear fraction. It is assumed that the increase in Evans blue in the nuclear fraction was due to an accumulation within large heterolysosomes.

One might argue that the increase of Evans blue in the nuclear fraction was not due to accumulation within large heterolysosomes but due to increased binding of Evans blue to nuclear proteins. However, the results in Table IX (distribution of Evans blue after addition of free Evans blue to homogenate of a non-injected rat) and Table X (distribution of Evans blue and acid phosphatase in nuclear fraction after treatment with triton X-100) disprove this possibility and strongly suggest that the Evans blue present in the nuclear fraction was contained within vacuolar bodies.

The amount of Evans blue recovered from the supernatant for the first 3 hours after injection was constant but rather high (Figure 6). Rupture of Evans blue containing phagosomes and/or Evans blue containing large heterolysosomes during tissue preparations is a possible source of this Evans blue. Approximately 16% of the total acid phosphatase activity was found in the supernatant (Table VIII); accordingly, rupture of vacuoles containing acid phosphatase and Evans blue could be a source of Evans blue in the supernatant for the first 3 hours after injection.

At 12 hours after injection the amount of Evans blue extracted from the supernatant increased substantially (Table III and Figure 6); however, the quantity of Evans blue in the serum declined with time (Figure 7). This indicates that less Evans blue was available for pinocytosis at 12 hours after injection, so the increased Evans blue in the supernatant was not due to increased pinocytosis of Evans blue. Also the percent of acid phosphatase activity in the supernatant did not increase at 12 hours (Table VIII), which indicates that increased rupture of Evans blue containing vacuoles was not a source of the increased Evans blue in the supernatant. Therefore, it is suggested that the elevation of Evans blue in the supernatant at 12 hours after injection was the result of digestion within large heterolysosomes. Pinocytosed Evans blue was originally bound to serum albumin; however, hydrolytic enzymes within large heterolysosomes degraded the albumin and released free Evans blue which then diffused through the vacuolar membrane into the cytoplasm.

Effects of Low Magnesium Diet - As seen in the RESULTS, five major differences from control kidney studies were prominent in the magnesium deficiency studies: increased total kidney Evans blue at 3 hours after injection, increased heavy mitochondrial Evans blue at 3 hours, increased supernatant Evans blue at 3 hours, elevated serum Evans blue, and increased urine Evans blue at 6 hours after injection. It is suggested that magnesium deficiency caused certain changes in rat kidney cells which resulted in the accumulation of Evans blue into intermediate sized phagosomes which sedimented in the heavy mitochondrial fraction. This could be the source of the increased Evans blue in total kidney and heavy mitochondrial fraction at 3 hours after injection. The increase in supernatant Evans blue indicates an increased release of Evans blue into the cytoplasm by some manner, possibly from intermediate phagosomes. A difficulty in interpretation of these data is posed by the lack of information concerning the form of Evans blue in the supernatant. It would be very helpful in explaining these data if it were known whether the Evans blue was free or bound to albumin or some other protein. However, possible explanations of the data will be suggested.

One suggestion is that the effect of a magnesium deficiency caused an increase in the rate of pinocytosis in kidney cells. This assumption might not seem plausible on first glance since an elevation in serum Evans blue was also observed with magnesium deficient rats (Figure 12). Nevertheless, it is suggested that the various rat tissues reacted differently to the deficiency; some decreased their rate of pinocytosis which caused the elevation in serum Evans blue, and others such as the kidney cells increased their rate of pinocytosis. This is entirely possible since the kidney only accounts for 4.75% of the total uptake of Evans blue in the first 6 hours. The increased rate of pinocytosis increased the number of small Evans blue containing phagosomes. As stated earlier these normally fuse with large heterolysosomes thus disgorging Evans blue into these large vacuoles. However, the rate of fusion of small phagosomes with large vacuoles may be the rate limiting step in the vacuolar apparatus. It is postulated that the increased intake overcame the capacity of the rate limiting step and thus produced a surplus of small Evans blue containing phagosomes. In order to releave the cell of this surplus the small phagosomes merged with each other to form an increased number of intermediate sized phagosomes. Many small Evans blue containing phagosomes probably fused with primary lysosomes forming intermediate heterolysosomes (which sediment in the heavy mitochondrial fraction).

As mentioned earlier the increase in Evans blue in the supernatant indicates release of Evans blue into the cytoplasm. Digestion of albumin within intermediate heterolysosomes could have released free Evans blue which diffused through the vacuolar membrane into the cytoplasm. Another possibility is that the intermediate phagosomes formed as a result of the magnesium deficiency were more fragile than normal vacuoles, and upon tissue preparations many ruptured releasing Evans blue, probably bound to albumin, into the cytoplasm.

The increased amount of Evans blue in the supernatant at 3 hours after injection could be a source of the increased amount of Evans blue found in the urine during the first 6 hours post-injection. Cytoplasmic Evans blue could have been released from the cells by diffusion across the plasma membrane. The released Evans blue was either taken up by the blood compartment or emptied into the renal tubules where it was removed with urine. Regurgitation of intermediate sized phagosomes containing Evans blue into the tubular lumen was another possible source of the increased Evans blue in the urine. The kidney cells not being able to cope with the increased population of phagosomes might have, therefore, turned to regurgitation as a mechanism of equilibration.

The differences in the kidney cells were seen at 3 hours following injection; however, at 12 hours post-injection data for control and deficiency studies were very similar (Table XI). By 12 hours the amount of Evans blue in the blood stream had decreased greatly; therefore, much less Evans blue was being pinocytosed, and accordingly, much less Evans blue was accumulating in intermediate phagosomes. The accumulation of Evans blue in the nuclear fraction persisted due to continued fusion of preexisting intermediate Evans blue laden phagosomes with large heterolysosomes.

Further experiments are necessary in order to explain the differences observed with a greater degree of confidence. Fractionations at 2, 4, 6, and 9 hours post-injection of Evans blue to deficient rats should reveal the sequential formation of the intermediate Evans blue containing vacuoles. An isolation of intermediate vacuoles is necessary in order to determine if they contain hydrolytic enzymes. This would indicate whether they had fused with lysosomes. Another experiment would be to determine the form of the Evans blue (free, bound to albumin, etc.) in the cytoplasm and urine. This would enable one to suggest with more accuracy from where the Evans blue evolved. Also cytochemical studies using both light and electron microscopy of deficient rat kidney would aid in identifying the intermediate phagosomes.

SUMMARY

It has been suggested that a matrix for kidney stone formation may originate in the vacuolar apparatus of kidney cells and that imbalances in intracellular calcium and magnesium concentrations may stimulate its egress into the tubular lumen. It was the purpose of this thesis to study the normal behavior of Evans blue in the vacuolar apparatus of rat kidney and to determine changes in this normal behavior imposed by a magnesium deficiency. Since Evans blue binds tightly to serum albumin, and since Evans blue-albumin complexes are reabsorbed from the glomerular filtrate by pinocytosis by the cells of the renal proximal tubules, Evans blue served as a suitable marker of the vacuolar apparatus for these studies.

Pinocytosis of Evans blue-albumin complexes by rat kidney cells resulted in the formation of small Evans blue containing phagosomes. In normal rat kidney these small phagosomes either fused with each other to form intermediate phagosomes which then fused with large heterolysosomes, or fused directly with large heterolysosomes. In either case, however, the Evans blue-albumin complexes in the small phagosomes were disgorged into large heterolysosomes where digestion could take place. By fractional centrifugations at different time periods post-injection, the net uptake of Evans blue by the total kidney and each subcellular fraction was observed. The amount of Evans blue taken up by the total kidney was shown to increase with time. A gradual increase in the amount of Evans blue in the nuclear fraction with time was also seen. This was interpreted as an accumulation of

Evans blue in large heterolysosomes. The other fractions remained rather unchanged throughout the 12 hour test period except the supernatant which rose sharply at 12 hours post-injection. This was believed due to a release of Evans blue from larger heterolysosomes after digestion of the albumin in the Evans blue-albumin complexes. A decline in the Evans blue in the serum was also noted. These patterns of uptake of Evans blue by the total kidney and each subcellular fraction represent the normal behavior of Evans blue in the vacuolar apparatus of rat kidney.

The same experiments were applied to three day magnesium deficient rats. The net uptake of Evans blue by deficient rat kidney was higher than the net uptake by control rat kidney only at 3 hours post-injection. The net uptake of Evans blue by the nuclear, light mitochondrial, and microsomal fractions were similar for both the deficient and control rat kidneys. Much more Evans blue was extracted from the heavy mitochondrial fraction and the supernatant of the deficient rat kidney at 3 hours post-injection than the control heavy mitochondrial fraction and supernatant at this time period. However, at 12 hours post-injection the amount of Evans blue extracted from the heavy mitochondrial fraction of both deficient and control rat kidney was similar, and the amount extracted from the supernatants of both was similar. Therefore, magnesium deficiency caused certain changes in the normal uptake of Evans blue which were apparent at 3 hours post-injection. The large increase of Evans blue in the heavy mitochondrial fraction at 3 hours after injection is suggested to be due to the accumulation of

Evans blue in an increased number of intermediate phagosomes. The increase in the supernatant may be due to either release of Evans blue after digestion of the albumin within the intermediate heterolysosomes or rupture of intermediate phagosomes due to experimental procedures. Much Evans blue was also found in the urine of deficient rats at 6 hours after injection. This increase may have resulted from diffusion of Evans blue into the tubular lumen from the cytoplasm or regurgitation of intermediate Evans blue containing phagosomes back into the tubular lumen. The results are not inconsistent with the possibility that regurgitated phagosomes could serve as the matrix for kidney stone genesis.

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THE ACCUMULATION AND DISTRIBUTION OF EVANS BLUE IN THE KIDNEY OF RATS FED NORMAL OR LOW MAGNESIUM DIETS

by

George Williams Seignious, IV

(ABSTRACT)

The normal behavior of Evans blue in the vacuolar apparatus of rat kidney and the changes in this normal behavior imposed by a magnesium deficiency were investigated. Since Evans blue binds tightly to serum albumin, and since Evans blue-albumin complexes are reabsorbed from the glomerular filtrate by pinocytosis by the cells of the renal proximal tubules, Evans blue served as a suitable marker of the vacuolar apparatus for these studies.

Rats were injected intravenously with Evans blue and sacrificed at either 1/2, 1, 3, or 12 hours post-injection. Kidneys were removed and homogenized. By fractional centrifugations at the different time periods post-injection, the net uptake of Evans blue by the total kidney and each subcellular fraction was observed. The amount of Evans blue taken up by the total kidney was shown to increase with time. Also a gradual increase in the amount of Evans blue in the nuclear fraction with time was seen. This was interpreted as an accumulation of Evans blue in large heterolysosomes.

The same experiments were applied to three day magnesium deficient rats. Differences observed from control kidney studies were: increased total kidney Evans blue at 3 hours after injection, increased heavy mitochondrial Evans blue at 3 hours, increased supernatant Evans blue at 3 hours, elevated serum Evans blue, and increased urine Evans blue at 6 hours after injection. Possible explanations of these differences are discussed in the thesis.