COLORIMETRIC INVESTIGATION OF THE UPTAKE OF AN INTRAVENOUSLY INJECTED PROTEIN (HORSERADISH PEROXIDASE) BY RAT KIDNEY AND EFFECTS OF COMPETITION BY EGG WHITE

W. STRAUS, Ph.D.

From the Laboratory of Physiological Chemistry, University of Louvain, Louvain, Belgium. Dr. Straus's present address is Department of Physiology, University of North Carolina, Chapel Hill

ABSTRACT

After intravenous injection of horseradish peroxidase into rats, the foreign protein appeared in the kidney first in the small phagosomes and its concentration there decreased quickly; it then was concentrated and "stored" for several days in the large phagosomes. After injection of 10 mg of peroxidase per 100 gm of body weight, the concentration of peroxidase in blood and urine decreased exponentially during the first 6 hours; small amounts of peroxidase were excreted in the urine for several days. When 0.05 to 1.0 mg of peroxidase per 100 gm were administered, most of the peroxidase was taken up by the liver and little by the kidney, and a portion was excreted in the urine even at the lowest dose. At doses above 1.5 mg per 100 gm, the liver cells were saturated, and large reabsorption droplets appeared in the tubule cells of the kidney. With further dosage increase, the concentration of peroxidase in the phagosomes of the kidney increased rapidly until saturation was reached at doses of 13 mg per 100 gm. After intraperitoneal injection of egg white 18 hours prior to the administration of peroxidase, the concentration of peroxidase in all kidney fractions was only 10 to 25 per cent of the values for the untreated animals, the disappearance of peroxidase from the blood was delayed, and 81 per cent more peroxidase was excreted in the urine. The treatment with egg white had no effect on the uptake of peroxidase by the liver. The ability of kidney tissue to degrade and adsorb peroxidase *in vitro* was tested.

It was reported previously (1, 2) that considerable amounts of intravenously injected horseradish peroxidase are taken up by the kidney and liver; lesser amounts were found in certain other organs, of which the spleen, bone marrow, thymus, prostate, epididymis, pituitary, pancreas, small intestine, and heart have been tested. Approximately 20 per cent of the injected protein was excreted in the urine and 6 per cent in the feces (1). In these experiments, the colorimetric analysis of peroxidase in homogenates (1) and in isolated fractions (3) was correlated with cytochemical tests for peroxidase in squash preparations of these same tissues or in smears of the isolated fractions (2). A good agreement between the cytochemical and colorimetric data was attained.

The cytochemical observations had shown that much of the peroxidase was concentrated in cytoplasmic granules ("phagosomes") varying in diameter from the limit of microscopic visibility up to 5 μ. Many phagosomes were identified cytochemically in the epithelial cells of the kidney and in the cells of the reticuloendothelial system (macrophages and endothelial cells) of the organs

mentioned (2). Relatively pure phagosomes were isolated from kidney cells after the administration of egg white (4–6) and peroxidase (3), and from the kidneys of untreated animals (4, 7). These "droplets" were found to contain high concentrations of hydrolytic enzymes (4, 7), the same that de Duve *et al.* (8) had discovered in the lysosomes of liver. The injected egg white (5) and horseradish peroxidase (3) were also concentrated in the phagosomes.

In recent cytochemical investigations on the kidney, the importance of the cell membranes as the primary site of uptake of the injected protein and for the formation of the phagosomes has been demonstrated. Large phagosomes were observed to develop on "strands" at the base of the brush border or close to the apical cell membranes ("canaliculi") of the convoluted tubule cells. Very small phagosomes were located in the region of the brush border and along fine fibrils or membranes extending from the base of the convoluted tubule cells to the lumen (9–11). The hypothesis was proposed that the small phagosomes serve in transport, and that the large phagosomes develop as a protective reaction of the cells against the invasion by foreign materials (9–11). The localization of phagosomes and lysosomes in kidney and liver has also been investigated by Novikoff (12, 13) using peroxidase and acid phosphatase for the cytochemical detection of these granules.

In the present investigation, the changes in the concentration of peroxidase in kidney homogenates and fractions isolated therefrom were determined in relation to the dose, in relation to the time following administration over a period of several days, and in competition with another protein (egg white). Corresponding concentration changes of peroxidase in blood serum, urine, and liver, and the ability of the kidney to take up and degrade horseradish peroxidase *in vitro* have also been tested.

I. METHODS

a) Animals and Reagents

Peroxidase, dissolved in physiological saline, was injected into the femoral vein of male albino rats weighing 200 to 250 gm. For the competition experiments, 7 ml of egg white, mixed with 7 ml of physiological saline, were injected intraperitoneally 18 hours prior to the administration of peroxidase.

Peroxidase was obtained from the Sigma Chemical Co., St. Louis, Missouri.[1] The activities of different samples varied somewhat after transport overseas. For most experiments, except those described in section II(*e*), "type II" preparations of horseradish peroxidase were used, showing activities between 5000 and 6000 units per milligram. N,N-dimethyl-*p*-phenylenediamine hydrochloride was purchased from Eastman Organic Chemicals, Rochester, New York. The reagents for the cytochemical tests of peroxidase were indicated previously (2).

b) Fractionation of the Kidney Homogenates

After periods varying from a few minutes to several days following administration of peroxidase, the kidneys were perfused from the aorta with cold 30 per cent sucrose solution, and excised. The cortices were dissected as well as possible from the medullae, and the capsules were removed. The kidneys (cortices) were homogenized in 30 per cent sucrose solution in a glass homogenizer with a motor-driven Teflon pestle (A. Thomas and Co., Philadelphia). An incompletely homogenized residue containing tissue fragments, whole cells, nuclei, and large phagosomes was separated by centrifugation for 5 minutes at 500 RPM (56 *g*) in an International centrifuge, model PR-2. The sediment was rehomogenized and resedimented twice under the same conditions, and the supernatant fluids were added to the homogenate. The residue, containing 20 to 25 per cent of the nitrogen of the homogenate, was not included in the fractionation.

Fraction NDrI, containing nuclei (N) and large phagosomes (DrI, Dr for "droplets") and also some large mitochondria, was prepared by centrifugation of the above homogenate for 20 minutes at 2000 RPM (814 *g*). Fraction MDrII, containing mitochondria (M) and intermediate sized "droplets" (DrII), was obtained by centrifugation of the supernatant fluid of sediment NDrI for 20 minutes at 15,000 RPM (19,600 *g*) in a Spinco ultracentrifuge, model L. Fraction PDrIII, containing microsomes (P) and small "droplets" (DrIII), was prepared by centrifugation of the supernatant fluid of sediment MDrII for 45 minutes at 40,000 RPM (105,400 *g*). The final supernatant fluid (S) was decanted and saved. The particulate fractions NDrI, MDrII, and PDrIII were washed once by resuspension in 30 per cent sucrose solution and resedimentation. The final sediments were resuspended in distilled water.

The fractions, as well as an aliquot of the original homogenate (after removal of the incompletely homogenized residue) and the incompletely homoge-

[1] The supply of horseradish peroxidase from the Sigma Chemical Co. under their "Cooperative Allowance" plan is gratefully acknowledged.

nized residue itself, were analyzed for peroxidase and total nitrogen (micro-Kjeldahl method). The recovery of nitrogen and of peroxidase in most experiments was between 95 and 100 per cent.

The fractionation procedure was similar to the one used previously (6) except that fraction DrII (6) was not separated but was sedimented together with the mitochondrial fraction MDrII, and that higher centrifugal forces were used for the separation of the microsomal fractions. As was shown previously (2) and will be mentioned in section I(*d*), the size and number of phagosomes in tissue fractions isolated from peroxidase-treated animals can be estimated rapidly by treating air-dried smears of the isolated sediments with benzidine, and observing the blue-stained granules with the microscope. This test was applied routinely to all sediments isolated during the present investigation. The loss of minute amounts of sedimented material used for the smears was neglected.

In the above fractionation procedure, an artifact, which could not be avoided, exaggerated the values for peroxidase in the supernatant fluids. In order to minimize the possible damage to the phagosomes and release of peroxidase during the homogenization, the tissue was homogenized by just a few up and down strokes with a loosely fitting Teflon pestle, leaving 20 to 25 per cent of the tissue incompletely homogenized. The "free" peroxidase of this tissue residue was released to the homogenate, whereas the peroxidase bound to the phagosomes was separated and was not included in the fractionation. The values for the peroxidase content of the supernatant fluids were therefore 10 to 15 per cent too high. In addition, approximately 10 per cent of the peroxidase was released during the fractionation procedure itself. This was estimated from control experiments in which the tissue was homogenized cautiously and the supernatant fluid separated immediately by high speed centrifugation.

c) Colorimetric Determination of Peroxidase

This method is based on the formation, proportional to the amount of enzyme and to the time of incubation, of a red pigment from *N,N*-dimethyl-*p*-phenylenediamine by peroxidase in the presence of H_2O_2 (1). Previously used with a colorimeter (1), the method was adapted to the Beckman spectrophotometer without significant changes. The following reagents were added to a Beckman cuvette: 1.2 ml of a 0.2 M solution of phosphate buffer, pH 6.2; highly diluted samples of tissue fractions from peroxidase-treated animals; H_2O to make a final volume of 3.0 ml; and 0.2 ml of a freshly prepared, ice-cooled 0.05 per cent solution of *N,N*-dimethyl-*p*-phenylenediamine hydrochloride. The reaction was started by the addition of 0.2 ml of a 0.15 per cent solution of H_2O_2, and the formation of the red pigment was followed with the spectrophotometer, by noting the optical densities at 520 mμ and the times at which the readings were made, over a period of 2 minutes. The thermostat for the Beckman compartment was set at 20°C. A blank containing the reagents without tissue samples was run under the same conditions, and the slight color formed by the autoxidation of the amine was deducted from the enzyme values. The activity of peroxidase was expressed as the increase in optical density per minute per milligram of nitrogen of tissue samples, or per milligram of the commercial peroxidase preparation, under the above conditions. These units were 2.5 times higher than the "QDI-units" defined and used previously (1, 14).

Possible interference by reducing or oxidizing agents, among the latter especially hemoglobin, has been discussed in detail in a previous paper (1). As was mentioned there, such interference has to be taken into account only when the concentration of peroxidase is very low or the concentration of the interfering substance very high. During most of the present experiments, the high concentrations of peroxidase necessitated high dilutions of the test solutions, so that interference was negligible. Interference by hemoglobin, which has a slight peroxidasic activity, was minimized by the perfusion of the kidneys. Kidney fractions from untreated animals showed very slight color development due to cytochrome oxidase. It has been shown (1, 14) that the color formation by cytochrome oxidase is very weak under the conditions applied for the peroxidase test, *i.e.* at pH 6.2, in the presence of high concentrations of buffer salts, and without the addition of cytochrome *c*. A correction for cytochrome oxidase had to be made only in the 90 hour experiment (section II(*c*)) when the concentration of peroxidase in the kidneys had decreased to very low levels. No appreciable color development due to an endogenous peroxidase (leucocytes) was observed in the kidney fractions of untreated animals.

d) Cytochemical Tests for Peroxidase

These tests were made with small pieces of the same kidneys that were used for the colorimetric analysis. The tissue was squashed and was incubated for 1 to 3 minutes in an ice-cooled solution of benzidine in 70 per cent ethyl alcohol as reported previously (2). As was mentioned in section I(*b*), smears of the isolated sediments were examined by the same procedure, and the size and number of the peroxidase-containing phagosomes were estimated.

e) Effects of Perfusion

The blood and urine contained high concentrations of peroxidase during the first few hours following

administration (section II(e)). The kidneys were perfused in order to remove this extracellular peroxidase. The possible effects of the perfusion on the fractionation of the kidney homogenate were tested by perfusing only one kidney and by clamping off the other kidney from the circulation with a hemostat. Each kidney was then homogenized and fractionated separately during the same runs of the centrifuge. It was found that the specific activities of peroxidase were practically the same in both kidneys in fractions MDrII and PDrIII, and also in fraction NDrI when correction had been made for adsorption (see section II(a)). However, the concentration of peroxidase in the homogenate and in the supernatant fluid was higher in the non-perfused than in the perfused kidney owing to contamination by peroxidase from blood and urine. The perfusion also caused an approximately 10 per cent decrease of the nitrogen content of the kidney. Whereas most blood cells were removed with the incompletely homogenized residue, the serum proteins remained in the supernatant fluids of the non-perfused kidneys. The removal of the serum proteins by perfusion caused a somewhat lower nitrogen content and higher activities for peroxidase per milligram nitrogen in the supernatant fluids.

II. RESULTS

a) *Uptake of Peroxidase by Isolated Fractions in Vitro*

Before the uptake of peroxidase *in vivo* by the phagosomes could be determined, it was necessary to ascertain whether appreciable amounts of peroxidase were adsorbed by the granules *in vitro*. Varying amounts of peroxidase (0.1 to 7.0 mg of the commercial preparation) were added to 10 ml of the same pooled kidney homogenate from untreated rats. The homogenates were submitted to the same fractionation procedure as in the *in vivo* experiments. As was mentioned, this included the washing of each sediment by resuspension and resedimentation.

Fig. 1 shows that appreciable amounts of peroxidase were taken up *in vitro* by fraction NDrI and by the incompletely homogenized residue and that the same amounts were taken up by both, when up to 1000 units of peroxidase per milligram nitrogen of the homogenate had been added. It should be noted that the concentration of peroxidase in the homogenates of the *in vivo* experiments generally did not exceed 600 units per milligram nitrogen of the homogenate (Fig. 4 e). The cytochemical tests with benzidine showed that the nuclear membranes were a main site of adsorption of peroxidase *in vitro*. Much less peroxidase was adsorbed by fractions MDrII and PDrIII than by the nuclear fraction and by the tissue fragments (Fig. 1). Within the range of concentrations shown in Fig. 1, the amounts of peroxidase which were adsorbed to the cell particles were practically proportional to the amounts of added peroxidase. With further increase of the added peroxidase, however, the curves seemed to reach saturation levels. The concentrations of peroxidase in the following *in vivo* experiments were corrected for the adsorption *in vitro* according to Fig. 1, by deducting 20 per cent from the peroxidase activities in fraction NDrI, and 3.5 per cent in the fractions MDr II and PDr III.

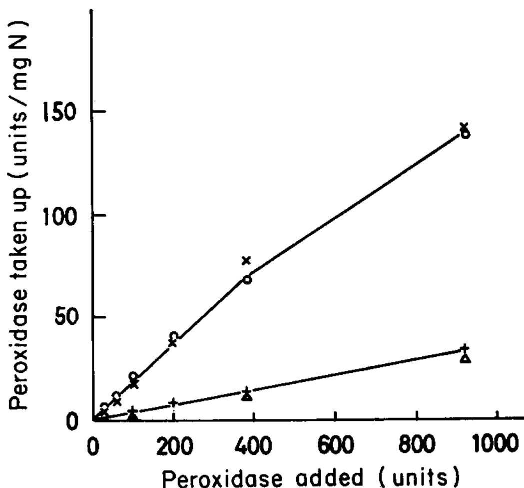


FIGURE 1

Uptake of peroxidase by isolated fractions and by tissue fragments of kidney *in vitro*. Fraction NDrI, ×; tissue fragments (incompletely homogenized residue), ○; fraction MDrII, +; fraction PDrIII, △.

b) *Concentration of Peroxidase in Fractions of Kidney Homogenate in Relation to Dose and Pretreatment with Egg White*

Doses of peroxidase varying from 1.7 to 23 mg per 100 gm of weight (commercial preparation; 5000 units per milligram) were injected intravenously. The kidneys were fractionated 1 hour later, and the specific activities of peroxidase in each fraction were determined. Figs. 2 and 3 show that the *in vivo* uptake of peroxidase increased rapidly with increasing dose until satura-

FIGURE 2

Concentration of peroxidase in isolated fractions of rat kidneys in relation to dose and pretreatment with egg white, 1 hour after administration. Fraction NDrI: from egg white-treated animals, □; from control animals, ■. Fraction MDrII: from egg white-treated animals, ○; from control animals, ●. Fraction PDrIII: from egg white-treated animals, △; from control animals, ▲.

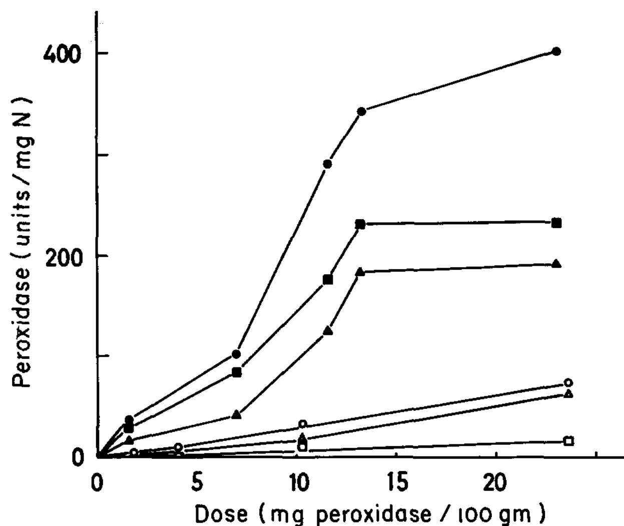
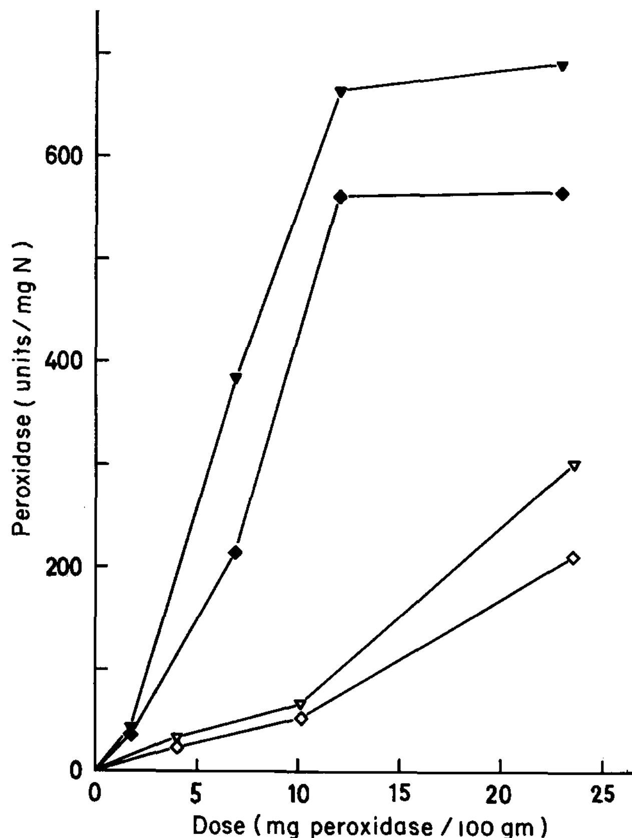


FIGURE 3

Concentration of peroxidase in homogenates and supernatant fluids of kidneys in relation to dose and pretreatment with egg white, 1 hour after administration. Homogenate: from egg white-treated animals, ◇; from control animals, ◆. Supernatant fluid: from egg white-treated animals, ▽; from control animals, ▼.



tion was reached at doses approaching 12 to 14 mg per 100 gm of weight. The changes in peroxidase concentration in relation to dose in the homogenates and supernatant fluids (Fig. 3) were similar to those in the particulate fractions (Fig. 2). Some fluctuations were observed in the concentration of peroxidase in the homogenates and supernatant fluids after incomplete perfusion.

This was caused by contamination with peroxidase from blood and urine (section I(*e*)).

When the animals had been treated with egg white 18 hours before the administration of peroxidase, the uptake of the foreign protein in all fractions was only 10 to 25 per cent of the uptake observed without egg white treatment (Figs. 2 and 3). After treatment with egg white, the uptake of peroxidase in the particulate fractions did not reach saturation levels with doses up to 23.0 mg per 100 gm of weight.

It should be noted that the data shown in Figs. 2 and 3 refer only to the interval of 1 hour after administration. At longer intervals, the uptake of peroxidase in fraction NDrI probably would be higher, and in fraction PDrIII lower, than that at the 1 hour interval (see following section).

c) Concentration of Peroxidase in Fractions of Kidney Homogenate at Different Intervals following Administration and Effects of Pretreatment with Egg White

A single dose of 12.0 mg of peroxidase per 100 gm of weight, which almost saturated the granules (section II(*b*)), was injected intravenously, and the kidneys (cortices) were fractionated at intervals varying from 10 minutes to 90 hours following administration. Figs. 4 *a* to 4 *e* show the changes in the specific activities of peroxidase in the four fractions and in the homogenate. The results will be discussed in section III.

It was preferred to express the uptake of peroxidase by the specific activities rather than by the intracellular distribution of peroxidase. It should be noted that the simple fractionation

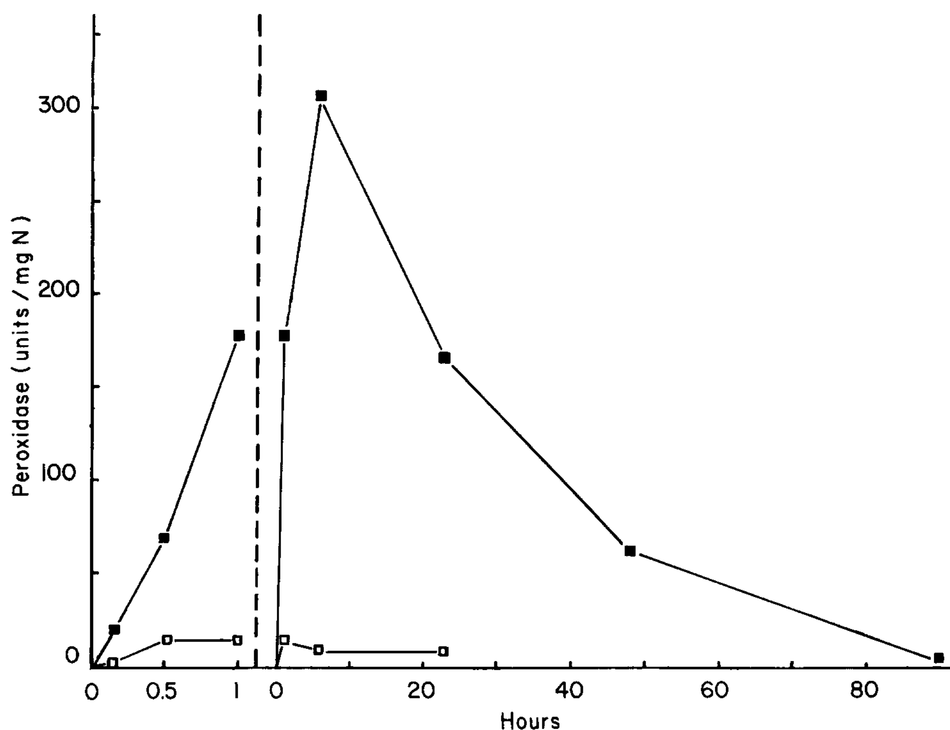


FIGURE 4 *a*

Concentration of peroxidase in fraction NDrI in relation to time after administration and pretreatment with egg white. Egg white-treated animals, □; control animals, ■.

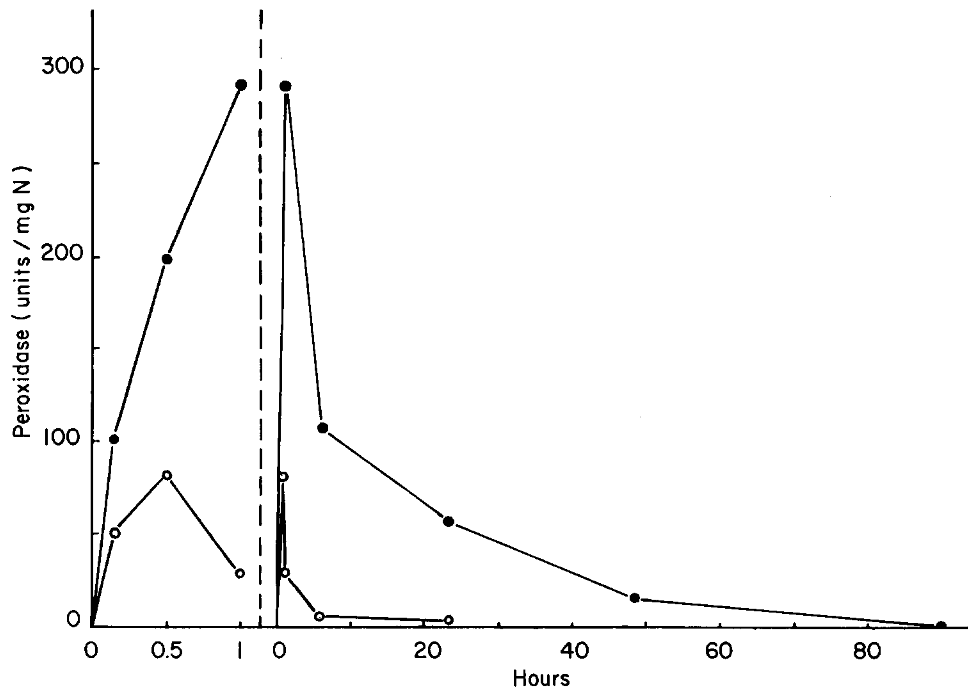


FIGURE 4 *b*

Concentration of peroxidase in fraction MDrII in relation to time after administration and pretreatment with egg white. Egg white–treated animals, ○; control animals, ●.

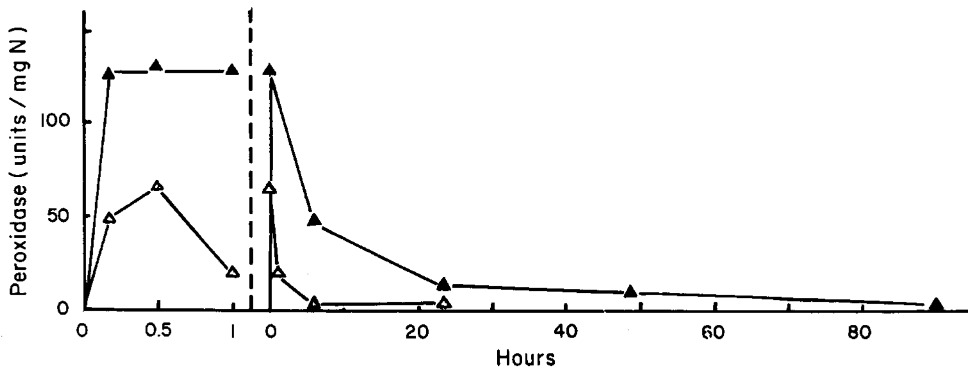


FIGURE 4 *c*

Concentration of peroxidase in fraction PDrIII in relation to time after administration and pretreatment with egg white. Egg white–treated animals, △; control animals, ▲.

procedure applied did not permit a good separation of the large phagosomes in fraction NDrI. A portion of the large phagosomes was removed with the incompletely homogenized residue, and the intracellular distribution showed too low a value for peroxidase in fraction NDrI. During the 90 hour interval, the peroxidase content of fraction NDrI varied between 2 and 15 per cent, of fraction MDrII between 8 and 15 per cent, of fraction PDrIII between 2 and 15 per cent, and of the supernatant fluid between 70 and 75 per cent of the homogenate. The intracellular distribution of

peroxidase was similar in the egg white–treated animals and in those not treated, except that the granular fractions of the former contained less, and the supernatant fluid more, peroxidase (79 to 88 per cent) than those of the latter. The reasons for the high peroxidase content of the supernatant fluids were indicated in section I(*b*), and corrected values will be mentioned in section III.

standard dose of 12.0 mg (60,000 units) per 100 gm of weight: 10 minutes after administration of peroxidase, the cortices contained 4.6 per cent of the injected dose, 30 minutes 7.8 per cent, 60 minutes 14.2 per cent, 3 hours 10.7 per cent, 6 hours 8.1 per cent, 23 hours, 4.0 per cent, 48 hours 1.3 per cent, and 90 hours 0.1 per cent. In egg white–treated animals the following values were found: 10 minutes after the administration of 12.0 mg of peroxidase per 100 gm of weight, the cortices contained 4.2 per cent of the injected dose, 30 minutes 5.4 per cent, 60 minutes 2.2 per cent, 6 hours 0.7 per cent, and 23 hours 0.2 per cent.

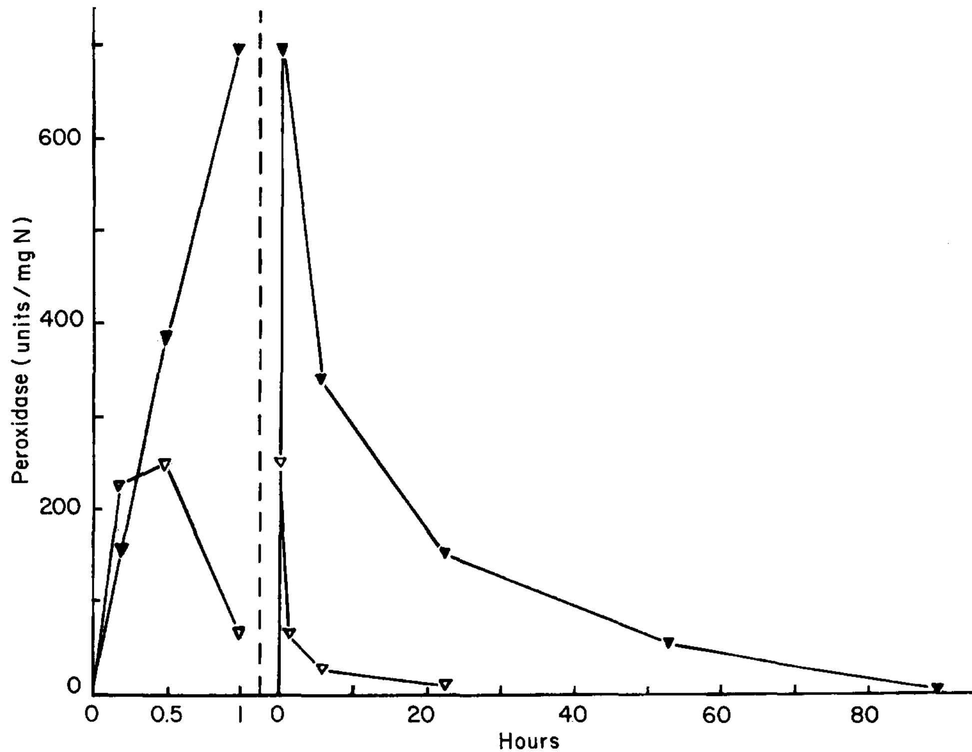


FIGURE 4 *d*

Concentration of peroxidase in supernatant fluid in relation to time after administration and pretreatment with egg white. Egg white–treated animals, ▽; control animals, ▼.

d) Peroxidase Content of the Total Kidney

The changes in the peroxidase content of the total kidneys (homogenates plus incompletely homogenized residues) at different intervals following administration were similar to those of the homogenates (total kidneys minus incompletely homogenized residues) shown in Fig. 4 *e*. The following values were found for the peroxidase content of the total kidneys at different intervals after administration, expressed as percentages of peroxidase taken up in reference to the injected

e) Concentration of Peroxidase in Blood Serum and Urine and Effects of Pretreatment with Egg White

A single dose of 10 mg of peroxidase per 100 gm of weight was injected. The commercial preparation of peroxidase used in these experi-

ments had a lower activity (approximately 3,000 units per milligram) than that used for the other experiments (5,000 units per milligram), and the injected dose was 20 per cent lower. However, the slopes of the curves shown in Figs. 5 and 6 were not much affected by this difference. Although no regular clearance tests for peroxidase could be made with rats, the analysis of the blood and urine samples at various intervals following administration was sufficient to establish the general character of the curves. They showed that the concentration of peroxidase in blood serum and urine decreased exponentially and that the plant enzyme was cleared from the blood during the first 5 to 6 hours (Figs. 5 and 6). This confirmed the data reported previously (1). After the exponential phase, small amounts of peroxidase were excreted in the urine up to several days (Fig. 6), as long as peroxidase activity was measurable in the kidney homogenates and as long as peroxidase-containing phagosomes could be detected cytochemically in the cortex.

Fig. 5 shows that the concentration of peroxidase in blood serum, 1 hour after the administration of 10 mg per 100 gm, was considerably higher in the egg white–treated than in the control animals. The delay in the disappearance of peroxidase from the blood in the egg white–treated animals was also apparent when the concentration of peroxidase in the serum was plotted against the dose administered (curves not shown here).

In experiments summarized in Table I, the urine excreted over a period of 24 hours was collected in metabolic cages, and the peroxidase content was compared for egg white–treated rats and for those not treated. In the treated rats, almost twice as much peroxidase was excreted during the first 7 hours (exponential phase) as in the untreated rats (Table I).

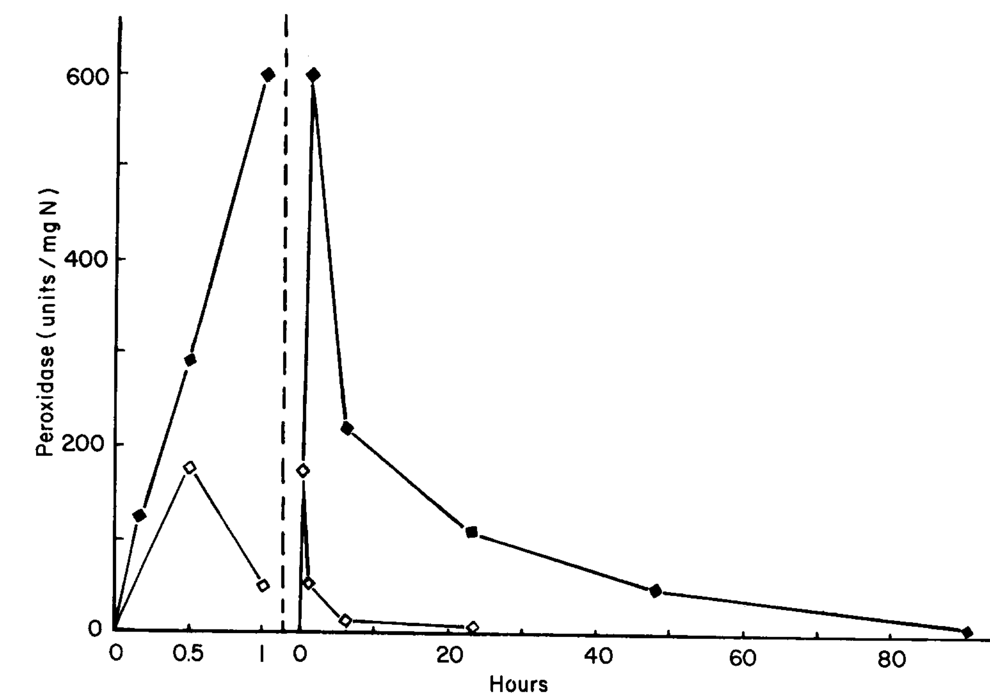


FIGURE 4 *e*

Concentration of peroxidase in homogenate in relation to time after administration and pretreatment with egg white. Egg white–treated animals, ◇; control animals, ◆.

f) Uptake of Peroxidase by Kidney and Liver after Administration of Scaled Doses and Effects of Pretreatment with Egg White

In these experiments, the dose of the injected peroxidase was increased from very low to higher

levels, and the activity of peroxidase was determined in kidney and liver homogenates, blood serum, and urine, 1 hour following administration. Cytochemical tests for peroxidase were performed on the same kidneys to determine whether phagosomes containing peroxidase were present in the tubule cells.

Table II shows that there was a sudden increase in the peroxidase content of the kidneys when phagosomes first appeared in the tubule cells. This occurred at the same low levels as those at which the liver cells were just saturated with peroxidase. It may also be seen from Table II that peroxidase appeared in the urine after the administration of very low doses, and before phagosomes containing peroxidase could be detected in the tubule cells. The concentration of peroxidase in the urine of some animals, as shown in Table II, may appear excessive. It should be noted that the figures refer to the amount of peroxidase per milliliter, whereas only a few drops of urine were collected from these animals. It was often observed that the first few drops of urine given by rats after the administration of peroxidase had the dark brown color of peroxidase and contained very high concentrations of the enzyme. Since it was difficult to obtain urine from rats during the first 30 minutes following treatment, the medullae of some animals were dissected from the cortex and assayed separately. It was found that 3 minutes after the administration of 0.2 mg of peroxidase per 100 gm, the concentration of peroxidase in the medulla was 3 times higher than in the cortex.

Since the concentration of peroxidase in isolated fractions and homogenates of the liver had been determined by colorimetry (1, 2), and the presence of phagosomes in smears of isolated liver fractions had been confirmed cytochemically (2), it was of interest to test the effect of egg white on the phagosomes of the liver. The results, summarized in Table III, show that the uptake of peroxidase by the liver, in contradistinction to the kidney, was not depressed by a preceding administration of egg white.

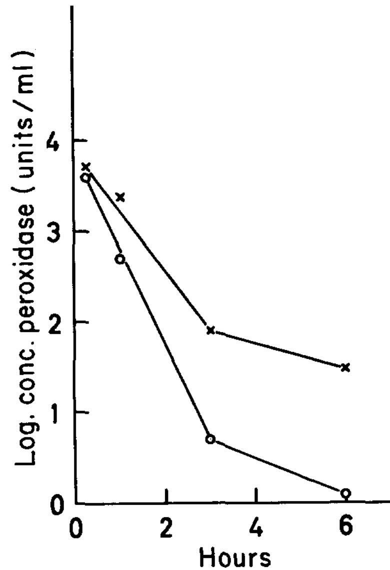


FIGURE 5

Concentration of peroxidase in blood serum in relation to time after administration of 10 mg (30,000 units) per 100 gm of weight, and pretreatment with egg white. Egg white-treated animals, ×; control animals, ○.

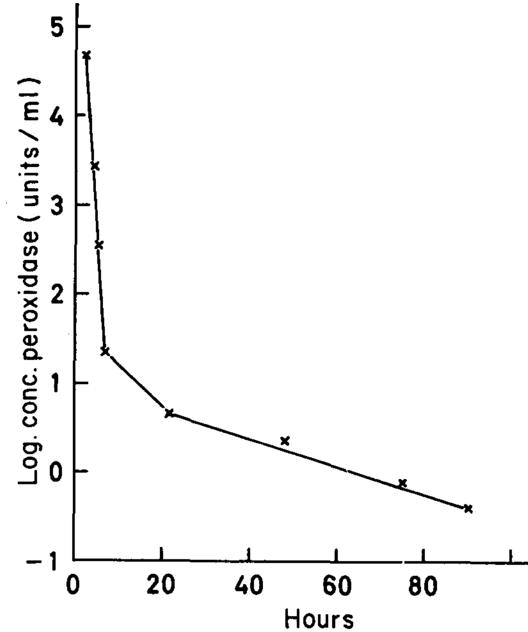


FIGURE 6

Concentration of peroxidase in urine at varying periods following administration of 10 mg (30,000 units) per 100 gm of weight.

g) Degradation of Peroxidase in Kidney Slices and Homogenates in Vitro

The slow disappearance of peroxidase from the kidney cortex over a period of several days raised the question whether peroxidase was degraded, or was secreted into the urine. It was of interest,

therefore, to test the ability of kidney tissue to degrade peroxidase *in vitro*, especially since catheptic enzymes are concentrated in the phagosomes (4, 7).

Kidney slices and kidney homogenates from peroxidase-treated animals were incubated at 37°C for several days at pH 6.5, pH 5.0, and pH 4.0, and the activities of peroxidase in the homogenates and slices (after homogenization) were determined colorimetrically. The incubation of the tissue slices took place in 0.2 ml of a saline-buffer mixture containing traces of toluene to prevent bacterial contamination. The incubation at pH 4.0 was discontinued when it was observed that peroxidase itself was not stable at this pH, although it was stable over a period of several days at pH 5.0 and pH 6.5.

It was found that no decrease in the activity of peroxidase occurred in the tissue slices and homogenates at pH 6.5, and that at pH 5.0 the decrease in activity over a period of 48 hours was slight (Fig. 7). At this time, the tissue still contained an appreciable amount of catheptic activity as tested separately according to Anson's method with hemoglobin as substrate.

TABLE I

Amounts of Peroxidase Excreted in Urine of Egg White–Treated and Control Rats after Administration of a Constant Dose (20 Milligrams = 60,000 Units per 200 Grams of Weight)

	Peroxidase excreted (units)			
	Egg white-treated		Controls	
	0–7 hours	7–24 hours	0–7 hours	7–24 hours
Exp. I	26,200	560	12,500	660
Exp. II	25,600	820	16,100	112
Exp. III	27,600	160	15,800	1170
Exp. IV	26,500	800	13,600	930
Exp. V	24,800	750	14,000	—
Average	26,140	616	14,400	718
Per cent of injected amount	43.5	1.0	24.0	1.2

TABLE II

Comparison of Concentration of Peroxidase in Kidney, Liver, Blood Serum, and Urine, 1 Hour after Administration of Low Doses, with the Cytochemical Appearance of Phagosomes in Tubule Cells

Dose injected (mg/100 gm)	Specific activity of peroxidase: Kidney (units/mg N)	Liver (units/mg N)	Serum (units/ml)	Urine (units/ml)	Phagosomes in tubule cells
0.22	0.25	7.1	0.1	425	−
0.52	0.95	14.0	1.1	1,674	−
0.95	0.97	18.8	0.9	5.400	−
0.89	4.7	14.9	2.9	2,000	+
1.43	12.2	23.5	6.7	45,500	+
2.1	32.3	20.1	53.3	*	+
4.2	80.6	17.8	360.6	*	+
12.0	562.8	16.7	2,160	*	+

* Not tested.

III. DISCUSSION

The colorimetric analysis of exogenous peroxidase in homogenates and in isolated fractions can only give values on the whole kidneys. It cannot clarify

such questions as to what types of kidney cells (proximal, distal, collective tubules, or glomerular cells) participate in the uptake of peroxidase, and whether the size, number, and peroxidase content of the phagosomes and of the other peroxidase-containing structures (canaliculi, membranes, and strands (9–11)) differ in the same or in different types of kidney cells in relation to dose and to time following administration. A cytochemical investigation on these questions using frozen sections is in progress, and the results will be reported separately (11).

TABLE III

Comparison of Uptake of Peroxidase by Kidney and Liver of Egg White-Treated and Control Rats at Different Intervals Following Administration of 12 Milligrams per 100 Grams of Weight

Hours following injection	Specific activity of peroxidase in homogenates (units/mg N)			
	Kidney		Liver	
	Egg white-treated	Control	Egg white-treated	Control
0.5	180.3	283.7	28.7	21.3
1	52.5	650.4	27.8	16.4
3	23.9	263.1	37.3	42.2
6	17.1	259.8	67.2	63.5
23	7.3	117.2	20.2	3.2
48	2.4	36.3	1.9	0.7

As may be seen from Figs. 4 *a* to 4 *e*, the concentration of peroxidase at various periods following injection is quite different for the fractions containing the small phagosomes and for those containing the large ones. The "microsomal" fraction PDrIII, containing phagosomes of 0.2 μ or smaller (Fig. 4 *c*), was saturated with peroxidase soon after administration, and its peroxidase content decreased quickly after 1 hour. In fraction NDrI (Fig. 4 *a*), containing large phagosomes with diameters of 1 to 5 μ, on the other hand, the concentration of peroxidase increased from low to very high levels during the first few hours, and it decreased only slowly afterward over a period of several days. The phagosomes in fraction MDrII (Fig. 4 *b*), with diameters of 0.2 to 2 μ, showed an intermediate behavior. These different properties are compatible with the hypothesis that the small phagosomes take part in the formation of the large ones. The same conclusion was reached from previous fractionation experiments showing that the number of the large "droplets" increased and that the number of the small ones decreased after treatment with egg white (6). An increase in the size of the granules after treatment with peroxidase has also been found in the case of the liver, using basic fuchsin or benzidine for the

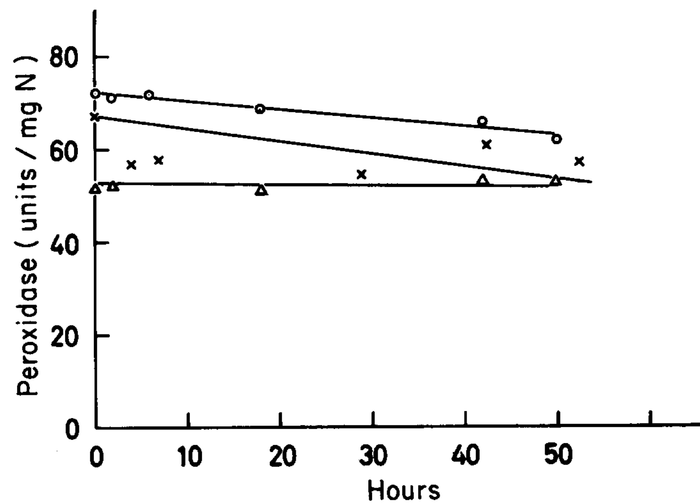


FIGURE 7

Degradation of horseradish peroxidase by tissue slices and homogenates of kidney *in vitro*, at 37°C and pH 5.0. Tissue slices, ×; homogenate, ○; peroxidase alone in buffer solution, pH 5.0, △.

microscopic identification of the phagosomes (2). A detailed analysis of the changes taking place in liver fractions after administration of peroxidase will be reported by Jacques *et al.* (15*b*).

It was concluded from several cytochemical observations that the *small* phagosomes serve in the transport or exchange of materials between the blood and special regions of epithelial cells (2, 10, 11). This hypothesis finds support in the observation of a simultaneous decrease of peroxidase in the small phagosomes of fraction PDrIII (Fig. 4 *c*) and in blood serum (Fig. 5) during the first hours following administration. Reference may be made in this connection to the electron microscopic investigations of Farquhar and Palade (16) and Miller (17). These investigators showed that in the glomerular and tubular cells of the kidney, foreign proteins (ferritin and hemoglobin) are transported by small pinocytotic vesicles into large vacuoles which gradually transform into dense bodies. It is suggested that many of the phagosomes in our "microsomal" fractions PDrIII are related to the micropinocytotic vesicles or to intermediate forms between vesicles and dense bodies observed in kidney cells by Farquhar and Palade and by Miller. In recent cytochemical experiments (9–11), very small peroxidase-positive granules were seen in the tubule cells in the region of the brush border, and along fine parallel membranes extending from the base of the cells to the lumen; similar small granules were observed in the glomerulus along capillary walls. These are probably the same small granules which appeared in smears of the isolated "microsomal" fractions PDrIII, after incubation with benzidine, as tiny blue points when the microscope was focused up and down (section I(*d*), and (2)). It should be noted that some of these granules may have submicroscopic size but may become visible in the microscope by the formation of the blue pigment after incubation with benzidine.

It was suggested (2, 10, 11) that the development of the *large* phagosomes represents a protective reaction of the cells against the invasion of foreign materials. This reaction consists in the segregation of the foreign material from the rest of the cytoplasm by enclosing it within membranes together with hydrolytic enzymes, and by the subsequent extrusion of a part of the inclusion bodies. The protective segregation of foreign materials might be essential for the survival of the cells, since the intracellular digestion of native, foreign proteins, for example horseradish peroxidase, if often quite slow. The uptake and storage of foreign matter by epithelial and reticuloendothelial cells has been known to histologists for many years. Möllendorff (18), Chlopin (19), and Parat (20) demonstrated the segregation of neutral red, trypan blue, and other dyes in granules or vacuoles of epithelial cells; Kedrowski (21) investigated the concentration of colloidal matter in granules of macrophages; Gérard and Cordier (22) and Lambert (23) described "athrocytosis" (*Speicherung*) of colloids or proteins in granules of kidney cells. The "phagosomes" in which peroxidase was found to be concentrated in various organs of absorption, secretion, and excretion (2) are probably identical with the granules in which other foreign materials have been shown, by the above-mentioned and many other investigators, to be segregated in epithelial and reticuloendothelial cells. The relationship of these cell structures to the acid phosphatase–containing α and β granules of egg cells, investigated by Pasteels (24), Dalcq (25), and Mulnard (26), and their relationship to the metachromatic vacuoles of ameba, investigated by Quertier and Brachet (27), should also be mentioned. According to several electron microscopic observations, the uptake of fluid material (pinocytosis) and solid material (phagocytosis) occurs by similar submicroscopic processes in which the cell membranes play an important role. The recent work of Brandt and Pappas (28) may be mentioned, showing that the uptake of macromolecular ferritin and of colloidal ThO_2 occurs in ameba by the same mechanism, *i.e.* by attachment to fine hair-like extensions of the plasmalemma. Although particle size is not critical for the uptake by the cell membranes, the particle size may determine whether the foreign materials can pass through the basement membranes of the blood capillaries before they are taken up by the epithelial cells (16).

The hypothesis, stated above, that the development of the large phagosomes is related to the protective segregation of foreign substances, seems to be contradicted by the large proportion of "free" peroxidase found in the supernatant fluids of the kidney homogenates (section II(*c*)). With a 10 per cent correction for the peroxidase released during the fractionation procedure, and a 10 to 15 per cent correction for the "free" peroxidase

released from the incompletely homogenized residue (section I(*b*)), it can be estimated that the supernatant fluids contained 40 to 60 per cent of the peroxidase of the homogenates. The values were higher after the administration of high doses than after low doses of peroxidase. Recent cytochemical observations have shown that a relatively large portion of the peroxidase in kidney cells is localized in "canaliculi" (intercellular spaces), membranes, and cytoplasmic strands adjacent to the canaliculi (9–11). It is suggested that a portion of this peroxidase is released during the experimental procedures and then appears in the supernatant fluids. Such an interpretation is preferred to the previous conclusion (5) that kidney cells take up foreign proteins in a soluble, diffusible form (except in the canaliculi and strands mentioned) before concentrating it in "droplets." A further possibility also should be considered. Peroxidase may be released *in vivo* from the phagosomes *after* they have been formed, owing to changes in the permeability of their membranes. Such a possibility was discussed for other cases of pinocytosis by Holter (29). Support for the existence of such a process may be seen in the release of hydrolytic enzymes from the phagosomes and their increase in the supernatant fluid after treatment with egg white as suggested previously (6), and in the perhaps analogous release of the same hydrolytic enzymes from the granules of leucocytes during phagocytosis of bacteria (30). However, in the case of the segregation of peroxidase in kidney cells, it is difficult to decide whether the release of peroxidase from the phagosomes *in vivo* is a normal process or whether it occurs only when the cells have been damaged by an excessive load of the foreign protein. Cytochemical observations, to be reported in more detail later, show that, in the latter case, cell fragments, nuclei, and whole cells containing large amounts of diffused peroxidase are being extruded or desquamated into the lumen (10). The presence of diffused proteins in damaged cells and in the nuclei of these cells has also been reported by Holtzer and Holtzer (31) using fluorescent groups for the marking of the proteins.

It is known that 18 hours after the intraperitoneal injection of a massive dose of egg white, the kidney cells are filled with many large "droplets" containing egg white (5, 32). The present experiments show that these cells are no longer able to take up much peroxidase, *i.e.* not more than 10 to 25 per cent of the amount taken up by the controls (Figs. 2 to 4). The exact nature of this "competition" has to be analyzed further. Since normal kidney cells always contain a certain number of "droplets" (4, 7) in which proteins may be segregated under physiological conditions, a less pronounced competitive effect may also exist in normal animals.[2] In pathological cases, the depressed reabsorption may cause a pronounced proteinuria. In the present experiments, a marked proteinuria also occurred. The strong depression of the uptake of peroxidase by the kidney cells, and perhaps by other organs, after treatment with egg white, has delayed the disappearance of peroxidase from the blood (Fig. 5). This increased concentration of peroxidase in the blood, in its turn, has increased the excretion of peroxidase into the urine (Table I), although an increased diuresis may also have contributed.

It is interesting that egg white did not compete with the uptake of peroxidase by the liver (Table III). Cytochemical tests have shown that peroxidase is mainly localized in the reticuloendothelial cells of the liver and, to a lesser extent, in the epithelial cells close to the cell membranes (11). Perhaps the predominant localization of the phagosomes in the reticuloendothelial cells in the case of the liver, and in the epithelial cells in the case of the kidney, is related to the different effects of egg white on the uptake of peroxidase by the two organs. The much higher specific activities of peroxidase in the kidney than in the liver (Table III) also seem to reflect the localization of peroxidase in different types of cells in the two organs.

The data shown in Table II indicate that peroxidase is not reabsorbed by the convoluted tubule cells of the kidney before the reticuloendothelial cells of the liver have been saturated. The predominant uptake of low doses of peroxidase by the liver was first observed by Jacques (15). It is probable that the differential uptake of peroxidase by the kidney and liver is related to the level of peroxidase in the blood serum. It should also be noted that the excretion of very low doses of peroxidase in the urine (Table II) before phagosomes appear in the tubule cells disproves the opinion that a reabsorption mechanism has to

[2] It should also be noted that the commercial preparation of peroxidase may contain inactive proteins which may compete with the uptake of the active peroxidase itself.

be saturated before the protein is excreted into the urine.

As reported in section II(*c*), the disappearance of peroxidase from the kidney *in vivo* is a relatively slow process extending over several days. The period of "storage" depends, of course, on the dose injected. Little peroxidase was degraded by kidney slices *in vitro* (Fig. 7). It can be estimated from Figs. 4 and 6 that the low amounts of peroxidase excreted in the urine during several days, after its complete clearance from the blood, represent only a small fraction of the peroxidase remaining in the tubule cells. This was also verified by cytochemical tests to be reported later. It may be concluded, therefore, that most of the peroxidase is degraded slowly in the kidney cells.[3]

The uptake of peroxidase *in vitro* by the nuclear fraction and by the cell fragments, and the linear character of the adsorption curves (Fig. 1), raise the question whether the uptake of peroxidase *in vitro* and that *in vivo* are related processes. The cytochemical tests with benzidine have shown that peroxidase, added *in vitro* to the homogenate, is mainly taken up by the nuclear membranes and by the basement membranes. The observation that the nuclear fraction took up the same amount *in vitro* as the cell fragments (incompletely homogenized residue), and the mitochondrial fraction the same amount as the microsomal fraction (Fig. 1), points to relatively specific sites of adsorption. On the other hand, the injected peroxidase is also adsorbed by the membranes of the kidney and liver *in vivo*, and the phagosomes later develop in relation to the same membranes (10, 11). Thus, the uptake of peroxidase *in vitro* and *in vivo* may be related processes, especially in the initial phase of the adsorption by the cell membranes. The experiments also point to the importance of testing in each case whether a foreign protein has been taken up by cell structures *in vivo*, or whether the uptake occurred *in vitro* after the tissue had been fixed or homogenized. During recent cytochemical experiments, certain cases were observed in which peroxidase penetrated into the nuclei (10, 11). This had not been found in earlier investigations (2). As was mentioned above, diffuse nuclear staining may be a sign of damage.

For technical reasons, it was not possible to correlate the present analysis of peroxidase uptake with enzyme studies, as was planned and as had been done in previous experiments with egg white (6). Some important investigations reported in the recent literature throw new light on the enzymes involved. Cohn and Hirsch (30, 33, 34) found that the granules isolated from leucocytes contain the lysosomal enzymes (8). This shows the close relationship between lysosomes and phagosomes which our previous experiments (3–7) had led us to expect.

[3] In the above considerations, the enzymatic activities were taken as a criterion for the amounts of peroxidase present in the tissue samples. It is possible that peroxidase was only inactivated without being much degraded. It may be assumed, however, that the observed disappearance of peroxidase (Figs. 4 *a* to 4 *e*) was due mainly to its degradation.

This work was done during the tenure of an Established Investigatorship of the American Heart Association.

The author would like to express his appreciation to Dr. C. de Duve of the University of Louvain, and to Dr. J. Brachet of the University of Brussels, for their interest and for the facilities which they have accorded him in their laboratories.

Received for publication, May 6, 1961.

BIBLIOGRAPHY

1. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 541.
2. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 193.
3. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 1037.
4. Straus, W., *J. Biol. Chem.*, 1954, **207**, 745.
5. Straus, W., and Oliver, J., *J. Exp. Med.*, 1955, **102**, 1.
6. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 933.
7. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 513.
8. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, 1955, **60**, 604.
9. Straus, W., *Exp. Cell Research*, 1960, **20**, 600.
10. Straus, W., *Exp. Cell Research*, 1961, **22**, 282.
11. Straus, W., *Exp. Cell Research*, in press.
12. Novikoff, A. B., *in* Biology of Pyelonephritis, (E. L. Quinn and E. H. Kass, editors), Boston, Little, Brown and Co., 1960, 113–144.

13. Novikoff, A. B., *in* The Cell, (J. Brachet and A. E. Mirsky, editors), New York and London, Academic Press, 1961, **2,** Chapter 6.
14. Straus, W., *Biochim. et Biophysica Acta*, 1956, **19,** 58.
15. (*a*) Jacques, P., *Arch. internat. physiol. biochim.*, 1960, **68,** 683; (*b*) Jacques, P. *et al.*, in preparation.
16. Farquhar, M. G., and Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 297.
17. Miller, F., *J. Biophysic. and Biochem. Cytol.*, 1960, **8,** 689.
18. Möllendorff, W. von, *Anat. Hefte*, 1915, **53,** 87; *Ergebn. Physiol.*, 1920, **18,** 141; *Ergebn. Anat. u. Entwcklngsgesch.*, 1923, **24,** 278; *Z. Zellforsch. u. mikr. Anat.*, 1925, **2,** 127.
19. Chlopin, N. R., *Arch. exp. Zellforsch.*, 1927, **4,** 462.
20. Parat, M., *Arch. anat. micr.*, 1928, **24,** 73.
21. Kedrowski, B., *Z. Zellforsch. u. mikr. Anat.*, 1933, **17,** 547, 587.
22. Gérard, P., and Cordier, R., *Biol. Revs.*, 1934, **9,** 110.
23. Lambert, P. P., *Arch. biol.*, 1936, **47,** 125.
24. Pasteels, J. J., *Arch. biol.*, 1959, **69,** 591.
25. Dalcq, A., *Arch. biol.*, 1959, **71,** 93.
26. Mulnard, J., *Arch. biol.*, 1959, **69,** 645.
27. Quertier, J., and Brachet, J., *Arch. biol.*, 1959, **70,** 153.
28. Brandt, P. W., and Pappas, G. D., *J. Biophysic. and Biochem. Cytol.*, 1960, **8,** 675.
29. Holter, H., *Internat. Rev. Cytol.*, 1959, **8,** 481.
30. Cohn, Z. A., and Hirsch, J. G., *J. Exp. Med.*, 1960, **112,** 1015.
31. Holtzer, H., and Holtzer, S., *Compt.-rend. trav. Lab. Carlsberg*, 1960, **31,** 373.
32. Oliver, J., MacDowell, M., and Lee, Y. C., *J. Exp. Med.*, 1954, **99,** 589.
33. Cohn, Z. A., and Hirsch, J. G., *J. Exp. Med.*, 1960, **112,** 983.
34. Hirsch, J. G., and Cohn, Z. A., *J. Exp. Med.*, 1960, **112,** 1005.