COMPARATIVE ANALYSIS OF THE CONCENTRATION OF INJECTED HORSERADISH PEROXIDASE IN CYTOPLASMIC GRANULES OF THE KIDNEY CORTEX, IN THE BLOOD, URINE, AND LIVER

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ABSTRACT

The concentration of horseradish peroxidase in total particulate fractions from the kidney cortex did not change much during the first few hours after injection, as long as most of the injected protein was not yet cleared from the blood. It decreased at a rate of 6–8% per hr afterwards. The concentration of peroxidase in total particulate fractions increased in proportion to the load (dose) over a wide range, suggesting that a constant fraction of the protein was reabsorbed by micropinocytic vesicles into the tubule cells from the glomerular filtrate. The amount of peroxidase excreted in the urine also increased in proportion to the injected dose. The proportion of peroxidase taken up by the liver, however, decreased several times when the dose was increased. A marked decrease of protein uptake into the kidney cortex and an increase of urinary excretion were observed when rats received a second, equal dose of peroxidase 4 hr after the first injection, and the rate of clearance of peroxidase from the blood was decreased after the second injection. The liver, on the other hand, took up almost twice as much peroxidase after two injections as after one. The uptake of peroxidase by the kidney cortex increased with age. Cytochemical observations on the preferential absorption of peroxidase by certain cell types and segments of the renal tubules in relation to dose are reported.

INTRODUCTION

The process by which horseradish peroxidase is reabsorbed by the proximal tubule cells of the kidney has been the subject of several investigations by light microscopy (17, 18, 20) and by electron microscopy (3). These studies have shown that a portion of the injected protein is reabsorbed by the tubule cells from the glomerular filtrate and is concentrated in apical phagosomes. The latter fuse with preexisting lysosomes and the protein is broken down there.

Quantitative biochemical analysis of exogenous peroxidase in tissue homogenates or fractions, in the blood and urine may help to clarify certain questions which cannot be resolved by cytochemical observations alone. Such questions, which are the subject of the present study, concern: (a) the relationship between the load and the amounts of the marker protein reabsorbed into the tubule cells and excreted in the urine, and changes of this relationship under certain experimental conditions; (b) the possible saturation of phagosomes and phagolysosomes with injected peroxidase or with other “competing” macromolecules and its effect on the renal excretion of the protein; (c) the influence of the reticuloendothelial system on these
functions; and (d) the rate of digestion of the marker protein within the phagolysosomes.

Previous biochemical work (16), in which the concentration of injected peroxidase was measured in fractions isolated from the kidney cortex in relation to dose and to time after injection, provided only partial answers to these questions so that an extension of the work was considered useful. In the present study, the concentration of injected peroxidase was compared under certain experimental conditions in the renal cortex, the medulla, the urine, and the liver 3–4 hr after injection, when most of the peroxidase had reached the lysosomes and the concentration of the protein in the blood and urine had decreased to low levels. These data were correlated with certain cytochemical observations. A related study on the early stages of the absorption of horseradish peroxidase will be reported later.1

MATERIALS AND METHODS

Animals and Reagents

Male rats of the Sprague-Dawley strain were used. The rats were supplied with Purina chow (Ralston Purina Company, Inc., St. Louis, Mo.) and water ad lib. For most experiments, animals weighing 220–240 g each were selected.

Horseradish peroxidase, type II, from Sigma Chemical Co., St. Louis, Mo., was used (estimated purity 30–40%; activity 4000–5000 units per mg). In some experiments, type VI from Sigma Chemical Co. (approximate activity 10,000 units per mg) was used. Lots of commercial preparations which were not completely soluble in 0.9% NaCl solution were clarified by high speed centrifugation. Because peroxidase uptake and excretion varied with different commercial preparations, and even with different batches of the same type, the same lot of type II from Sigma Chemical Co. was used for the same series of experiments. The plant enzyme, dissolved in 0.3 ml of physiological saline, was injected into the femoral vein and the animal was sacrificed a few hours later. For each experiment, the peroxidase solution was prepared in excess of 0.1–0.2 ml, and the enzymatic activity of a sample (0.025–0.1 ml) was determined.

Collection of Urine and Blood

The rats were kept in metabolism cages and the urine was collected during 4 hr in most cases. In a few experiments, in which very low doses of peroxidase were given, the experimental period was decreased to 1–2 hr. In this case, separate rats were used to estimate peroxidase contents in the tissues 1–2 hr, and in the urine 3–4 hr, after injection.

Early in the work, several experiments had to be abandoned because less than 1 ml of urine could be obtained from the rats during a period of 4 hr. A slight water diuresis was then induced by giving 1.5 ml of tap water per 100 g of body weight by stomach tube under light ether anesthesia immediately after injection of peroxidase. In this way, 1.5–3 ml of urine could be collected during a 4 hr period from most animals. Before sacrifice, it was often possible to induce the rats to empty their bladders once more into a beaker. At the end of the experimental period, the volume of collected urine was recorded. The urine, supplemented by small amounts rinsed with water from the metabolism cage, was filtered quantitatively through folded filter paper into a graduated cylinder, and the filter paper was washed extensively with distilled water. The final volume (usually between 100 and 150 ml) was recorded and the peroxidase activity of an appropriately diluted aliquot was determined by the colorimetric method mentioned below.

In experiments in which the disappearance of peroxidase from the blood was followed at regular intervals after injection, small amounts of blood were obtained from the tip of the tail. After centrifugation in capillary tubes (made from polyethylene tubing), the plasma was separated and the peroxidase content in the plasma was determined after appropriate dilution. In other experiments blood was taken from the heart only at the end of the experimental period (after 3–4 hr) and the peroxidase activity in the blood serum was determined. As was to be expected from previous observations (16), it was below 1% of the original value.

Preparation of Tissue Homogenates and Total Particulate Fraction

After sacrifice, one kidney was cut in half longitudinally and the cortex was separated from the medulla. The cortex and a sample of the liver (1.2–1.5 g wet weight) were homogenized with distilled water in glass homogenizers with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.), transferred quantitatively to 100-ml volumetric flasks, and distilled water was added up to the mark. In some experiments, the reddish-brown “outer medulla” was separated from the white-appearing inner medulla, the two layers were homogenized separately in small volumes of distilled water in microhomogenizers (Kontes Glass Co., Vineland, N. J.), and the suspensions were transferred quantitatively to 5-ml graduated cylinders.

1 W. Straus. Manuscript in preparation.
Total particulate fractions from the renal cortex were prepared by homogenizing the cortex in 7.5% sucrose solution. The homogenate was centrifuged for 5 min at 50 g in an International refrigerated centrifuge (International Equipment Company, Needham Heights, Mass.), and the sediment was resuspended and centrifuged once more in the same way. The combined supernatant fluids were centrifuged for 1 hr at 30,000 g by means of the high speed attachment of the International centrifuge. The sediment was resuspended in distilled water, transferred quantitatively to a graduated cylinder (with ground glass stopper), and the volume was recorded. This suspension is referred to as the "total particulate fraction"). It should be noted that the sediments were not washed and were contaminated, therefore, with small amounts of supernatant fluid.²

Biochemical Tests

The protein content of the homogenates and fractions was determined according to Lowry et al. (7). The peroxidase activities of homogenates, particulate fractions, blood serum, and urine were determined by measuring with the Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) the rate of formation of red pigment from N,N-dimethyl-p-phenylenediamine-monohydrochloride (Matheson, Coleman, and Bell, Cincinnati, Ohio) after the addition of H₂O₂ (16). After subtraction of the reagent blank, peroxidase activity was expressed as the increase of absorbance at 520 mμ/min per milligram protein under the conditions of the test. The peroxidase content of the cortices from both kidneys was estimated by doubling the value found for one kidney. The peroxidase content of the liver was calculated from the peroxidase content of the measured sample (wet weight) and the wet weight of the whole liver. The specific activities of acid phosphatase, deoxyribonuclease, and cathepsin in the homogenates were determined according to the procedures mentioned previously (13).

Cytochemical Tests

For the cytochemical tests, a 2-3 mm thick slice of the kidney from peroxidase-injected rats was fixed for 24 hr at 4°C in 100 ml of 4% formaldehyde solution (freshly prepared from paraformaldehyde [11]) containing 0.02 M cacodylate buffer, pH 7.0, and 7.5% sucrose. The tissue was washed twice, for 24 hr each time, with a 30% sucrose solution, slightly buffered at pH 7.0. Cryostat sections, 6 μ thick, were stained for peroxidase with benzidine (19), with diaminobenzidine (3), or with 3-amino-9ethylcarbazole (4).

RESULTS

Uptake and Excretion of Peroxidase in Relation to Dose

Tests with over 100 rats showed that the concentration of injected peroxidase in total homogenates of the kidney cortex 3-4 hr after injection, and the amounts excreted in the urine during this
time, increased in approximate proportion to the injected amounts over a wide range of doses. The uptake of peroxidase by the liver, on the other hand, tended toward saturation with increasing doses. Two types of deviations from this general trend were observed. Some rats excreted much more peroxidase in the urine and absorbed less into the cortex than the "average" rat. Different lots of commercial peroxidase preparations (type II from Sigma Chemical Co.) differed in regard to peroxidase uptake and excretion, although the approach to proportionality between the dose and the concentrations in the cortex and urine was observed in all cases.

Fig. 1 shows the renal uptake and excretion of a highly purified commercial peroxidase preparation (type VI from Sigma Chemical Co., estimated purity over 90%). When rats received amounts of peroxidase varying between 0.3 and 16.5 mg/100 g body weight, an almost constant proportion, amounting to 30-35% of the injected protein, was excreted in the urine during 4 hr and 4.5-6% of the protein was found in the cortex after injection of amounts varying between 4 and 16.5 mg/100 g body weight (Fig. 1). The renal concentration decreased at lower dose levels. The liver contained 79% of the injected amount at the lowest, but only 10% at the highest, dose tested (Fig. 1). In most experiments, less pure preparations (type II from Sigma Chemical Co., estimated purity 30-40%) were given. In experiments with 52 rats, for example, 2-3% of the peroxidase contained in such a preparation was concentrated in the kidney cortex and 20-30% was excreted in the urine when the dose was varied between 2 and 25 mg/100 g body weight. With other lots of type II from Sigma, 3-4% of the injected peroxidase was recovered in homogenates of the renal cortex over a wide range of dosages.

Concentration of Peroxidase in Total Particulate Fractions in Relation to Dose and Time after Injection

As may be seen from Table I, the proportionality between the concentration of peroxidase in the kidney cortex and the load correlates with the uptake of peroxidase by cytoplasmic granules. Table I also shows that the specific activities of peroxidase in the total particulate fractions from the kidney cortex did not change much during the first few hours after injection when most of the peroxidase was not yet cleared from the blood. The concentration of peroxidase in the supernatant fluids varied at different times after injection since these fractions were contaminated with peroxidase from the blood and urine.

At later times after injection, when most of the foreign protein had been cleared from the blood, the concentration of peroxidase in the cytoplasmic granules from the kidney cortex decreased at a rate of 6-8% per hr (Fig. 2). It may also be seen from Fig. 2 that the initial concentrations of peroxidase in the cytoplasmic granules were propor-

| Dose | Activity of peroxidase in fractions (units/mg protein) and in blood serum (units/ml) |
|------|----------------------------------------------------------------------------------|
| units/100 g body weight | 0.5 hr | 1 hr | 2 hr | 3 hr | 4 hr |
| 11 X 10⁶ | 7.4 (450) | 6.9 (11) | 7.2 (1) | 4.2 (1) | — |
| 33 X 10⁶ | 37.2 (2500) | 21.2 (720) | 18.5 (24) | 18.2 (5) | 16.8 (1) |
| 65 X 10⁶ | 51.0 (1910) | 42.0 (283) | 41.9 (9) | 20.7 (5) |
| 130 X 10⁶ | — | 80.5 (6300) | 85.2 (750) | 96.5 (1120) |

Male rats weighing 230-250 g each were injected with horseradish peroxidase in amounts indicated above and were sacrificed 0.5-4 hr later. The peroxidase and protein contents of the total particulate fractions separated from the kidney cortex were determined. The specific activities of peroxidase showing similar values after injection of the same doses or showing rough proportionality to the dose after injection of different doses are italicized. The figures in parentheses indicate the concentration of peroxidase in the blood serum at the time of sacrifice. The activity of the injected peroxidase preparation (Sigma Chemical Co., type II) was close to 5000 units per mg.

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FIGURE 2 Peroxidase activity in total particulate fractions prepared from the kidney cortex 2–14 hr after injection of four different doses. Three animals weighing 250 g each received a dose of $78 \times 10^3$ units per 100 g body weight, and the specific activities of peroxidase in total particulate fractions prepared from the cortex 5, 9, and 13 hr after injection, respectively, were determined (curve I). On the same day, three other animals, weighing 250 g each, received a six-times lower dose, prepared from the same peroxidase solution, and the particulate fractions obtained 2, 4, and 6 hr after injection, respectively, were analysed (curve IV). Note that the initial concentration of peroxidase in curve IV is close to six times lower than that in curve I, and that the initial activities decreased at an approximate rate of 6–8% per hr.

In a similar experiment, three animals received a dose of $58 \times 10^3$ units/100 g body weight and the particulate fractions were prepared 5, 11, and 14 hr later (curve II). On the same day, three other animals received a 1.8-times lower dose, and the peroxidase activities in particulate fractions obtained 6, 9, and 12 hr after injection, respectively, were determined (curve III). Note that the initial value for peroxidase in curve III is close to 1.8 times lower than that in curve II, and that the initial values decreased at an approximate rate of 6–8% per hr. The concentration of peroxidase in the blood serum at the time of sacrifice was below 0.01% of the original values in all animals.

Note also that the initial concentrations of peroxidase shown in curves I and II are not exactly proportional to the load, but that the initial value on curve I is 12% higher than the theoretical value. Such small differences in the proportionality between the load and the renal uptake were observed frequently when different batches of peroxidase preparations, prepared on different days, were injected.

Unexpectedly, the disappearance of peroxidase activity from the cytoplasmic granules seemed to be accelerated at later intervals after injection (Fig. 2). In order to test this point further, the rate of disappearance of peroxidase activity was determined in 12 rats, all of which received the same dose of 39,000 units of peroxidase/100 g body weight, by measuring the specific activities of peroxidase in the total particulate fractions from three rats for four different time intervals. The mean values for three rats, measured 3, 7, 11, and 15 hr after injection, respectively, were found to be 19.8, 14.3, 10.0, and 3.3 units of peroxidase per mg protein, respectively, thus again showing an accelerated decrease of peroxidase activity at later times (between 11 and 15 hr) after injection.

Concentration of Peroxidase in the Renal Cortex, the Renal Medulla, and in the Liver

In the experiments shown in Table II, the specific activities of peroxidase were compared for homogenates, prepared 4 hr after injection, of the cortex, of the outer medulla and inner medulla of the kidney, and of the liver. As may be seen from the table, the concentration of peroxidase in the inner medulla was much lower than in the cortex, and the concentration in the outer medulla showed intermediate values. The specific activity of peroxidase in the renal cortex was higher than in the liver at high dosage levels but was lower at low dosages.

Spontaneous Changes in Renal Absorption and Excretion of Peroxidase

It was observed repeatedly that some animals excreted 70–120% more peroxidase into the urine...
TABLE III
Reabsorption of Peroxidase by Kidney Cortex and Excretion in Urine after Two Successive Injections

| No. of injections | First urine | Second urine | Cortex | Liver |
|-------------------|-------------|--------------|--------|-------|
| 1                 | 23.2        | 21.5         | 2.7    | 7.3   |
| 1                 | 24.2        | 28.6         | 2.9    | 7.4   |
| 2                 | 18.0        | 16.4         | 1.3    | 13.8  |
| 2                 | 42.1        | 54.1         | 1.1    | 12.4  |

Three male rats weighing approximately 240 g each were injected twice with $65 \times 10^3$ units of horseradish peroxidase/100 g body weight. Peroxidase determinations were made on the urine excreted during 4 hr after the first injection and during 5 hr after the second injection. Peroxidase determinations on the kidney cortex and liver (total homogenates) were made at the end of the experimental period (9 hr). The figures for the first and second urine were calculated as percent of a single injected dose ($65 \times 10^3$ units/100 g body weight); the figures for the cortex and liver were calculated as percent of the total injected dose (twice $65 \times 10^3$ units/100 g body weight). The percentages of peroxidase in the kidney cortex, liver, and urine of the three once-injected animals were obtained 9 hr after the injection of $65 \times 10^3$ units/100 g body weight.

and absorbed 50–100% less into the cortex than the “average” rat. These changes occurred in 25% of animals when certain lots of commercial peroxidase preparations showing relatively low cortical uptake (2–3% of the injected amount) were used.

Effects of Double Injections of Peroxidase

When a second, equal dose of peroxidase was injected into the same animal 4 hr after the first injection, the proportion of the protein excreted in the urine after the second injection was approximately double the amount excreted after the first injection (Table III). In the twice-injected animals the proportion of the marker protein reabsorbed by the kidney cortex was approximately one-half, and the proportion taken up by the liver was approximately double, the amount found in the once-injected animals (Table III).

For reasons discussed below, it was of interest to compare the rate of clearance of peroxidase from the blood after two successive injections. As may be seen from Table IV, the rate of clearance of peroxidase from the blood during the first 2 hr following the second injection was only one-half of that observed after the first injection.

Uptake and Excretion of Peroxidase in Relation to Age

The uptake of peroxidase by the kidney cortex but not by the liver, increased with age, and the excretion of the protein in the urine decreased. In experiments in which rats weighing 60, 160, 240, and 400 g received the same dose of 62.5 units of peroxidase per 100 g body weight, the proportion of peroxidase found in homogenates of the kidney cortex of the rats weighing 60 and 160 g each was 1.6% of the injected amount (specific activity, 8.5 units per mg protein); it was 3.2% of the injected amount for the rats weighing 240 g each (specific activity, 17.4 units per mg protein); and 6.5% of the injected amount for the rats weighing 400 g each (specific activity, 40.5 units per mg protein).

The increase of peroxidase uptake by the kidney cortex with age raised the question of whether lysosomal enzymes also increased. The activities of three lysosomal enzymes were tested. As may be...
TABLE V
Specific Activities of Acid Phosphatase, Deoxyribonuclease, and Cathepsin in Kidney and Liver Homogenates in Relation to Age

| Weight | Acid P-ase | Acid DNAase | Cathepsin |
|------|------------|-------------|-----------|
|      | Kidney     | Liver       | Kidney    | Liver       | Kidney    | Liver       |
|      |            |             |           |             |           |             |
| 9    |            |             |           |             |           |             |
| 60-70 | 104        | 81          | 0.25      | 0.13        | 0.77      | 0.38        |
| ±5.5 | ±8.8       | ±0.037      | ±0.015    | ±0.06       | ±0.03     |
| 200-220 | 97        | 84          | 0.25      | 0.13        | 0.74      | 0.25        |
| ±5.4 | ±4.6       | ±0.013      | ±0.022    | ±0.06       | ±0.03     |
| 400-420 | 82        | 72          | 0.21      | 0.11        | 0.66      | 0.17        |
| ±3.6 | ±4.9       | ±0.017      | ±0.022    | ±0.03       | ±0.02     |

The figures for enzyme activities are means from eight animals in the case of acid phosphatase and deoxyribonuclease, and from five animals in the case of cathepsin. Acid phosphatase (acid P-ase) activity is expressed as µg P liberated per 15 min incubation at 37°C and pH 5.0 per mg protein; acid deoxyribonuclease (acid DNAase) as increase in absorbance at 260 µm after 15 min incubation at 37°C and pH 5.0 per mg protein; cathepsin as nEq tyrosine per min per mg protein X 10⁶, after 30 min incubation at 37°C and pH 3.2 with denatured hemoglobin as substrate according to Anson (see reference 13).

seen from Table V, the activities of acid phosphatase, acid deoxyribonuclease, and cathepsin did not increase with age but seemed to decrease slightly.

Cytological Observations

Only a few cytological observations, not reported previously or extending previous data (17, 18, 20), will be mentioned here. It should be noted that the tissue was obtained 3-4 hr after injection when most of the peroxidase originally present in phagosomes (endocytic vesicles or vacuoles) had reached the lysosomes (phagolysosomes).

Cortex

At very low dose levels (0.1-0.2 mg of “pure” peroxidase per 100 g body weight), marked staining of phagolysosomes, after 10 min incubation with dianinoazobenzidine and H₂O₂ (3), occurred mainly in one segment of the proximal tubules (Fig. 3). At higher doses, all three segments of the cortex showed marked staining (Figs. 4-5). At very high doses (7.6 mg peroxidase per 100 g body weight), phagolysosomes were seen not only in the proximal tubules but also in the outer medulla (Fig. 6).
FIGURE 7  Inner medulla of the kidney, 3 hr after injection of 7.6 mg horseradish peroxidase/100 g body weight; section stained for peroxidase with benzidine and \( \text{H}_2\text{O}_2 \). Note that certain cells of the collecting tubules in the outer zone of the inner medulla are enlarged and contain more and larger phagolysosomes than neighboring cells. Note also that the collecting tubule cells in this zone have lower height than those closer to the tip of the papilla (Fig. 6) and in the cortex. See also Young and Wissig (22). Inset: Enlarged cells at higher magnification. Fig. 7, \( \times 500 \); inset, \( \times 1120 \).

FIGURE 8  Inner medulla of the kidney, 3 hr after injection of 7.6 mg horseradish peroxidase/100 g body weight; section stained for peroxidase with diaminobenzidine and \( \text{H}_2\text{O}_2 \). Note peroxidase-positive phagolysosomes and phagolysosomes in interstitial cells (arrow) of outer zone of the inner medulla, and the characteristic ladder-like arrangement of these cells. \( \times 500 \).

FIGURE 9  Same preparation as seen in Fig. 8, showing interstitial cells (arrow) at higher magnification. \( \times 1120 \).
proximal tubules contained great numbers of phagolysosomes, although differences in uptake existed between the two segments in the outer cortex and the terminal segment in the inner cortex (see Discussion and reference 18). As will be mentioned in the following paragraph, the cells of the collecting tubules in the inner cortex also took up peroxidase at very low doses (0.2–0.3 mg/100 g body weight).

**Outer Medulla**

The cells of the collecting tubules in the outer medulla and in the inner cortex, but not those of the distal tubules, absorbed peroxidase at low dosages (Fig. 4). Endothelial cells which were more numerous in the outer medulla than in the cortex also contained peroxidase-positive granules at very low dosages. At doses above 2 mg of pure peroxidase/100 g body weight, the cells of the thick ascending limbs took up peroxidase into many cytoplasmic granules (Fig. 5). At higher doses, certain “thin” segments also ingested peroxidase. These segments were located in the inner zone of the outer medulla, adjacent to the thick ascending limbs (Fig. 5).

**Inner Medulla**

Uptake of peroxidase into the collecting tubule cells of the inner medulla occurred at doses that were higher than the doses that produced uptake of peroxidase into collecting tubule cells of the outer medulla and cortex. Fig. 6 shows the collecting tubules of the inner medulla after injection of a relatively high dose (7.6 mg of pure peroxidase/100 g body weight). Certain cells of the collecting tubules absorbed much more peroxidase than neighboring cells (Fig. 7). The larger size and greater number of phagolysosomes in these cells could also be distinguished after staining for acid phosphatase. These enlarged cells were located predominantly in the outermost zone of the inner medulla, close to the transition to the outer medulla. Peroxidase was also taken up by interstitial cells in the outer zone of the inner medulla (Figs. 8 and 9). Although some of the peroxidase in these cells was present in phagolysosomes (acid phosphatase reaction), most of the peroxidase-positive granules in the interstitial cells seemed to be small phagosomes.

**DISCUSSION**

It was known from previous experiments (15, 16) that most of the injected peroxidase was cleared from the blood in 2–4 hr (depending on the dose), and that its concentration in the phagolysosomes had reached a high level at that time. An experimental period of 4 hr, therefore, was selected for most of the present experiments. The rate at which horseradish peroxidase is degraded by lysosomal cathepsin in vivo (16) and in vitro (2) is relatively low. The experiments summarized in Table I and Fig. 2 seem to indicate that the degraded peroxidase was replaced by newly ingested peroxidase during the first few hours, and that the gradual loss of peroxidase activity from the cytoplasmic granules began only after most of the peroxidase had been cleared from the blood. According to Fig. 2, the peroxidase activity then decreased in the cytoplasmic granules at a rate of 6–8% per hr. Although the disappearance of peroxidase from the granules seemed to be accelerated later, a statistical evaluation of data obtained from many animals would be required to determine whether the rate of disappearance deviated from the expected, exponential course. It should also be noted that the analytical method did not establish the degradation of peroxidase molecules but only the loss of enzymatic activity.

In order to obtain sufficient urine, a slight water diuresis was induced in animals by giving them tap water by stomach tube. Comparison of these animals with animals not receiving tap water showed that the renal uptake and excretion of peroxidase was not altered significantly by the slight diuresis. Fluctuation of urine flow also did not seem to have a noticeable effect on these values. Whether or not changes of the glomerular filtration rate influenced the renal uptake and excretion of peroxidase could not be tested. Since the concentration of peroxidase in the blood decreased exponentially, the urine formed during the first 2 hr contained the bulk of the excreted enzyme. A relatively small proportion was found in the late urine obtainable in most cases before sacrifice or taken from the bladder by puncture. If, therefore, the animals had voided 1.5–2 ml of urine during a 3–4 hr period, the bulk of the excreted protein had been recovered.

The data illustrated by Fig. 1 indicate that 4.5–6% of the injected peroxidase (highly purified preparation) was recovered in homogenates of the renal cortex, and that 30–35% of it was recovered in the urine over a wide range of doses. With less pure preparations of peroxidase, the renal uptake and excretion also increased in approximate proportion to the injected dose, although the
values varied with different lots of the commercial preparations. The proportionality between the load and the tubular concentration was related to the uptake of peroxidase by cytoplasmic granules. This could be seen when the concentration of peroxidase was determined in total particulate fractions separated from the kidney cortex at various loads (Table I). It may be assumed from previous work (14, 17) that the ingested peroxidase was present in phagosomes and phagolysosomes of the particulate fractions, and that mitochondria and microsomes were free of the exogenous protein. The concentration of peroxidase in the supernatant fluids varied considerably with time, as will be discussed later, due to the contamination with peroxidase from the blood and urine.

The following interpretation of these data may be proposed. It is known from observations made with the electron microscope (3) that microphagocytic vesicles containing ingested peroxidase are formed at the apical poles of the proximal tubule cells. It is suggested that a constant fraction of peroxidase is reabsorbed by these vesicles into the tubule cells from the glomerular filtrate. The concentration of peroxidase in individual granules thereby increases in proportion to the concentration of peroxidase in the blood and glomerular filtrate. As suggested by the experiment summarized in Table I, a steady state may exist between the transfer of peroxidase to lysosomes and the digestion of the protein therein. Perhaps the transfer of peroxidase from phagosomes to lysosomes is regulated, in a way not yet understood, in relation to the rate of lysosomal digestion.

No saturation of the cytoplasmic granules with injected peroxidase was observed up to dosage levels of 20 mg of pure peroxidase (Sigma Chemical Co., type VI) per 100 g body weight, or 50 mg of a less pure preparation/100 g body weight (Sigma, type II). It could not be determined at what dosage a saturation occurred, since the animals became sick at the highest doses tested. After repeated injections of a high dose of peroxidase, however, the cytoplasmic granules appeared to become saturated, as is discussed below. At a very low dosage, the proportion of peroxidase absorbed into the cortex and excreted in the urine decreased, probably due to the almost complete capture of the protein by the liver.

When rats received a second, high dose of peroxidase 4 hr after the first injection, the uptake by the renal cortex was not increased but the urinary excretion was approximately doubled (Table III). These results call to mind earlier experiments (16) in which the uptake of peroxidase into the phagolysosomes of the proximal tubules was strongly depressed by a preceding injection of egg white. It should be noted, however, that the excess of urinary excretion of peroxidase could only partially be accounted for by a depression of tubular absorption. It was shown in earlier work (15) that the reticuloendothelial cells throughout the body took up a considerable proportion of injected peroxidase. A partial blockade of the reticuloendothelial system might delay the removal of the protein from the blood and thereby increase urinary excretion. This interpretation was supported by the finding that the rate of removal of peroxidase from the blood was slower after the second injection than after the first injection of the same amount into the same animal (Table IV). A strong delay of peroxidase clearance from the blood was also observed after preinjection of egg white (see Fig. 5 in reference 16).

In contradistinction to the protein uptake by the kidney cortex, the proportion of peroxidase taken up by the liver decreased from 80% at a very low dose to 10% at a high dose (Fig. 1). Jacques (5) reported similar findings. Previous cytochemical observations (20) had shown that endothelial cells and Kupffer cells took up much more injected peroxidase than liver epithelial cells. The decrease of fractional peroxidase uptake by the liver with increasing dose may be related to the saturation of endothelial cells and Kupffer cells at relatively low dose levels (see also Table II in reference 16). After two successive injections of the marker protein into the same animal, the liver again reacted in a different way than the kidney. As was also reported by Jacques (5), the liver took up almost twice as much peroxidase after two injections as after one. It should be noted that the uptake by the liver was not increased when the double dose was injected at once (Fig. 1). This reaction of the liver is not understood. It was suggested from cytochemical observations (20) that a transformation of endothelial cells into Kupffer cells occurred after injection of peroxidase. Perhaps a proliferation of phagocytic cells or an invasion of macrophages from other parts of the body occurred after the first injection and caused the increased protein uptake by the liver after the second injection.
The ability of the kidney cortex to reabsorb peroxidase increased considerably with age. It was important, therefore, to use animals of the same age groups for the present study. It is known that the excretion of serum proteins is increased in older rats (12). Perhaps the increased excretion of serum proteins and the increased tubular absorption of peroxidase in old rats are related to an increased permeability of the glomerulus to proteins. In view of the known relationship between protein uptake and lysosomes, experiments were carried out to test whether lysosomal enzymes also increased with age. No increase, but rather a slight decrease, was observed (Table V). These observations may indicate that the lysosomal enzymes already present are adequate for all later needs.

Although few conclusions of a quantitative nature can be drawn from cytochemical observations, the biochemical analysis was facilitated by the correlation with cytochemical observations. These showed, for example, that peroxidase in the homogenates of the kidney cortex was derived mainly from the three segments of the proximal tubules and that the cortical segments of the distal and collecting tubules, of endothelial cells and glomeruli (mesangial cells), contributed relatively little peroxidase to these homogenates. It could also be seen from cytochemical observations that peroxidase in the homogenates of the inner medulla originated mainly from collecting tubule cells but that peroxidase in homogenates of the outer medulla came from several types of cells. Most of the peroxidase was present in the granules of the thick ascending limbs, but appreciable amounts were also located in collecting tubule cells of the outer medulla, and a minor portion in thin segments (see below) and endothelial cells.

In the context of the present study, it may be of interest to report cytochemical observations showing preferential uptake of peroxidase by certain cell types or segments in relation to the dose. Even when as little as 0.1–0.2 mg of peroxidase per 100 g body weight was injected, numerous phagolysosomes in the cells of one segment of the proximal tubules in the outer cortex reacted for peroxidase (Fig. 3). This was probably the same segment which previously (18) was observed to be more active in peroxidase uptake than another segment in the outer cortex and the terminal segment in the inner cortex. On the basis of observations by Oliver et al. (10) and by Maunsbach (8), the cells which absorbed extremely low doses of peroxidase may be interpreted tentatively as belonging to the middle segment of the proximal tubules.

The cells of the collecting tubules in the inner cortex and outer medulla (but not those in the inner medulla) also ingested peroxidase at very low doses (Fig. 4). The absorption of the protein by the cells of the distal tubules, however, required higher loads. Certain cells of the collecting tubules in the inner medulla ingested much more peroxidase than did neighboring cells (Fig. 7). These cells may be related to the “dark” cells known from electron microscopy (6). Although dark cells usually are observed only in the cortical segments of the collecting tubules, Young and Wissig (22) also saw dark cells in the inner medulla.

The capacity of certain thin segments located in the inner zone of the outer medulla to ingest the marker protein (Fig. 5) was overlooked in previous work. If the cells in question should belong to the thin segments of Henle’s loop, their ingestion of the protein in only a narrow zone of the outer medulla would be remarkable. The ability of interstitial cells in the renal medulla to ingest peroxidase (Figs. 8 and 9) is in agreement with observations by other investigators (1, 9), who reported the phagocytic ability of these cells. An increase in the number of lysosome-like granules in the interstitial cells after K⁺ depletion has also been described (9, 21).

In view of the function of the renal medulla as a counter-current multiplier for the concentration of urine, it may be of interest to note that peroxidase uptake by enlarged collecting tubule cells, by interstitial cells, and by the cells of thin segments was limited to narrow zones of the medulla. Protein uptake by these cells was more pronounced in some animals than in others, thus suggesting an influence of different metabolic states. It may also be noted that the concentration of peroxidase in the collecting tubule cells of the inner medulla often increased towards the tip of the papilla, thus following the osmotic gradient.

This work was supported by research grants from the United States Public Health Service (GM 12123) and from the American Heart Association.

Received for publication 22 June 1970, and in revised form 24 August 1970.
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