Colorimetric Analysis with *N*,*N*-Dimethyl-*p*-phenylenediamine of the Uptake of Intravenously Injected Horseradish Peroxidase by Various Tissues of the Rat*

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ABSTRACT

1. A method is described for the colorimetric determination of peroxidase with *N*,*N*-dimethyl-*p*-phenylenediamine. The amount of red pigment formed by peroxidase is proportional to the concentration of enzyme and to the time of incubation during the first 40 to 90 seconds. The influence of the concentration of enzyme, *N*,*N*-dimethyl-*p*-phenylenediamine, H_2O_2, the time of incubation, pH, the temperature, and the possible interference by oxidizing and reducing agents of tissues has been tested.

2. The method has been used to follow the uptake of intravenously injected horseradish peroxidase by 18 different tissues of the rat over a period of 30 hours. The highest concentration of the injected tracer enzyme was found in extracts of kidney, liver, bone marrow, thymus, and spleen. Considerable amounts were taken up by pancreas, prostate, epididymis, and small intestine. Lower concentrations were found in extracts of lung, stomach, heart, and skeletal muscle, aorta, skin, and connective tissue. No uptake was observed by brain and peripheral nerve tissue.

3. Tissue homogenates containing high concentrations of the injected peroxidase, in general also showed high or average activity of acid phosphatase.

4. Six hours after intravenous administration, the liver contained 27 per cent, the kidney 12 per cent, and the spleen, 1.4 per cent of the injected dose.

5. Approximately 20 per cent of the injected peroxidase was excreted in the urine during the first 6 hours, and the concentration of peroxidase in blood serum and urine fell exponentially during this time. After 6 hours, only low concentrations were excreted in the urine but low enzyme activity was still detectable after 30 hours. Approximately 6 per cent of the injected dose was excreted in the feces from 6 to 20 hours after administration.

6. After feeding through a stomach tube, low concentrations of peroxidase were found in blood serum and urine. Considerable variations in the extent of absorption from the gastrointestinal tract were observed in individual rats.

Particles of colloidal or macromolecular size as well as protein molecules can be incorporated by animal cells of many types through a process described as pinocytosis or athrocytosis. Certain cytoplasmic granules appear to be functionally related to the material thus incorporated. Granules of this type, probably identical with the "hyaline droplets" of the pathological literature, have been isolated from kidney homogenates of rats (1, 2), and a characteristic property of these granules is their high concentration of acid phosphatase, acid ribonuclease, desoxyribonuclease, cathepsin, and β-glucuronidase (1, 2). Fractions containing high concentrations of the same enzymes had previously been isolated from liver homogenates by de Duve *et al.* (3) who called small granules in which these enzymes are probably concentrated "lysosomes" (3).

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In order to study the ability of these granules to segregate proteins, a new method has been developed. It consists of injecting intravenously a few milligrams of a plant enzyme, horseradish peroxidase, as a tracer, and then determining colorimetrically the concentration of the injected enzyme in isolated cell fractions. The principle of this method and its first application to the "droplets" isolated from kidney cells has been briefly communicated earlier (4). The present paper gives a more detailed account of the method and reports the results obtained by measuring the concentration of the tracer protein, at varying intervals after intravenous administration, in homogenates of 18 tissues of the rat and in the blood serum, urine, and feces. The absorption of the protein from the gastro-intestinal tract after feeding through a stomach tube has also been briefly investigated. In a later report, a cytochemical application will be presented.

The assay for peroxidase is a modification of a method for cytochrome *c* oxidase reported previously (5). It makes use of the transformation, by oxidative enzymes, of *N*,*N*-dimethyl-*p*-phenylenediamine into a red pigment of semiquinone character. Microgram amounts of cell material, resuspended or diluted with distilled water, develop only a faint color during the first few minutes of incubation. They will, however, develop a deeper color, proportional to the amount of enzyme and to the time of incubation if an excess of the appropriate substrates is added. This property has been utilized previously in the microdetermination of cytochrome *c* oxidase by adding cytochrome *c* to the cell material.[1] Polyphenol oxidase has also been briefly tested by adding 3,4-dihydroxyphenylalanine to a potato extract (5). In the routine procedure, enzyme samples and reagents were incubated for ½ to 2 minutes at 37°C., after which time the enzyme was inactivated by the addition of alcohol, and the color intensity was measured in the colorimeter. As mentioned (5) the reaction can be followed directly in the colorimeter or spectrophotometer after appropriate modifications.

I. Reagents

0.2 M phosphate buffer, pH 6.2; *N*,*N*-dimethyl-*p*-phenylenediaminehydrochloride (Eastman Organic Chemicals, Rochester, New York), 0.1 per cent solution, freshly prepared and kept on ice; 0.18 per cent H_2O_2 solution (Merck & Co., Inc., Rahway, New Jersey); horseradish peroxidase, "30 units per mg." (Nutritional Biochemicals Corporation, Cleveland, Ohio).

II. Colorimetric Assay of Peroxidase

(*a*) *Method.*—The following reagents were added directly to the colorimeter tube: 2.0 ml. of 0.2 M phosphate buffer solution, pH 6.2; aliquots of tissue samples of peroxidase-treated animals containing 20 to 100 μg. of N, or other peroxidase-containing solutions; distilled water to make a final volume of 10.0 ml. After keeping the tube in a water bath at 20°C. for 2 to 3 minutes,[2] 0.4 ml. of a 0.18 per cent H_2O_2 solution and 0.3 ml. of a freshly prepared, 0.1 per cent solution of *N*,*N*-dimethyl-*p*-phenylenediamine hydrochloride were added. The tube was thoroughly shaken and then inserted into an Evelyn colorimeter (filter No. 515), the galvanometer needle of which had been adjusted previously to 100 against distilled water. After about 3 seconds, timing was started with a stop watch and the position of the galvanometer needle was noted (time zero). The position of the shifting galvanometer needle was recorded every 10 seconds, up to 70 to 90 seconds. When very high concentrations of peroxidase were tested, the position of the needle was recorded every 5 seconds, up to 30 seconds.

A blank was run subsequently containing the reagents alone (without tissue material). Since the value of this blank did not vary significantly, it served as control for a whole series of samples. This blank was used with samples of purified peroxidase (Figs. 1 and 2). When a tissue fraction from a peroxidase-treated animal was tested for the first time, an analogous fraction was prepared from an untreated animal, assayed in the same way, and the color value deducted as blank. The use of such a blank is essential for the few animal tissues which naturally contain peroxidase (leucocytes, bone marrow, spleen, lung). For most other tissues

[1] It was mentioned (reference 5, footnote page 61) that the cytochrome *c* preparation used in the early experiments with cytochrome oxidase was not pure and that an excess of it inhibited the color formation with *N*,*N*-dimethyl-*p*-phenylenediamine but that the cytochrome *c* preparations (Sigma Company, St. Louis, Missouri) used later did not cause any inhibition. It may be noted here that the method for cytochrome oxidase has now been applied as routine for several years, and that reliable results are obtained by using 0.3 ml. of a 0.04 per cent solution of this cytochrome *c* preparation (Sigma Chemical Company) together with the other reagents in the concentrations indicated (5). The time of incubation is usually shortened to 45 seconds since the rate of color development might fall off slightly afterwards.

[2] If the room temperature was close to 20°C. and if small errors could be tolerated, the use of a water bath was often omitted.

which do not contain measurable amounts of peroxidase, the blank prepared with the reagents alone (without tissue material) may be used resulting in only a slight error (see section III (*e*)).

(*b*) *Calculation of Results.*—The photometric density of samples and blanks corresponding to the colorimeter readings at 0, 10, 20, 30, etc., seconds, was read from a conversion table. The increase in photometric density per 10 seconds was determined by taking the difference between successive readings. The increase per 10 seconds in the range in which it was constant over a period from 20 to 60 seconds, was extrapolated to 60 seconds.

The readings were corrected with the appropriate blanks. When fractions prepared from untreated animals from tissues which naturally contained peroxidase (bone marrow, spleen, lung) were used as blanks, the color values were extrapolated to the same amount of N as was present in the corresponding sample from the peroxidase-treated animal. However, when the tissue blanks did not contain measurable amounts of peroxidase (most animal tissues), no extrapolation of their color value was made (see section III (*e*)).

The photometric densities per minute per milligram N were expressed in "quinonediimonium" (QDI) red units,[3] by dividing by 0.75 when the Evelyn colorimeter with filter 515 was used. These values served as a basis of comparison for different samples (Tables I and II).

(*c*) *Comparison of the Method of Willstätter and Stoll with the Present Method.*—In Willstätter and Stoll's (6) conventional method for determining peroxidase, purpurogallin, formed by the reaction of peroxidase with pyrogallol, is extracted with ether and measured colorimetrically. The commercial preparation of peroxidase used in our experiments was assayed according to the method of Willstätter and Stoll (using concentrations of the reagents 1/10 of those indicated by the authors), and by the present method. The "purpurogallin number" (defined as milligrams of purpurogallin formed by 1 mg. of the enzyme sample in 5 minutes (6)) of the commercial preparation was found to be 135. When tested with the present method, the same preparation showed 695 "quinonediimonium" red units per minute per milligram.[4] Thus, a purpurogallin number of 100 approximately corresponds to 515 QDI red units.

[3] This unit is defined as the photometric density of a 10 ml. solution of the pigment formed at pH 6.5 by the oxidation of 0.12 mg. of *N*,*N*-dimethyl-*p*-phenylenediamine hydrochloride with $K_2Cr_2O_7$ (5). By using a commercial preparation of the amine, a photometric density of 0.75 was found (5).

[4] This preparation was less pure than two others purchased from Nutritional Biochemicals Corporation after completion of the present experiments. One of these preparations, designated "30 units per mg.," showed 950 QDI red units per mg. The other one designated "60 units per mg." showed 1685 QDI red units per mg. These preparations formed clear solutions in water and seemed to be better tolerated by the rats than the earlier preparation.

TABLE I

Concentration of Peroxidase in Homogenates of Various Tissues at Varying Intervals after Intravenous Injection

Intravenous injection of 23 mg. of a commercial peroxidase preparation containing ~ 2.5 mg. of pure peroxidase. Specific activity of peroxidase expressed as QDI-red units per mg. N per minute. Specific activity of acid phosphatase expressed as mg. P liberated (from β-glycerophosphate) per mg. N per 15 minutes.

	Specific activity of peroxidase						Specific activity of acid phosphatase
	Controls	Treated (Controls deducted)					
Hrs. after injection	0	1	3	6	15	30	0
Kidney	0	38.5	34.1	21.5	14.0	1.0	74.2
Liver	0	9.3	8.3	8.6	3.9	1.2	64.5
Bone marrow	6.8	12.1	9.7	7.0	7.4	0.5	35.2
Thymus	0	7.5	6.9	7.6	2.9	0.5	43.2
Spleen	4.4	7.1	4.7	3.6	2.7	0	92.2
Prostate	0	4.5	—	4.8	1.6	0.5	30.6
Epididymis	0	3.3	—	3.0	0.9	0.2	189.7
Pancreas	0	3.0	—	3.8	1.5	0.4	20.9
Lung	2.9	1.7	—	0.9	1.3	0	28.9
Stomach	1.2	2.3	—	1.7	0	—	30.9
Intestine (jejunum)	0.5	4.6	—	3.2	1.3	—	25.0
Heart muscle	0.8	1.0	—	1.4	0	—	11.2
Aorta	0.5	0.4	—	1.3	0	—	9.7
Skeletal muscle	0	0.8	—	0.9	0.3	—	7.5
Connective and fat tissue	0.6	1.1	—	0.7	0	—	17.5
Skin	0	0.7	—	1.5	0.2	—	21.5
Brain	0	0	0	0	0	—	13.5
Peripheral nerve	0	0.3	0	0	0	—	12.2

TABLE II

Concentration of Intravenously Injected Peroxidase in Blood Serum, Urine, and Feces

Administration of 23 mg. of a commercial peroxidase preparation containing ~ 2.5 mg. of pure peroxidase. Concentration of peroxidase in blood serum and urine expressed as QDI-red units per minute per milliliter; concentration of peroxidase in feces expressed as QDI-red units per minute per gram wet weight. Total amount excreted expressed as per cent of injected dose.

	Concentration of peroxidase							Total amount excreted		
Hrs. after injection	0.25	1	2	3	6	15	30	0–6	6–20	20–30
Blood serum	715	111	35	12	2	0	0			
Urine	—	—	6310	1780	63	4	0.8	19.2	0.5	0.04
Feces	—	—	—	—	228	6.2		0	5.8	0.4

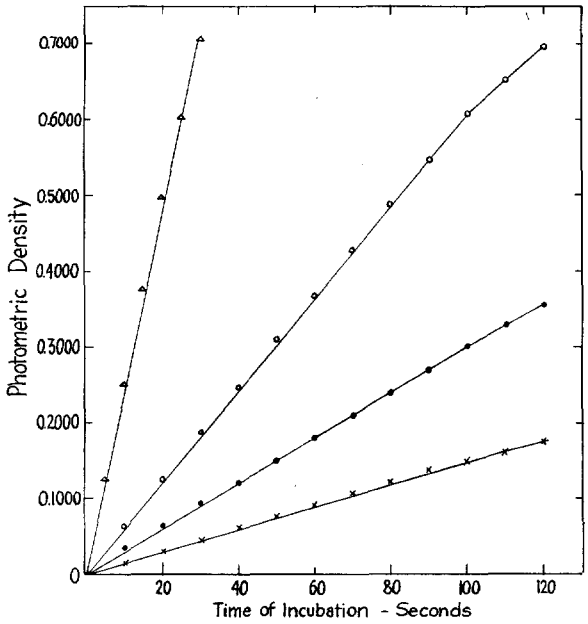

FIG. 1. Formation of red pigment by 0.25 (×), 0.5 (•), 1.0 (○), and 4.0 (△) μg. of a commercial peroxidase preparation. Incubation at 20°C.; pH 6.25.

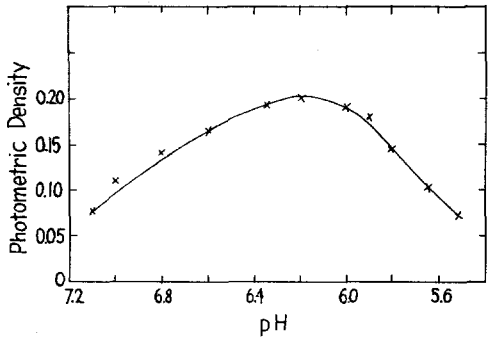

FIG. 2. Formation of red pigment by peroxidase in relation to pH.

Since pure peroxidase is reported to have a purpurogallin number of 1220 (7), the commercial preparation was approximately 11 per cent pure.

III. Test of Method

The effects of the following factors on the enzymatic color development were tested: the concentrations of the enzyme, of *N,N*-dimethyl-*p*-phenylenediaminehydrochloride and of H_2O_2, the time of incubation, pH, the temperature, and the possible interference by oxidizing and reducing agents present in the tissues.

(*a*) *Concentration of N,N-dimethyl-p-phenylenediaminehydrochloride and of* H_2O_2.—Varying concentrations of these two reagents were tested systematically employing 0.2 to 4.0 μg. of the commercial peroxidase preparation as the enzyme source. On the basis of these experiments, concentrations of 1.74×10^{-4} M dimethyl-*p*-phenylenediamine hydrochloride (0.3 ml. of a 0.1 per cent solution in 10 ml.) and of 2.1×10^{-3} M H_2O_2 (0.4 ml. of a 0.18 per cent solution in 10 ml.) were chosen for the standard procedure.

(*b*) *Time of Incubation and Concentration of Enzyme.*—As may be seen from Fig. 1, the color intensity produced by 0.25, 0.5, 1.0, and 4.0 μg., respectively, of the commercial peroxidase preparation increased in proportion to the concentration of enzyme and to the time of incubation during the first 20 to 90 seconds. Samples containing higher concentrations of peroxidase were diluted until the color that developed fell within the above range.

(*c*) *Influence of the pH.*—Fig. 2 shows that the color obtained with horseradish peroxidase with *N,N*-dimethyl-*p*-phenylenediamine was optimal between pH 6.0 and 6.4. In the routine procedure, phosphate buffer solution, pH 6.2, was used.[5]

[5] In the early experiments (4), peroxidase activity was measured at pH 7.0. Since the color formation at pH 7.0 is approximately 56 per cent lower than at pH 6.2 (see Fig. 2), the activities found previously (reference 4, Table I) are proportionally smaller.

(*d*) *Influence of Temperature.*—When the activity of 0.25 μg. of peroxidase (commercial preparation) was measured at 5, 10, 15, 20, etc., up to 40°C., photometric densities of 0.0581, 0.0731, 0.0938, 0.1196, 0.1392, 0.1428, 0.1405, and 0.1446, respectively, were observed. These figures were obtained by deducting the color values of the blanks containing the reagents alone from those obtained with the enzyme sample. With temperatures rising above 25°C., the color formation by the small enzyme sample increased only slightly whereas the color development in the blanks continued to increase. It was mentioned previously (5) that the autoxidation of dimethyl-*p*-phenylenediamine increases strongly with rise of temperature but that it is greatly inhibited by the presence of tissue material containing traces of reducing agents (see also section III (*e*)).

As a routine procedure, incubation at 20°C. was adopted.

(*e*) *Blanks; Interference by Oxidizing and Reducing Agents.*—Since the method described has been worked out with the aim of tracing injected peroxidase in tissues, the possible interference by oxidizing and reducing agents and the importance of the tissue blanks will be discussed in some detail.

Relatively little color is formed by autoxidation of the blank containing the reagents alone (without tissue material) using the concentrations employed as routine and incubating for 1 minute at 20°C. The colorimetric density of the reagents blank, amounting to approximately 0.065 QDI red units per minute, was used to check the tissue blanks in the case of untreated animals. If addition of the tissue material caused a decrease in color formation as compared with that produced by the reagents alone, this was taken as an indication that traces of reducing agents predominated over traces of oxidizing agents in the tissue. If it caused an increase, it was taken as an indication that the oxidizing agents predominated.

In the case of normal animals, the following tissue homogenates (containing approximately 100 μg. of N) developed *less* color than the reagents alone[6] (0.065 units per minute): kidney, 0.060, thymus, 0.060; pancreas, 0.058; prostate 0.055; peripheral nerve, 0.052; skeletal muscle, 0.050; epididymis, 0.048; liver, 0.038; brain, 0.032. All figures are expressed as QDI red units per 0.1 mg. N. These tissues were considered, for practical purposes, to have a peroxidase content equal to zero (Table I, second column). In the calculation of peroxidase activity (Table I), these values for normal animals were deducted from those obtained with the corresponding tissue fraction from a peroxidase-treated animal. If instead of these blanks, the color value obtained with the reagents alone (0.065 units per minute) is deducted as blank, a small error is made. However, the procedure is much simplified since no tissue fractions from control animals have to be prepared.

The following tissue homogenates, containing 100 μg. of N, developed *more* color than the reagents alone (0.065 units per minute): bone marrow, 0.745; spleen, 0.505; lung, 0.355; stomach, 0.185; heart muscle, 0.145; connective tissue, 0.125; aorta, 0.115; small intestine, 0.115. All figures are expressed as QDI red units per minute per 0.1 mg. N. Using these figures, the "peroxidase activity" of the above homogenates from untreated animals was calculated by deducting the reagent blank (0.065) and by extrapolating to 1 mg. N (Table I, second column). The activity of these tissue extracts from normal animals was partially due to peroxidase derived from broken-up white blood cells,[7] partially to hemoglobin derived from hemolyzed red blood cells.

Hemoglobin has a slight peroxidatic activity. Hemolysis of red blood cells should therefore be avoided when low concentrations of injected peroxidase are to be measured in blood serum. Using the routine procedure outlined, 0.01 to 0.5 ml. of fresh blood serum from normal rats showed values of 0.05 to 0.08 QDI-red units per minute thus indicating no predominance of either reducing or oxidizing agents. These figures from normal rats were deducted as blank values from those obtained from the peroxidase containing sera (Table II).

It may be mentioned that under the conditions of the peroxidase assay, cytochrome *c* oxidase does not contribute significantly to color formation by tissue samples (pH 6.2, no addition of cytochrome *c*, relatively high concentration of buffer (5)).

The reducing agents present in tissues may interfere with the color formation if the concentration of peroxidase is very low and the concentration of the reducing agents relatively high. Interference by reducing agents can be recognized by assaying two to three different concentrations of the sample and by verifying whether the development of color is proportional to the concentration of the sample (peroxidase). In the

[6] The results varied somewhat with the age and size of the samples. Most observations were made with tissue suspensions which had remained overnight in the refrigerator. Variations of the N-content of the samples between 50 and 120 μg. caused only small changes in the colorimetric densities.

[7] In the analysis of tissues which naturally contain peroxidase or of pathological tissues which show infiltration with leucocytes, a certain error may result from the fact that the experimental and control values are derived from tissue samples of different animals. The possible error decreases with the decrease in concentration of the natural peroxidase and with the increase in concentration of injected peroxidase in the tissues under study. Since most of the natural peroxidase content of animal tissues is probably derived from leucocytes, the possible errors may be lessened by separating the leucocytes by low speed centrifugation and by using only the supernatant fluids in the preparation of the fractions.

analysis of homogenates from various tissues shown in Table I, at least two different concentrations of the suspensions containing between 20 and 100 μg. of N, were tested as routine. The results agreed in general within 5 per cent, and no significant interference by reducing agents was observed at these low concentrations of tissue material.[8]

With some urine samples from rats treated with peroxidase, a delay in color formation from ½ to 3 minutes was observed when volumes of urine above 0.1 ml. were tested and when the concentration of peroxidase in the urine was low. After the latent period, the color formation proceeded normally. It is probable that a reducing agent was oxidized before the reaction started. Most urine samples had to be diluted highly before the color formation could be measured (Table II).

(*f*) *Sensitivity of Method.*—As may be seen from Fig. 1, 0.1 μg. of the commercial peroxidase preparation developed sufficient color for accurate measurement. Assuming that the purity of the commercial preparation was approximately 11 per cent (section II (*c*)), 0.011 μg. of pure peroxidase can be assayed. The color intensity (sensitivity) can be increased further by using 6.0 ml. instead of 10.0 ml. as the final volume (and by adjusting the compartment for the colorimeter tube to this volume). The data discussed in section III (*e*) permit an estimation of the extent to which this sensitivity can be maintained in the presence of tissue samples.

[8] In order to determine the degree of interference by reducing agents, the activity of peroxidase shown in the presence of relatively high concentrations of tissue samples was compared with the activity of the same amount of peroxidase alone. 0.1, 0.2, 0.3, and 0.5 μg., respectively, of the commercial peroxidase preparation were added to homogenates of brain and liver from normal rats containing 100, 200, 400, and 600 μg., respectively, of N. The slight color developed by the same amount of tissue suspension without added peroxidase was deducted as blank. The same amounts of peroxidase were then tested without tissue samples. These experiments showed that brain homogenates containing 100 to 600 μg. of N, and liver homogenates containing 100 to 400 μg. of N, did not cause any inhibition of the color formation if more than 0.2 μg. of the peroxidase preparation had been added. However, suspensions containing over 400 μg. of N, showed some inhibition if only 0.1 μg. of the commercial peroxidase preparation had been added. The effects of high concentrations of brain and liver suspensions differed, however, in the following points. With brain suspensions, maximal color formation was attained only after 1 to 2 minutes of incubation whereas with liver suspensions, maximal color development took place during the first 20 to 40 seconds, and then decreased. It should be noted that the concentration of the tissue samples (100 to 600 μg. of N) were much above those used as routine (20 to 100 μg. of N). As was mentioned above, no significant interference by reducing agents was observed with these low concentrations of tissue samples. A more detailed analysis of these effects should consider the possibility that H_2O_2 may be destroyed by catalase and that therefore the concentration of H_2O_2 may have to be increased.

IV. Application of Method

(*a*) *Concentration of Peroxidase in Tissue Homogenates Following Intravenous Administration.*—Twenty five milligrams of the commercial peroxidase preparation, corresponding to ~ 2.7 mg. of pure peroxidase (section III (*c*)) or 13000 QDI-red units, were dissolved in 1 ml. of saline. The solution was clarified by low speed centrifugation,[4] 0.05 ml. was retained for determination of the activity, and the rest injected into the femoral vein of male rats weighing approximately 400 gm. Animals were sacrified 1, 6, and 15 hours after administration. In the case of some tissues, samples were also obtained at 3 and 30 hours. Extracts of 18 different tissues were prepared by homogenization in distilled water. In each case, the non-homogenized residue was separated by low speed centrifugation, rehomogenized, and the supernatant fluid added to the main extract. Results showing the specific activity (concentration) of peroxidase are presented in Table I. If peroxidase was present in the tissues of control animals (Table I, second column), this activity was deducted from that shown by the experimental animals.

The last column of Table I shows the specific activity of acid phosphatase measured on tissue homogenates of normal rats (average of 2 to 3 experiments).

(*b*) *Peroxidase Content of Whole Liver, Kidney and Spleen.*—Six hours after intravenous administration of 12,000 units of peroxidase to a rat weighing 410 gm., the homogenate of the liver contained 3300 units, or 27.5 per cent of the injected amount, and the homogenate of the kidney contained 1440 units, or 12.0 per cent of the injected dose. In another experiment, the homogenate of the spleen was found to contain 1.4 per cent of the injected dose.

(*c*) *Concentration of Peroxidase in Blood Serum, Urine, and Feces.*—Table II shows the concentration of peroxidase in blood serum, urine, and feces at varying intervals after intravenous administration of 23 mg. of the commercial preparation

(12,000 units) into rats weighing approximately 400 gm. The total amounts excreted in urine and feces from 1 to 6, 6 to 15, and 15 to 30 hours after administration, expressed as per cent of the injected dose, are also tabulated. It should be noted that the rapid fall of peroxidase in blood serum excludes a significant contribution of peroxidase from contaminating blood to the peroxidase content of the homogenates (Table I) prepared later than 2 hours after administration.

(*d*) *Absorption of Peroxidase from the Gastro-intestinal Tract.*—A few exploratory experiments were made in order to determine whether peroxidase was absorbed from the gastro-intestinal tract. Approximately 50 mg. of the commercial preparation (26,700 units) were fed through stomach tube to rats weighing approximately 400 gm. Samples of blood (from the heart) and urine were taken at varying intervals after feeding.

The following observations were made. In 3 rats, the concentration of peroxidase in blood serum varied between 0.4 and 1.0 unit per minute per ml., in samples taken 1 to 8 hours after feeding. In total, approximately 20 units were excreted in the urine during this period. In another rat, very low concentrations of peroxidase were detected in the blood serum but none could be found in the urine. In a fifth rat in which only the urine was tested, relatively high concentrations appeared in the urine, varying from 11 to 120 units per minute per ml. In this rat, approximately 250 units of peroxidase were excreted in the urine during the first 10 hours after feeding. Thus, there was considerable variation in the degree of absorption from the gastro-intestinal tract among the rats tested.

V. Discussion

The localization in animal tissues of injected proteins has been studied by several investigators in relation to certain aspects of protein metabolism, antibody formation, and permeability. For such experiments, proteins, in most cases those prepared from blood serum or eggs, are labelled by conjugation with dye (8, 9), fluorescent (10, 11) or radioactive (12–15) molecules, and the distribution of the labels is traced in tissue slices or in isolated cell fractions. For reliability of results, these methods require that the label remain attached to the protein molecule *in vivo* and not be redistributed. Coons, Leduc, and Kaplan (16) avoid this possible complication by treating tissue slices with fluorescein-labelled antibodies and observing the localization of the injected antigen within the cells by fluorescence microscopy. Many investigators have analyzed antigenic, foreign proteins in tissue extracts with immune sera. A serological method was also used to demonstrate the segregation of intraperitoneally injected egg white in the droplets of rat kidney (17).

The peroxidase method described in this paper permits the tracer protein to be analyzed by a simple colorimetric assay. The reaction may be considered to be a property of the native protein. Analysis of the protein in isolated fractions can be correlated with cytochemical tests for peroxidase. Although horseradish peroxidase, molecular weight 44000 (18), may be especially favorable for use as a tracer, the possibility of selecting other enzymes as tracers should not be overlooked.

It was estimated in section III (*f*) that the method allowed the detection of approximately 0.011 μg. of peroxidase. Since 7.5 (4) to 25 mg. of the commercial preparation, corresponding to approximately 0.8 to 2.8 mg. of pure peroxidase, were administered, it follows that $\frac{1}{73,000}$ to $\frac{1}{250,000}$ of the injected dose could be detected by the method described. With this dosage, the concentration of peroxidase in most tissue homogenates was high enough for accurate measurement. If a higher concentration of peroxidase in the tissues is required, the amount injected may be increased. Although no toxic symptoms were observed with low dosage (4), some rats showed a few toxic signs (congestion of certain segments of the small intestine and dyspnea) when doses of the commercial preparation above 20 mg. were administered intravenously. The intravenous administration of preparations of peroxidase purer than the ones used in these experiments may be advantageous.[4]

By employing proteins tagged with dye, fluorescent or radioactive molecules, several investigators have observed the localization of foreign proteins in the parenchymal cells of kidney and liver, and in the reticulo-endothelial cells of bone marrow, spleen, lymph nodes, and liver. The present experiments point to a still more general ability of cells to segregate proteins. Such organs, for example, as pancreas, prostate, epididymis, and intestine were capable of taking up considerable amounts of peroxidase. Although tissues such as skin, and connective tissue showed relatively low concentrations (Table I), the total amount of

peroxidase taken up by these tissues throughout the body may be considerable. Practically no peroxidase was taken up by brain and peripheral nerve. The slight activity of the nerve tissue homogenate 1 hour after administration (Table I) was probably derived from adherent blood. Since brain tissue (19) and peripheral nerve tissue contain acid phosphatase (Table I, last column) and since other hydrolytic enzymes have been observed in brain (19), the failure of these tissues to take up peroxidase from the blood stream may be due to other factors affecting permeability ("blood-brain barrier"), and not to the lack of segregating ability as such.

As mentioned in the introduction, the uptake of proteins is probably the function of certain intracellular granules related to the droplets of kidney cells. The term "phagosomes" is suggested for these granules. (If these granules, characterized by segregating ability, are found to be identical with the "lysosomes" (3), the latter term can be used as a common nomenclature.) In homogenates of tissues, the phagosomes are of course diluted by an excess of other material. In order to analyze the concentrating ability of the phagosomes more accurately, they have to be separated from the homogenate and at least partially purified. It can then be determined how much more the injected peroxidase is concentrated in the fractions rich in phagosomes than it is in the original homogenate and in the other fractions separated from the homogenate (4). These observations can be correlated with the analysis of the hydrolytic enzymes and with cytochemical tests for peroxidase. The present experiments are only a first step toward this more detailed analysis.

In evaluating the data shown in Table I, it should be realized that the tissue homogenates are derived from several types of cells, such as epithelia, connective and muscular tissue, and that the incidence of the phagosomes is probably more frequent in one type of cell (epithelium) than in another. It is therefore desirable also to localize the injected peroxidase histochemically, and such work is now in progress. Some tissues such as muscle, skin, and connective tissue are difficult to homogenize. Since drastic means of homogenization were intentionally avoided, larger portion of these tissues than of other tissues remained unhomogenized. This may have caused small variations in the figures shown in Table I. Peroxidase taken up by phagocytic white blood cells probably did not contribute much to the peroxidase content of the homogenates since white blood cells are difficult to disrupt and many of them were probably removed together with the unhomogenized residue.

It may be expected that the acid phosphatase bound to the phagosomes will show some correlation with the segregation of proteins (see reference 4, Table I). However, this can be tested only after partial purification of the phagosomes and by the separation of the phosphatase bound to the granules from that "free" in the supernatant fluid. In spite of these limitations, the figures shown in Table I (last column) seem to indicate that the homogenates with high uptake of peroxidase in general also have high or average activity of acid phosphatase (kidney, liver, spleen, bone marrow, thymus, prostate, epididymis).

Table II shows that approximately 20 per cent of the peroxidase administered was excreted in the urine during the first 6 hours, and that the concentration of peroxidase in blood serum and urine fell exponentially during this time. Approximately 6 per cent of the peroxidase administered was excreted in the feces. It has not yet been determined whether a significant amount was degraded in the intestinal lumen. It may be assumed that the bulk of the injected peroxidase was metabolized within the cells of the various tissues after segregation by the phagosomes. This is in agreement with the hypothesis of de Duve *et al.* (3) concerning the function of the "lysosomes."

The observation that small amounts of peroxidase fed by means of a stomach tube were absorbed from the gastro-intestinal tract, is of interest in reference to problems of immunity and allergy. The absorption of orally fed I^{131}-labelled serum globulins in rats of different age groups was also reported by Bangham and Terry (20). The varying extent to which peroxidase was absorbed by different rats may have been caused either by the more or less effective degradation of the protein by the digestive enzymes of the gastro-intestinal tract, or by the varying degree of absorption by the intestinal epithelium in individual rats.

The question may be raised as to whether the phagosomes play a role in the process of immunity. It does not seem impossible that preliminary concentration of antigens by the phagosomes of many cells may be of some significance in antibody formation. The observations by Garvey and Campbell (21) that the retention of S^{35}-labelled protein

in liver was related to the antibody titer in the blood is of great interest in this connection.

In any discussion of the uptake of proteins by cells, reference should be made to studies of pinocytosis in ameba and tissue culture cells, and to the submicroscopic aspects of pinocytosis and phagocytosis revealed by electronmicroscopy. Lewis (22), Gey *et al.* (23), Rose (24), and others described the ability of tissue culture cells to ingest fluid droplets together with nutrients (pinocytosis). Holter and Marshall (25) observed the ingestion by ameba of large amounts of fluorescein-labelled protein and its segregation into vacuoles. Chapman-Andresen and Holter showed that C^{14}-labelled glucose is not taken up alone but is ingested together with proteins (26). Chapman-Andresen and Prescott (27) observed that pinocytosis in ameba was induced by serum albumin, lactoglobulin, peptone, tobacco mosaic virus, ribonuclease, cytochrome *c*, lysozyme, cystine, glutathione, and various solutions of inorganic salts, and that the uptake of S^{35}-labelled methionine was increased in the presence of bovine plasma albumin. The uptake of ribonuclease by ameba and other cells was also observed by Brachet (28). Reference should also be made to the interesting review by Schechtman (29) discussing the uptake of proteins by embryonic tissues.

As Palade (30) and Bennett (31) have pointed out in electron microscope studies of many cell types, the cell membrane forms narrow infoldings and invagination pockets of various sizes (usually $\sim$ 50 mμ). They have suggested that these infoldings establish connections with the membrane of the endoplasmic reticulum, and that in this way channels of communication are formed from the cell surface into the cytoplasm and in the opposite direction ("membrane flow"). Submicroscopic channels connecting the cell membrane and nuclear membrane have actually been shown to exist in Rous tumor cells by Epstein (32). If the infoldings of the cell membrane are pinched off, vacuoles containing ingested material are formed within the cytoplasm. It has been shown by electron microscopy that ingested materials such as colloidal gold particles (33), melanin particles (34), HgS, thorium oxide (35), and fat particles (36) are concentrated in vacuoles or in dense particles, and that the segregation of these materials is associated with the activity of the cell membrane. The possibility exists that the colloidal particles have to be coated with protein before being ingested (37).

It would not be surprising if this process of pinocytosis and phagocytosis on a submicroscopic level plays an important role in "active transport," in the permeability of cells, in such cellular activity as the absorptive function of the renal and intestinal epithelia, and in the secretory function of exocrine and endocrine glands. The role of the phagosomes in this process requires further analysis. In particular, it has to be ascertained whether the phagosomes of different cells contain the same enzyme spectrum as the renal droplets and the hepatic lysosomes; whether all of these granules have the ability to segregate intravenously injected proteins, and possibly other substances; and whether they all originate in the Golgi region of the cells. A point of special interest will be the study of hormonal effects. The existence of such effects is suggested by reports showing a marked influence of hormones on the intracellular levels of β-glucuronidase (38), acid RNAase and DNAase (39), and cathepsin (40); hormones also seem to have an effect on the absorption of proteins by the cells of the renal tubules (41). The observation that intravenously administered hormones, labelled with radioactive iodine, are bound to cytoplasmic granules of liver cells (42), is also pertinent to this question.

Application of the peroxidase method may facilitate the study of these problems.

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