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Studies on the Peroxidase Effect of Cytochrome c

I. The Peroxidase Activity in Subcellular Fractions from the Rat Kidney, and the Assay of Soluble Cytochrome c by Paper Electrophoresis

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Homogenates of perfused rat kidneys have been prepared in isotonic sucrose and in saline, and their subcellular fractions examined for peroxidase activity. Using a benzidine-peroxide-nitroprusside solution as screening reagent for peroxidase activity, the following results were obtained:

(1) A positive peroxidase reaction was demonstrated in all subcellular fractions.

(2) The peroxidatically active components of the soluble fraction were studied electrophoretically with paper or starch-gel as the supporting medium. The main peroxidase activity of the soluble fraction could be attributed to cytochrome c.

(3) Paper electrophoresis provided a simple and reliable routine method for the detection and assay of small amounts of soluble cytochrome c in tissue homogenates and extracts. As little as 3×10^{-2} μg cytochrome c was clearly visible by means of the peroxidase staining.

(4) Starch-gel electrophoresis constitutes a less sensitive method of detecting cytochrome c than paper electrophoresis.

(5) The peroxidase activities in the mitochondrial and the submicroscopic particle fractions are discussed.

The occurrence of peroxidase activity in the parenchymatous cells of various organs was extensively studied for the first time by Batelli and Stern [1] in 1908. They found that extracts of nearly all animal tissues were able to catalyze the peroxidation of formic acid, and one of the highest catalytic activities was demonstrated in the kidney. Maximum of activity was obtained at " a HCl concentration of about 1.5 ‰", and the reaction was believed to be due to a peroxidase derived from the parenchyma, and not to an unspecific peroxidase activity attributable to contaminant hemoglobin, since the former reaction demonstrated a temperature optimum different from the hemoglo-

bin-catalyzed reaction. Bancroft and Elliott,[2] however, could only find a weak peroxidase activity (at pH 6) in extracts from the kidneys of rabbits and rats, and since perfusion almost completely removed the peroxidase activity, they concluded that the activity was due to contamination by hemoglobin. Further, Neufeld *et al.* [3], using special methods to insure minimal interference by catalase and hemoglobin, demonstrated a low peroxidase activity (at pH 6) in homogenates from rat kidneys. They also found, however, that the total peroxidase activity of the soluble fraction could be accounted for by its content of hemoglobin, and that only a weak peroxidase activity was associated with the fraction sedimented at 75.000 × *g*, containing all the particulate fractions of the homogenate. A hydrogen donor specific peroxidase (glutathione) has also been demonstrated in rat kidneys,[4] but it showed no activity when tested with pyrogallol as hydrogen donor.

The histochemical literature on this problem also contains controversial results. Wachstein *et al.*[6] detected a labile, formalin sensitive peroxidase in the glomeruli, the collecting ducts and the thin limbs of Henle's loop of the rat kidney. This observation has in part been confirmed by Straus,[7] although Straus[8] and Drews and Engel[9] could not demonstrate any peroxidase staining in the rat kidney.

Thus, all these investigations leave the question unanswered whether or not the kidneys contain an endogenous peroxidase. The purpose of this paper is to describe a method by which peroxidatically active components can be demonstrated in all cell subfractions of the rat kidney. By means of zone electrophoresis, it is shown that the catalytic activity of the soluble fraction is attributable to cytochrome c.* Based on this technique, a new method is described for an assay of small amounts of soluble cyt. c, which is applicable to tissue homogenates and extracts.

MATERIALS AND METHODS

Experimental animals. Healthy, adult, albino rats (Wister strain from the stock of Institutt for Patologisk Anatomi, Rikshospitalet, Oslo) weighing 265—360 g were used for the experiments. They were fed a standard diet *ad libitum*, and showed a normal hemoglobin level.

Hemoproteins. Myoglobin was extracted from minced rat skeletal muscle by means of distilled water, and separated from contaminating hemoglobin by paper electrophoresis at pH 8.6 (see below). Horse radish peroxidase and hemin (bovine) was obtained from the Sigma Chemical Co., U.S.A. Lactoperoxidase from cows' milk was kindly supplied by A. Carlström, Department of Medical Chemistry, University of Umeå, Sweden. Crystalline rat hemoglobin was prepared according to Keilin and Hartree.[10] A highly purified preparation of cytochrome c from beef heart muscle was obtained by filtration on Sephadex G-75;[11] the iron content was 0.43 (8) %.

Rat serum. Serum from healthy, adult rats was used. Venous blood was allowed to clot spontaneously in a glass tube at 37°C for 1 h. It was then centrifuged (240 × *g*, 5 min, 20°C), and the serum pipetted off.

Chemicals. A. g. chemicals (from E. Merck AG, Germany, and the Sigma Chemical Co., U.S.A.) and glass distilled water were used throughout.

Preparation of kidneys for homogenization. The abdomen and pleural cavity were opened by a ventral section under ether anesthesia, and the descending thoracic aorta was isolated and transversely cut. A polyethylene tubing with an external diameter of

* The following abbreviation will be used: Cyt. c = cytochrome c.

1 mm was inserted into the distal section of the aorta, the tip of the tube reaching to about 5 mm above the right renal artery. A ligature was made just above the diaphragma and below the left renal artery. The kidneys and part of the liver and the gut were perfused at 37°C with about 100 ml of either 0.25 M sucrose or 0.15 M sodium chloride. Immediately upon their removal the kidneys, which appeared quite pale both on the surface and in sections, were chilled over crushed ice and trimmed of visible fat and connective tissue at the renal hilus.

Isolation of subcellular fractions. Each kidney was homogenized in 9 volumes of 0.25 M ice-cold sucrose with a teflon-glass Potter-Elvehjem homogenizer. The subcellular fractions obtained by fractionated centrifugation,[12] were examined by phase contrast microscopy. The $700 \times g$ fraction (unbroken cells, nuclei, *etc.*) was not re-homogenized. The $5000 \times g$ fraction (mitochondria) and the $57000 \times g$ fraction (submicroscopic particles) were washed with 3 × 8 ml 0.25 M sucrose [12] and finally resuspended in 4 ml of 0.25 M sucrose. The volume of the soluble fraction was approximately 24 ml.

To obtain a higher concentration of the soluble fraction for the electrophoresis experiments, the kidneys were homogenized with an equal volume of 0.25 M sucrose or 0.15 M sodium chloride. The homogenate in both media was centrifuged ($105000 \times g$, 30 min, 4°C) giving a tightly packed pellet and a clear yellow-brown supernatant with an almost colourless turbid top layer including some floating lipids. The clear fluid, which contained the soluble peroxidase activity from the homogenate, was pipetted off, care being taken to avoid contamination from the other fractions.

A Spinco model L ultracentrifuge with rotor No. 40 was used for this work.

Paper electrophoresis was carried out in a LKB apparatus (Type LKB-3276, Stockholm, Sweden), with Whatman No. 1, No. 3 MM, and No. 31 (acid cleaned) filter paper. Four 40 × 410 mm strips were run at the same time. Buffer solutions of different pH-values were used: (1) A tris-EDTA-boric acid buffer, pH 9.0;[13] (2) a veronal-acetate buffer, pH 8.6;[14] (3) a phosphate buffer, pH 7.0;[15] and (4) an acetate buffer, pH 4.0.[16] The samples were applied 5 cm from the middle of the paper on the cathodic side by drawing a fine capillary pipette repeatedly along each of the starting lines, and run over-night (14 h) at room temperature with about 5 V/cm. The strips were dried immediately in a tempera-ted air stream.

The proteins were stained with a 1 % (w/v) solution of Amido black 10 B.[17]

The peroxidase active components were located by a benzidine-peroxide-nitroprusside reagent, which was prepared exactly 1 min before use, and had the following composition: Benzidine 0.3 g, barium peroxide 2.4 g, sodium nitroprusside 0.15 g, and acetic acid (25 %, v/v) 60 ml, giving a final pH of 3.45. The paper strips were immersed for 1 min in this solution, and then washed thoroughly for 5 min in five successive baths with distilled water.

Catalatic activity was located by immersing the electrophoresis strips in a freshly prepared 0.1 M solution of hydrogen peroxide in 0.01 M phosphate buffer of pH 7.0. The active zones could easily be identified by the development of bubbles of oxygen. The zones were lightly traced on the filter paper with a pencil when still wet.

Starch-gel electrophoresis [18] was carried out with a discontinuous system of buffers (tris-citrate for the starch-gel and boric acid-sodium hydroxide for the bridge compart-ments [19]). A plexiglas tray with six parallel compartments (25 × 16 × 6 mm) was used. A piece of filter paper (Whatman No. 3 MM) was soaked in the test material and intro-duced by making transverse cuts in the gel 30 mm from the middle of the gel on the cath-odic side. The electrophoresis was run for 4 h at room temperature with 3 mA per gel and about 180 V. The heating of the gel during electrophoresis was negligible, but any evaporation was prevented by a thin layer of paraffin wax. Each gel was sliced into two layers, the top layer being stained for 1.5 min with a saturated solution of Amido black 10 B,[18] and the bottom layer with a freshly prepared benzidine reagent. The gel was covered with a filter paper which was moistened with the reagent using a cotton wool pencil. After 2 min the filter paper was removed, and the reagent in excess removed by washing in distilled water. After staining, the gels were preserved in methanol-distilled water-glacial acetic acid (5:5:1, v/v). In this way, good contrast was obtained for photo-graphy and the blue peroxidase-positive zones could be preserved for several days.

Continuous-flow paper electrophoresis was carried out using a modification of the apparatus described by Holdsworth.[20] A veronal-acetate buffer, pH 8.6, ionic strength 0.06, was used for the paper, the buffer reservoir and the electrodes. The electrophoresis

was run for 72 h at room temperature with about 4 V/cm, and the filter paper was fed continuously by means of a plastic tube. Twenty fractions were collected from the paper curtain (tube No. 1 at the cathode), and they were examined for materials absorbing in the visible and ultraviolet region of the spectrum (range: 650—380 mμ). At the end of the electrophoresis run, the paper was immediately dried, sprayed with a freshly prepared solution of the peroxidase reagent, and washed in distilled water.

An LKB apparatus (Type 3371 A, Stockholm, Sweden) served as power supply in all the electrophoresis experiments.

Preparation of ^{59}Fe-labelled hemoglobin. One adult, male albino rat weighing 324 g was bled to give a fall in the hemoglobin concentration from 15.5 to 8.9 g/100 ml. Two days later, the rat was injected intraperitoneally with 106 μC (156 μg iron) of [^{59}Fe] citrate "Abbott". Four days after the injection the rat was killed by exsanguination through the inferior vena cava under light ether anesthesea, and the blood collected in 3.1 % (w/v) citrate. The blood was centrifuged (320 × *g*, 5 min, 20°C), and the plasma discarded. The erythrocytes were washed three times in 0.15 M saline and then preserved. The radioactivity was found to be 733.140 counts/min/ 1 ml red cell mass.

Radioautography. Dried electrophoresis strips were placed in contact with an X-ray film (Kodak) in suitable cassettes. The film was exposed for 3 weeks.

Assay of radioactivity. The zones on the paper electropherograms containing the peroxidase active components, were cut out and put into a glass tube containing 3 ml of a solution of sodium dichromate in concentrated sulphuric acid. The paper strips were digested, and the ^{59}Fe activity was measured in a well scintillation counter. The volume of the sample counted was always 3 ml.

Spectrophotometry. Absorption spectra were obtained using a Beckman DU spectrophotometer with 1 cm quartz cells. The concentration of cyt. c was determined after reduction with sodium dithionite in 0.065 M phosphate buffer, pH 6.8 using the extinction coefficient $E^{1\,\%}_{1\,cm}$ 550 mμ (red) = 22.6.[21]

Photoelectric scanning. The electropherograms and radioautograms were scanned using a Beckman Model RB Analytrol. The areas under the densitometer curve were measured by cutting and weighing the paper. The integral record was not used for quantification.

pH-meter. A glass electrode pH-meter (Radiometer, Copenhagen, Denmark, Model 22) was used. Accuracy ± 0.05 pH-unit.

RESULTS

Investigations on the subcellular fractions

Peroxidase activity, as well as catalase activity, was demonstrated in all the cell subfractions. When, *e.g.*, 15 μl of the respective fractions were applied on a Whatman No. 1 filter paper, dried and stained with a freshly prepared benzidine-peroxide-nitroprusside reagent, precipitates of "benzidine blue" were immediately obtained at each fraction; the most intensive colour appeared at the mitochondrial fraction. No colour was seen without the addition of peroxide to the reagent, and the blue colour obtained must be due to peroxidase active components and not to the action of benzidine oxidases. Further, when *e.g.* 100 μl of the different fractions were added to 5 ml of the hydrogen peroxide solution (0.01 M in phosphate, pH 7.0), the latter was rapidly decomposed, filling the solution with bubbles of oxygen. This indicated the presence of catalase activity in all the cell subfractions.

Investigations on the soluble fraction

Separation of peroxidase active components by means of zone electrophoresis. The migration pattern of the soluble fraction in isotonic saline on paper

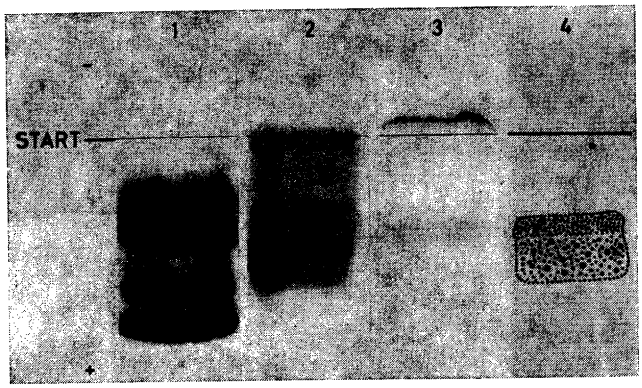
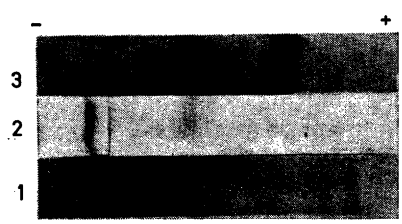


Fig. 1. Migration patterns on paper electrophoresis of proteins: (1) Normal rat serum stained with Amido black; (2), (3), and (4) the soluble fraction of perfused kidneys homogenized with an equal volume of isotonic saline. (2) Amido black staining shows no distinct zones, but only a "diffuse" pattern without serum albumin. (3) Benzidine-peroxide-nitroprusside staining shows two peroxidase active zones; one migrating towards the anode (free hemoglobin) and one migrating towards the cathode (cytochrome c). (4) Site for catalatic reaction. The dotted area corresponds to the development of gas bubbles on the filter paper. The size of the dots indicate the intensity of gas development. 10 μl of the material were applied to each paper strip. Veronal-acetate buffer, pH 8.6.

electrophoresis is demonstrated in Fig. 1. Amido black staining showed no distinct zones, but only a "diffuse" pattern mostly of tissue proteins, since no zone attributable to albumin could be seen when compared with rat serum run in parallel. Two zones migrating in opposite directions regularly appeared with the peroxidase staining. At increasing acidities (pH 8.6, 7.0, and 4.0) both fractions increased the mobility towards the cathode. It can be seen from Fig. 1 that in the positively charged zone (at pH 8.6) there is a feebly stainable zone travelling in front of the main portion. The migration pattern on starch-gel (Fig. 2) was in good agreement with that on paper, but the "cathodic" component always appeared homogeneous, and the staining reactions were weaker for equal quantities. No colour developed on paper strips or on starch-gel slices when peroxide was omitted. Thus, it is obvious that the zones of "benzidine blue" were the products of peroxidase and not of benzidine oxidase reactions. Amido black staining resulted in a weak colour correspon-

Fig. 2. Migration pattern on starch-gel electrophoresis of the proteins in the soluble fraction (the same preparation as in Fig. 1). Amido black staining (1) shows several distinct zones migrating towards the anode. Peroxidase staining (2) shows one zone migrating towards the anode (free hemoglobin) and one zone migrating towards the cathode (cytochrome c). Normal rat serum (3) stained with Amido black. Tris-citrate buffer, pH 8.65.



ding to the "cathodic" component when 50 μl, but not 10 μl of the fraction were applied.

At the completion of electrophoretic separation one of the dried paper strips was immediately placed in hydrogen peroxide. An evolution of oxygen bubbles occurred only from one zone (Fig. 1). The reaction was slight over the hemoglobin band, and intense over the zone just in front of the latter band. This zone, from which vigorous evolution of oxygen occurred, is considered to contain catalase. No reaction was seen on the cathodic side.

Continuous-flow paper electrophoresis revealed good resolution with three zones stainable with the peroxidase reagent. The collected materials in tubes Nos. 13 and 14 (on the anodic side) demonstrated the absorption bands of methemoglobin, but in tubes Nos. 9 and 10 (on the cathodic side) only a weak Soret band without measurable bands in the visible was obtained (tube No. 9, $A_{415} = 0.059$). The electrophoretic mobility of the weakly stainable peroxidase active zone, converging towards tube No. 16, corresponds well with that obtained for the complex of hemoglobin and the serum protein haptoglobin.[22] This was confirmed in a separate experiment by addition of rat hemoglobin to rat serum. Because of the low concentration of haptoglobin in the soluble fraction after the perfusion of the kidneys, the conventional electrophoresis technique (see above) failed to disclose it.

When the soluble fraction from a homogenate in 0.25 M sucrose was subjected to zone electrophoresis, the same results were obtained, although the peroxidase active zone on the cathodic side was weaker.

The isolation and identification of the peroxidase active component on the cathodic side. The elution of this zone succeded only when zone electrophoresis were performed on an acid-cleaned Whatman No. 31 filter paper and with a tris-EDTA-boric acid buffer of pH 9.0. One ml of the soluble fraction was applied to the filter paper, and the electrophoresis was run for 16 h at a potential gradient of 4.5 V/cm. A narrow pink-coloured zone, well separated from the other protein fractions, appeared on the cathodic side and could be

Table 1. The radioactivity and peroxidase activity of the two peroxidase active components separated by means of paper electrophoresis as demonstrated in Fig 3. Experimental details are given in the text.

Material[a]	Radioactivity[b]		Peroxidase activity (peak area, cm²)
	($n_s \pm y$) counts/min[c]	Net counts/min	
Anodic (Hb) component	4333 ± 174	4104	25.4
Cathodic (cyt. c) component	244 ± 56	15	5.4

[a] 100 μl of the soluble fraction were applied to a Whatman No. 3 MM filter paper.
[b] The total counts collected were always sufficient to obtain a coefficient of variation < 1 %, calculated from the formula $C.V.$ (%) = $(100 \times n^{\frac{1}{2}})/n$ (where n = total counts collected).
[c] The probable error, $y = 2.576\ (n_s + n_b)^{\frac{1}{2}}$ ($p = 0.01$); the background (n_b) = 229 counts/min.

eluted with distilled water. It was spectrophotometrically identified as ferrocytochrome *c*. Only a minimal increase in the intensity of the absorption bands was observed upon the addition of dithionite.

Radio-iron studies. The absorption spectrum indicates that cyt. c had been obtained at a high degree of purification. To exclude the possibility of contamination by hemoglobin, an experiment with ^{59}Fe-labelled hemoglobin was performed. Perfused kidneys from one rat were homogenized in an equal volume of saline together with 0.3 ml of the suspension of ^{59}Fe-labelled erythrocytes. The soluble fraction was subjected to paper electrophoresis, and the paper

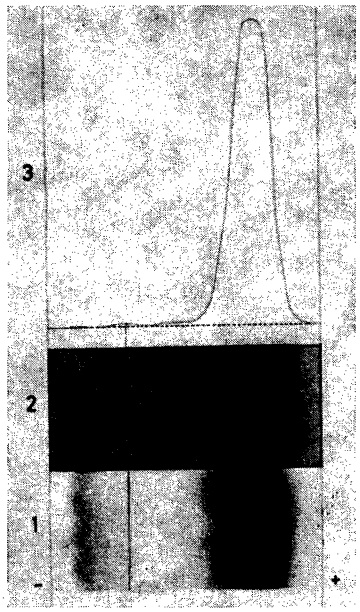


Fig. 3. Migration pattern on paper electrophoresis of the peroxidase active components of the soluble fraction in the radioisotope experiment (see also Table 1). 100 µl of the soluble fraction were applied to a Whatman No. 3 MM filter paper. (1) Two zones were stainable with the peroxidase reagent; one migrating towards the anode (free hemoglobin) and one towards the cathode (cytochrome c). (2) Radioautogram of the electrophoresis strip (1), and (3) the curve obtained by scanning this radioautogram. Note that there is no measurable blackening of the film corresponding to the peroxidase active zone attributable to cytochrome c. Veronal-acetate buffer, pH 8.6. For details see text.

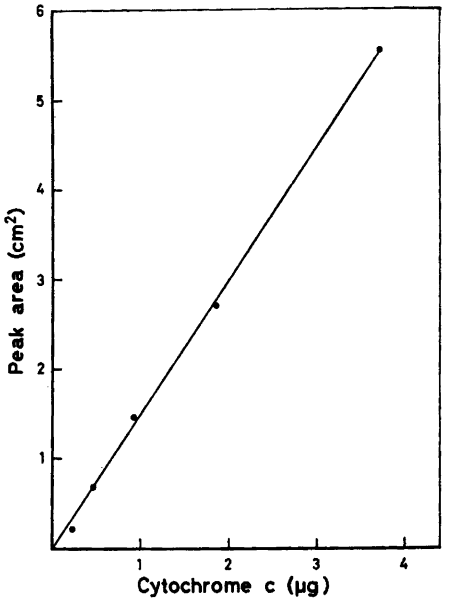


Fig. 4. Reference curve for the assay of cytochrome c. After electrophoresis (standard procedure, pH 8.6) the papers were stained with the peroxidase reagent, and the areas of the scanned peaks plotted against the amounts of cytochrome c (highly purified beef heart cytochrome c with Fe content 0.43 (8) %). Volumes of 10 µl with varying concentrations were consistently used. For details see text.

strips examined for radioactivity (Fig. 3 and Table 1). The discrepancy in the ratio between radioactivity and peroxidase activity of the two components (Hb and cyt. c) is clearly demonstrated, and did not support the possibility of any contamination of the cyt. c zone by hemoglobin.

The assay of soluble cytochrome c

Paper electrophoresis with Whatman No. 1 filter paper and a veronal-acetate buffer of pH 8.6 permitted the assay of microquantities of soluble cyt. c in crude homogenates of rat kidneys. After staining, cleaning and drying (see methods), the electropherograms were scanned, and the peak areas measured and compared with a standard reference curve (Fig. 4). There is a straight-line relationship between the amount of cyt. c and the peak area. As little as 3×10^{-2} μg of cyt. c was detected by the naked eye, and the densitometer was sensitive to a minimal amount of about 1×10^{-1} μg cyt. c. With a tris-EDTA-boric acid buffer for the electrophoresis, the peroxidase staining was less sensitive since EDTA inhibits the peroxidase reaction.

The lack of specificity of the staining reaction. Hemoglobin, free or bound to haptoglobin, myoglobin, and hemin as well as lactoperoxidase and horse radish peroxidase were applied to paper electrophoresis strips, and treated with the peroxidase reagent. All compounds gave a strongly positive reaction (permanent blue precipitate) with this stain.

DISCUSSION

A benzidine-hydrogen peroxide reagent, in various modifications, has been widely used for the staining of hemoglobin and its derivatives, free or bound to serum proteins, in paper chromatography,[23] and in paper [24] and starch-gel electrophoresis.[19,25,26] "True" peroxidases from animals and plants may also be demonstrated in this way, *e.g.* myeloperoxidase.[27] The pH of the reagent solution, however, is seldom controlled by means of buffers. In the present assay a low pH (3.45) was used, since optimum colour development was observed at this pH. The reaction product of oxidized benzidine, usually obtained,[23-26] is labile, changing in colour from dark blue to purple, brown or green and fading within minutes, and appreciable background staining is seen. Thus, photography must be carried out at the optimal development of colour, while the paper is still wet, and quantitation by photoelectric scanning is difficult. Pickworth,[28] however, stained erythrocytes in tissue sections, and obtained a stable blue product of oxidized benzidine by the addition of sodium nitroprusside. The observation was confirmed in the present investigation. The reagent, which also contained nitroprusside, gave a permanent blue precipitate of oxidized benzidine at the site of peroxidase reaction. The precipitate was insoluble in the staining solution and withstood thorough washing in distilled water, and the colour did not fade during storage for more than a year. Moreover, no background staining was observed. The optimum concentration of nitroprusside (8.4 mM) was found empirically. When a lower concentration was used, the blue reaction product partly dissolved in the staining and

rinsing solutions, and a higher concentration gave an inhibition of the colour development.

Highly reproducible electrophoretic patterns were obtained using the soluble fraction of homogenates of perfused rat kidneys, irrespective of whether paper or starch-gel was used as supporting medium. The inhomogeneity of the peroxidase staining of the cyt. c zone in some of the experiments (*e.g.* that in Fig. 1), is in good agreement with the different electrophoretical mobilities reported on ferro-and ferri-cytochrome *c*.[29,30]

In their fractionation studies on rat liver, Schneider and Hogeboom [12] were unable to demonstrate cyt. c spectrophotometrically [31] in the soluble fraction. However, with the same homogenization procedure on rat kidney and liver, the method described in the present paper demonstrated cyt. c in as little as 10 μl of the soluble fraction. As little as 3×10^{-2} μg of cyt. c was clearly visible after the peroxidase staining.

The subcellular distribution of catalase activity in all the cell subfractions of the rat kidney homogenate is in good agreement with earlier observations on rat liver.[32-34]

In the mitochondrial fraction a strongly positive peroxidase reaction was obtained. This fits in well with the known localization of cyt. c to the mitochondria,[12] and with the mitochondrial localization of peroxidase activity at about pH 3.6 which has recently been demonstrated in freeze-dried tissue sections of perfused rat kidneys.[35]

The submicroscopic particles also exhibited some peroxidase activity. In rabbit liver, a peroxidase activity with its optimum at pH 5.3, using guaiacol and benzidine as hydrogen donors, has been demonstrated in the ribonucleoprotein particles,[36] and the active component identified as cytochrome b_5. This cytochrome may possibly account for the weak peroxidase activity in the submicroscopic particles of the rat kidney. The question of whether the activity is partly attributable to cyt. c, as could be expected from radioisotope experiments on the re-distribution of this hemoprotein during homogenization,[37] cannot yet be answered.

In conclusion, the present studies have confirmed the observations made by Batelli and Stern [1] many years ago that kidney extracts from various animals are able to catalyze the peroxidation of a suitable hydrogen donor in acid solution. The catalytic activity is attributable to cyt. c. The reaction represents a new approach in the study as well as in the assay of cyt. c and attempts will therefore be made to elucidate its mechanism.

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