PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 322-324 (1977)

A Soluble Peroxidase in Heart and Skeletal Muscle of Rat and Mouse[1] (39663)

ZOILO GONZALEZ-LAMA[2] AND ROBERT N. FEINSTEIN[3]

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

The existence of peroxidase in both soluble and insoluble forms was first noted by Willstätter and Pollinger (1) in horseradish, and later by Bancroft and Elliott (2) in animal tissues. The demonstration of true peroxidase activity in many animal tissues is complicated by the concomitant presence of hemoglobin, which has peroxidatic activity, and of catalase, which not only has peroxidatic activity, but also actively degrades the H_2O_2 which is needed for the activity of peroxidase.

The relatively recent advent of techniques for isoelectric focusing on polyacrylamide gel now permits the definitive physical separation of these several proteins. We wish to report here the existence of soluble peroxidatic activity in rat and mouse heart and skeletal muscle, and the absence of comparable soluble peroxidatic activity from a variety of other tissues.

Materials and methods. Mice used were C3H/An1 adults of both sexes; no sex-dependent differences in peroxidatic activity were noted. Rats were adult Charles River Sprague-Dawley males. Mice were killed by cervical dislocation; rats, by ether inhalation. Desired tissues were removed, washed briskly in cold distilled water, blotted on filter paper, homogenized in glass-Teflon Potter-Elvehjem homogenizers with 9 vol of cold 0.25 *M* sucrose, and centrifuged at 48,000*g* for 30 min. The supernatant fractions were used to obtain the results described below; some work with the precipitate fraction is also discussed.

Isoelectric focusing on polyacrylamide gel was performed using the Multiphor apparatus (LKB Produkter, Bromma, Sweden), modified as described elsewhere (3). Peroxidase activity was detected by covering individual gel slabs with a mixture containing 0.06% H_2O_2 and unbuffered 0.2% benzidine hydrochloride. Homovanillic acid and *o*-dianisidine were also tested as substrate and gave comparable results; however, benzidine was by far the more sensitive.

Chemicals purchased were the best quality available from common sources and were not further purified.

Results and discussion. Figure 1 is a series of photographs, with counterpart drawings for clarity, demonstrating peroxidase activity in the soluble fraction of mouse heart, and its absence from the soluble fraction of mouse liver and lung. A sample of mouse hemoglobin (lysed erythrocytes) was included (first slide), and it can be seen from the figure that hemoglobin was present in each soluble fraction, but other peroxidase bands were detected only in heart and not in liver or lung.

Figure 2 shows that rat heart and skeletal muscle contain soluble peroxidase activities that are not hemoglobin. Two of the three heart muscle peroxidase isozymes appear to be identical to the two present in skeletal muscle.

Overlapping activities of the various heme proteins is a frequent source of difficulty and ambiguity in the measurement of peroxidase activity. Various techniques have been employed to remedy this situation. For example, the peroxidatic activity of hemoglobin is frequently corrected for by measuring the hemoglobin present and calculating the peroxidatic activity of that amount of hemoglobin (4). Also, 2,6-dichlorophenol has sometimes been added to peroxidase assay mixtures to inhibit catalase activity (5).

The clearest demonstration of peroxidase activity, however, would seem to be the physical separation of the various proteins, which is readily accomplished by isoelectric focusing on polyacrylamide gel. Hemoglobin has an isoelectric point (p*I*) spreading over the range of about pH 6.9-7.4 in the

[1] Work supported by the United States Energy Research and Development Administration, and by the Ministerio Educacion y Ciencia, Spain.

[2] Present address: Departamento Microbiologia, Facultad Ciencias, Universidad Granada, Spain.

[3] To whom correspondence should be addressed.

ISSN 0037-9727

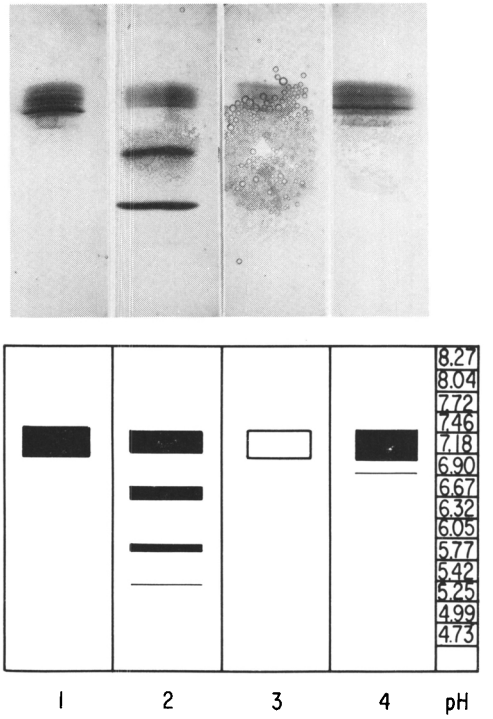

FIG. 1. Isoelectric focusing of mouse hemoglobin (lysed erythrocytes) and soluble fractions from heart, liver, and lung. Drawings below are counterparts of photographs above, for easier visualization. Slide 1: Lysed erythrocytes (diluted by visual inspection to the approximate color of the heart extract). Slide 2: Soluble fraction of heart. Slide 3: Soluble fraction of liver. Slide 4: Soluble fraction of lung. For each sample, 25 μl was applied to the gel. The cathode is at the top; anode, at the bottom.

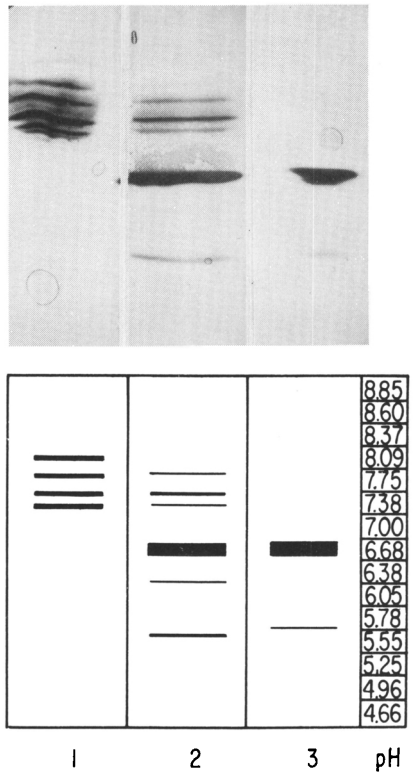

FIG. 2. Isolectric focusing of rat hemoglobin (lysed erythrocytes) and soluble fractions of heart and skeletal muscle. Slide 1: Lysed erythrocytes. Slide 2: Soluble fraction of heart. Slide 3: Soluble fraction of skeletal muscle. Details are as in Fig. 1, except that soluble fractions from 10% homogenates were used in Fig. 1, and from 20% homogenates in Fig. 2.

mouse, and about pH 7.4–8.1 in the rat. Catalase, which is readily identified on the photographs by the area of oxygen bubbles, has a somewhat lower p*I*. Catalase is well known for its diffuse banding, either isoelectrically or on standard polyacrylamide or starch electrophoresis gels; this diffuseness is readily to be seen in a multitude of recent papers (for examples, see 6, 7). The most anodal lines (pH 6.7, 5.8, and 5.3) in Fig. 1 must be considered to represent true peroxidase.

Commercial beef heart cytochrome *c*, at 0.1 mg/ml, produced no lines on the gels under the conditions employed. This is not an unexpected finding, because the p*I* of cytochrome *c* has been stated (8) to be 9.8 or higher; this is beyond the range of the gels used. The possibility that the benzidine staining is due to some nonperoxidase hemoprotein other than hemoglobin, catalase, or cytochrome *c* cannot be ruled out at present. We have not, however, been able to specify any other known hemoprotein that might be responsible.

Bancroft and Elliott (2), in measuring the peroxidase activity of a variety of tissues from the rat and the rabbit, used well-pulverized whole tissue. Neufeld *et al.* (9) used only the resuspended insoluble portion of tissues for peroxidase assays. Bancroft and Elliott (2) found lung and spleen to have the greatest peroxidase activity of any tissues tested. Neufeld *et al.* (9) found lung and spleen to have 20–30 times the peroxidatic activity of heart and skeletal muscle; Bancroft and Elliott (2) did not test these latter two tissues. After isoelectric separation on polyacrylamide gels, we found in mouse or rat lung, spleen, liver, kidney, or brain no

soluble peroxidatic activity that could not be accounted for as hemoglobin. We also attempted to solubilize the insoluble peroxidase of lung and spleen with urea or chlorhexidine, as we successfully did with dog and pig thyroid peroxidase; however, no true peroxidase lines could be demonstrated on Multiphor gels.

We suggest that the discrepancy between the present results and those of others lies in the fact that intact erythrocytes remain in insoluble fractions of tissues prepared with isotonic or hypertonic extractive agents. In our experience, the insoluble fractions of lung and spleen, prepared with 0.25 *M* sucrose, showed strong coloration and strong peroxidatic activity with benzidine as substrate when resuspended in distilled water; however, when the resuspended material was centrifuged, the new supernatant continued to exhibit color, but on Multiphor gels the only "peroxidase" bands were those cathodic lines (pH 6.9–7.4) that we associate with hemoglobin.

In mouse heart, on the other hand, soluble peroxidase activity was found by the benzidine assay. We measured the hemoglobin content of the preparation and corrected for the peroxidatic activity of the hemoglobin. It was found that the hemoglobin could account for *all* peroxidase activity, yet on Multiphor definite nonhemoglobin peroxidase lines were observed. This suggests that the commonly used correction for the peroxidatic activity of hemoglobin is at best a semiquantitative measure.

Summary. Several peroxidase isozymes have been demonstrated in the 0.25 *M* sucrose-soluble portion of mouse and rat heart and skeletal muscle. Isoelectric focusing on polyacrylamide gel was used to show that the peroxidatic activity is not due to hemoglobin, catalase, or cytochrome *c*. No true peroxidase activity was detected in either the soluble or insoluble fraction of mouse liver, kidney, brain, spleen, or lung.

1. Willstätter, R., and Pollinger, F., Untersuch. Enzyme **1,** 512 (1928).
2. Bancroft, G., and Elliott, K. A. C., Biochem. J. **28,** 1911 (1934).
3. Feinstein, R. N., Anal. Biochem. **72,** 533 (1976).
4. Banerjee, R. K., and Datta, A. G., Endocrinology **88,** 1456 (1971).
5. Neufeld, H. A., Lucas, F. V., Martin, A. P., and Stotz, E., Cancer Res. **15,** 550 (1955).
6. Matsubara, S., Suter, H., and Aebi, H., Humangenetik **4,** 29 (1967).
7. Jones, G. L., and Masters, C. J., Arch. Biochem. Biophys. **169,** 7 (1975).
8. Florkin, M., and Stotz, E., "Comprehensive Biochemistry," vol. 7, p. 25. Elsevier, New York (1963).
9. Neufeld, H. A., Levay, A. N., Lucas, F. V., Martin, A. P., and Stotz, E., J. Biol. Chem. **233,** 209 (1958).

Received September 1, 1976. P.S.E.B.M. 1976, Vol. 154.