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Renal Lysosomes: Role in Biogenesis of Erythropoietin

Abstract. *The "light" mitochondrial pellet obtained from the kidneys of rats previously treated with Triton WR-1339 and rendered hypoxic was separated into subcellular component fractions by sucrose density gradient centrifugation in a zonal rotor. Selected fractions were pooled, disrupted by osmotic lysis and repeated freeze-thawing, and incubated in the presence and absence of normal rat serum. The incubation mixtures were assayed for erythropoiesis-stimulating activity (erythropoietin). High specific activity was identified only in fractions rich in lysosomes. Biochemical analysis of reference enzymes for the identification of lysosomes and mitochondria, supplemented by electron microscopic examination of the various separated fractions, supports the observed requirement for lysosomal constituents in the formation of erythropoietin by the kidney.*

In several studies (*1*, *2*) the biosynthesis of the hormone erythropoietin (Ep) has been related to the interaction between a renal erythropoietic factor, erythrogenin, presumably enzymatic in nature, and a substrate present in normal serum. The association of the renal erythropoietic factor with a particulate fraction of the rat kidney (*1*) has led to attempts to characterize the subcellular entity involved in erythrogenin formation or sequestration. Recovery of the factor from both nuclear (*3*) and "light" mitochondrial (*4*) fractions of homogenates of hypoxic kidney has been reported. Recently the lysosome has been implicated as the site of erythrogenin localization (*5*).

Male Long-Evans rats weighing 250 to 400 g received a single intraperitoneal injection of the nonionic detergent Triton WR-1339 (Rohm and Haas) at a dose of 85 mg per 100 g of body weight (*6*). After 77 hours, the rats were exposed to 0.45 atm of air for 19 hours in a hypobaric chamber and then immediately killed by cervical dislocation. Kidneys were rapidly removed, freed of perirenal fat and fascia, decapsulated, and finally homogenized in a Potter-Elvehjem homogenizer in ten volumes of 0.25*M* sucrose solution. These and all subsequent preparative steps were performed at 4°C. The initial homogenization sequence was accomplished with a Teflon pestle precisely milled so as to establish a mortar-pestle clearance of 0.51 mm. A final homogenization was achieved by substituting a wider pestle with a clearance of 0.14 mm. The homogenizer drive motor was operated at 720 rev/min.

The light mitochondrial pellet employed in these experiments was the material sedimenting between 6000*g* (10 minutes) and 21,000*g* (30 minutes) and was prepared in a rotor possessing a fixed angle of 34°. Pellets were resuspended in 0.25*M* sucrose solution and introduced into a Spinco Ti-14 zonal rotor which had been previously filled with a linear sucrose gradient extending from 36 to 52 percent. Centrifugation in the gradient was at 102,000*g* for 20 hours (about $12 \times 10^7 g$-min). Fractions were collected from the rotor in 65 10-ml portions. Sucrose density and protein determinations by the biuret method (*7*) were immediately conducted on alternate fractions; on the basis of these data and irregularly spaced cytochrome oxidase measurements (*8*) for the localization of the mitochondrial population within the gradient, fractions were pooled. Five such pooled fractions were selected for Ep bioassay in exhypoxic, polycythemic mice (*9*) following hypotonic disruption of the particles in distilled water and ten freeze-thaw sequences. The pooled fractions were incubated with either saline or normal rat serum (NRS) before assay. Values for 48-hour incorporation of ^{59}Fe into red blood cells (RBC) were converted to international reference preparation (IRP) units of Ep by reference to a standard dose-response curve prepared simultaneously with the assay of the fractions. Specific activities of the pooled fractions were expressed as net IRP units of Ep formed per milligram of protein administered to the assay mice.

Portions of pellets of the pooled fractions were prepared for electron microscopic examination. Portions of fractions from the zonal rotor taken prior to pooling were assayed for both cyto-

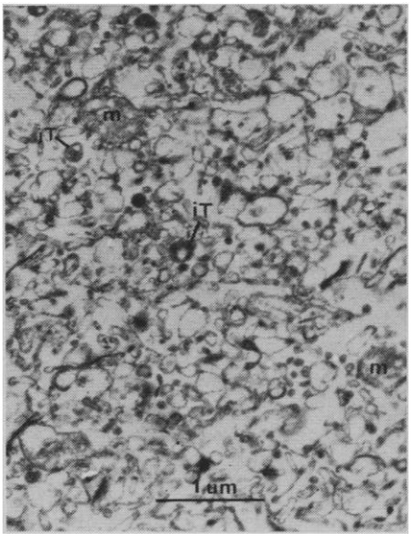

Fig. 1. Random electron micrograph through the midportion of the pellet of pooled fraction 18–22 from experiment B. The large, electron-lucid vesicles are Triton-treated lysosomes. While several mitochondrial profiles (*m*) are evident, the fraction is essentially free of other contaminants. Several lysosomes reveal dense matrices enclosing electron-lucid spaces. These are identified as lysosomes in which the Triton accumulation process is incomplete (*iT*). Fixation in 2.5 percent glutaraldehyde solution followed by 1 percent osmium tetroxide; sections stained with uranyl acetate.

chrome oxidase and acid phosphatase, with *p*-nitrophenyl phosphate as substrate for the latter enzyme (*10*). In a single experiment the acid phosphatase determination was performed in duplicate, in the presence and in the absence of 0.2 percent Triton X-100. Enzyme activity of the fractions was not significantly elevated in the presence of this detergent.

The three experiments reported in detail (Table 1) have been selected from among 23 zonal separations completed to date. The detergent Triton WR-1339 used in these experiments possesses no erythropoiesis-stimulating effects per se but is selectively absorbed by lysosomes, altering their median equilibrium density from 1.21 to 1.10 g/cm^3 (*6*). In experiment A, high specific activity was observed in pooled fractions 48–52, 53–57, and 58–63, extending across the density range 1.21 to 1.24 g/cm^3 with peak activity at 1.21 to 1.22 g/cm^3, the frequently reported density of the lysosome not treated with Triton (*11*). Also, the pooled fraction demonstrating highest specific activity contained primarily lysosomes and microbodies, as indicated by examination in the electron microscope.

In experiment B, high specific activity was observed in pooled fractions 18–22 and 46–50 isolated in the density regions 1.17 to 1.18 g/cm^3 and 1.21 g/cm^3, respectively. The density at which the former particles were recovered suggests the incompleteness of the Triton accumulation process. This might be accounted for by the absorption of the major portion of the delivered dose of Triton WR-1339 to hepatic lysosomes, which, because of the vascular drainage of the peritoneal cavity, may accumulate the detergent before it reaches the kidney. Alternatively, if lysosomes were made more fragile by the absorption of Triton WR-1339 and were then damaged either during homogenization or in the protracted zonal centrifugation, their sedimentation properties would probably be altered.

The observation that the major constituents of pooled fraction 18–22 in experiment B were Triton-treated lysosomes (Fig. 1) bearing striking resemblance to those depicted by Beaufay (*12*) lends further support to the contention that erythrogenin is a lysosomal constituent. No microbodies were found when pooled fraction 18–22 was examined with the electron microscope. Although this observation per se does not completely exclude the possible role of microbodies in the biosynthesis of Ep, material recovered from the gradient at a density of 1.23 g/cm^3 possessed considerably less Ep specific activity than did the particle population recovered from the gradient at a density of 1.21 g/cm^3. Supporting the lysosome concept still further is the observation that high acid phosphatase specific activity was identified in the rotor outflow fractions comprising pooled fraction 18–22.

The data of experiment C again suggest incomplete Triton treatment, because high specific activity was observed only in pooled fraction 42–46, which equilibrated in the gradient at a density of 1.21 g/cm^3. In this experiment, acid phosphatase specific activity was highest in fraction 12 and was virtually undetectable in the fractions comprising pooled fraction 42–46. The distribution of this reference enzyme suggests the existence of several biochemically discrete populations of lysosomes in the rat kidney. On the basis of the differential distribution of lysosomal hydrolases and of other properties of the particles, it has been suggested (*13*) that lysosomes comprise a biochemically and a physiologically heterogeneous population.

The identification of the particles of low density and high specific activity as Triton-treated lysosomes is thus based on four criteria: (i) they resemble the particles identified by Beaufay (*12*) as Triton-treated lysosomes and are limited by a single membrane possessing characteristic lysosomal thickness (about 90 Å); (ii) their equilibrium densities in sucrose gradients resemble those of lysosomes; (iii) these particles generally reveal high acid phosphatase activity; and (iv) high Ep specific activity does not correlate positively with either mitochondria-rich or microbody-rich pooled fractions.

When mitochondria recovered from the zonal rotor in high purity (> 90 percent, as judged from random electron micrographs) were incubated in the presence or absence of NRS, the Ep specific activity, although variable, was always low. Cytochrome oxidase determinations on fractions collected from the rotor revealed that, of the pooled fractions with high Ep specific activity, all but a few contained either undetectable or slight amounts of this reference enzyme.

To verify that the substance that was formed during incubation of the lyso-

Table 1. Erythropoiesis-stimulating activity of fractions recovered from a linear sucrose gradient in a zonal rotor. In the three experiments represented, the "light" mitochondrial fractions used for zonal centrifugation were prepared from the kidneys of hypoxic rats that had been treated with Triton WR-1339 before hypoxia was induced. In experiments A and B, values for the bioassay standards for Ep (given as percentage of ^{59}Fe incorporated into RBC in 48 hours) were 0.53 ± 0.15 for 0.9 percent NaCl, 3.28 ± 0.41 for 0.05 IRP unit of Ep, and 19.00 ± 1.58 for 0.20 IRP unit of Ep. In experiment C, the corresponding values for the three standards were 1.08 ± 0.17, 4.42 ± 1.61, and 15.20 ± 2.19. The specific activity of Ep is given as 10^3 times the net IRP units of Ep per milligram of protein; cytochrome oxidase specific activity is given as micromoles of cytochrome c oxidized per minute per milligram of protein; and acid phosphatase specific activity is given as micromoles of *p*-nitrophenyl phosphate hydrolyzed per minute per milligram of protein; NA, not available.

Pooled fraction	Density range, 4°C (g/cm^3)	^{59}Fe (%) incorporated into RBC in 48 hours		Ep specific activity	Cytochrome oxidase specific activity	Acid phosphatase specific activity
		Pooled fraction plus NRS	Pooled fraction plus saline			
		Experiment A				
4–8	1.04–1.12	5.11 ± 0.93	5.51 ± 0.30	0	0	NA
42–47	1.20–1.21	4.14 ± 0.64	2.18 ± 0.50	6	0.054	NA
48–52	1.21	3.40 ± 0.62	0.57 ± 0.05	16	0.038	NA
53–57	1.22	5.11 ± 0.43	1.48 ± 0.51	34	0.009	NA
58–63	1.22–1.24	3.63 ± 0.71	1.18 ± 0.33	20	0	NA
		Experiment B				
5–14	1.09–1.16	4.54 ± 0.64	3.58 ± 0.88	2	0	0.068
18–22	1.17–1.18	10.77 ± 1.51	1.69 ± 0.06	24	0.035	0.028
36–40	1.19–1.20	7.29 ± 0.33	2.21 ± 0.45	12	0.091	0.019
41–45	1.20–1.21	3.84 ± 0.49	1.57 ± 0.45	6	0.069	0.011
46–50	1.21	5.25 ± 0.79	0.75 ± 0.16	23	0.036	0.009
		Experiment C				
6–10	1.07–1.12	2.78 ± 0.23	1.44 ± 0.12	8	0	0.028
11–15	1.12–1.15	2.84 ± 0.50	1.23 ± 0.27	3	0.014	0.062
16–19	1.16	5.20 ± 0.60	1.45 ± 0.23	7	0.022	0.038
20–24	1.17–1.18	4.07 ± 0.21	2.52 ± 0.65	3	0.030	0.037
42–46	1.21	3.21 ± 1.22	0.95 ± 0.14	40	0.033	0.008

somal fractions and serum and evoked high incorporation of ^{59}Fe into RBC in the assay mice was actually Ep, a separate experiment was performed in which each pooled fraction was divided into three aliquots following membrane-disruptive procedures. One aliquot was incubated with saline, another was incubated with an identical volume of NRS, while the third was incubated with NRS followed by antiserum against Ep prepared from rabbits immunized against human urinary Ep as described by Schooley and Garcia (*14*). Excess antibody was removed by incubation of the mixtures with goat antiserum against rabbit gamma globulin before injection into the assay mice. The results indicate that treatment with antiserum against Ep abolishes the erythropoiesis-stimulating activity of the incubated mixtures in the assay animals. It is concluded, therefore, that the increased ^{59}Fe incorporation into RBC observed in these experiments is attributable to the formation and specific action of Ep. In this experiment with antibody against Ep, the Ep specific activity of the prezonal light mitochondrial fraction was 4 compared with values of 26 and 19 for the Triton-treated and -untreated lysosomes, respectively.

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Topology of the Outer Segment Membranes of Retinal Rods and Cones Revealed by a Fluorescent Probe

Abstract. *When* N,N′*-didansyl cystine binds to the cell membranes of vertebrate rods and cones its fluorescence efficiency increases about 20-fold. The entire outer segments of living cones become brilliantly fluorescent. Stained live rods, as well as most freshly detached outer segments, are only weakly fluorescent, but they become brightly fluorescent within a few seconds if their plasma membranes are osmotically ruptured. The difference in staining of rod and cones suggests that disk membranes of rods are not continuous with the plasma membranes are osmotically ruptured. The difference in staining of rod and cones plasma membrane on outer segments of photoreceptors in electrophysiological and biochemical experiments, and to study the infolding pattern of rod and cone disks.*

The outer segments of vertebrate rods and cones consist largely of stacks of phospholipid pleats formed by regular infolding of the plasma membranes during cell growth (*1*). Electron microscopy suggests that the topological connectivity of rod membranes is different from that of cones. The infolded membranes of rods apparently detach from the plasma membrane to form stacks of closed, flattened sacs suspended in the cytoplasm. The infoldings of cones appear to remain continuous with the plasma membrane so that the space between alternate membranes is an extension of the extracellular fluid space (*2*). Experiments on the cable properties of live rod outer segments of rats indicate that the electrical capacitances of their plasma membranes are those of smooth cylindrical lipid bilayers. Thus, an electrical measurement seems to confirm that the internal sacs of rods are not continuous with the plasma membrane (*3*). This difference in topology of rods and cones is not a minor curiosity; it implies that excitation of rods by photons absorbed in the rhodopsin-bearing sacs cannot spread to the plasma membrane by electrical polarization of a membranous connection between the two structures. Instead, a chemical transmitter substance released by the rod disks might be needed to explain the ability of light to reduce the Na^+ permeability of the overlying plasma membrane (*4*). Since it is important to know if the structural and electrical evidence is correct, we now report a simple procedure for demonstrating the different topologies of the plasma membranes of retinal rods and cones on the basis of their staining behavior in solutions of the fluorochrome, *N,N′*-didansyl cystine, denoted here by DDC (*5*). Unlike both electron microscopy and measurement of electrical cable constants, the method is simple, fast, and potenti-

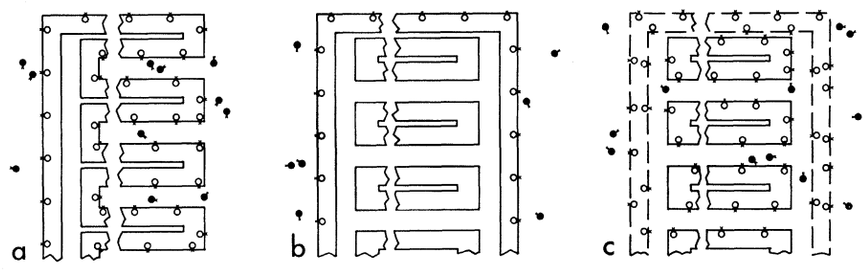

Fig. 1. Illustration of the principle of selective fluorescent staining of the outer segments of (a) cones, (b) intact rods, and (c) stripped rods with broken plasma membranes. Closed symbols indicate unbound, weakly fluorescent probe molecules. Open symbols denote bound, highly fluorescent probes.