*J. Biosci.,* Vol. 14, Number 3, September 1989, pp. 269-277. © Printed in India.

# A rapid procedure for the isolation of lysosomes from kidney cortex by Percoll density gradient centrifugation

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MS received 28 April 1988; revised 20 July 1989

**Abstract.** Highly pure lysosomes were isolated from buffalo (*Bubalus bubalis*) kidney cortex by a procedure involving differential and isopycnic Percoll density gradient centrifugations. Arylsulphatase, N-acetyl-$\beta$-glucosamindase and cathepsin D in the lysosomal preparation were 26-45-fold enriched over the homogenate. The purified lysosomes contained less than 0·06% of mitochondrial, microsomal and peroxisomal marker enzymes. In the electron micrographs the particles appeared as large dense granules of size 0·3–1·9 $\mu$m with no apparent structural features belonging to mitochondria or microsomes. The isolation procedure was also found to be suitable to obtain highly pure lysosome particles from renal cortex of other sources such as rat, lamb and beef. No ultracentrifugation steps were involved in the procedure.

**Keywords.** Lysosomes; kidney cortex; Percoll gradient; isolation procedure; buffalo kidney.

## Introduction

Lysosomes are recognized as an important site of intracellular catabolism (Gordon, 1973; Pontremoli *et al.,* 1984; Harikumar and Ninjoor, 1985). Isolation of these organelles in a highly pure form is an essential step in establishing their structure-function relationships. However, separation of lysosomes free from other subcellular contaminants has proved difficult mainly because their size and equilibration densities overlap considerably with that of mitochondria and microsomes (Beaufay, 1972). Most of the successful attempts at the isolation of lysosomes, therefore, involve the use of density altering agents (Leighton *et al.,* 1968; Beaufay, 1972; Arborgh *et al.,* 1974; Harikumar and Ninjoor, 1986), which often adversely affect the permeability characteristics of lysosomal membrane. However, currently available methods employing density gradient media such as metrizamide (Wattiaux *et al.,* 1978) and Percoll (Pertoft *et al.,* 1978; Yamada *et al.,* 1984) have made it possible to isolate pure lysosomes in unmodified form from organ tissues. Harikumar and Reeves (1983) demonstrated that highly enriched lysosomal preparations with minimum contamination of mitochondrial or microsomal marker enzymes could be obtained from rat kidney cortex by Percoll density gradient centrifugation. The present communication reports a rapid centrifugation procedure for the isolation of pure lysosomes from buffalo kidney cortex using Percoll gradient and indicates the efficiency of this method in the isolation of renal lysosomes of high purity from other sources such as rat, beef and lamb.

Abbreviations used: HL, Heavy lysosomes; RSA, relative specific activities.

## Materials and methods

Glucose-6-phosphate, 5′-adenosine-monophosphate, *p*-nitrocatechol sulphate, *p*-nitrophenyl-N-acetyl-*ß*-D-glucosaminide, haemoglobin type II, *p*-nitrotetrazolium violet and Percoll were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Density marker beads were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade quality. Kidneys of buffalo, lamb and beef were obtained fresh from the local abattoir immediately after slaughter and brought to the laboratory in chilled 0·3 M sucrose containing 1 mM EDTA adjusted to pH 7 with Tris (sucrose buffer). Rat kidney was obtained from male albino rats of Wistar strain weighing 300–400 g. Animals were anaesthetized with ether and kidneys were rapidly excised and chilled in sucrose buffer. Cortex tissues from the kidneys were removed with a razor blade, weighed, minced and rinsed thrice with excess sucrose buffer. This rinsing step is recommended to prevent contamination of the lysosomal preparation with erythrocytes.

### *Tissue homogenization*

All homogenates were prepared in sucrose buffer. Rat kidney cortex was homogenized with 3 up and down strokes in Potter Elyehjem glass homogenizer while kidneys of buffalo, lamb and beef were homogenized with two, 10 s bursts at speed 4 in a polytron tissue homogenizer. The volume of the homogenized tissue suspensions were made up to 10 ml/g tissue with sucrose buffer and then filtered through a single layer of cheese cloth.

### *Differential and isopycnic density gradient centrifugation*

Two of the important physical properties of kidney cortex lysosomes observed earlier (Straus, 1954) are their characteristic grey colour and sticky nature which enabled their visible differentiation from nonlysosomal constituents during the present tissue fractionation. The isolation procedure is schematically represented in figure 1. All centrifugations were carried out in transparent polycarbonate tubes using SS-34 fixed angle rotor fitted to a Sorvall RC-2 refrigerated centrifuge. Homogenates were spun at 120 g for 5 min. The sediment was discarded and the supernatant was centrifuged at 12,000 *g* for 20 min. The resulting sediment consisted of a dark grey zone of lysosomes overlaid with a whitish zone which predominantly consisted of mitochondria. The supernatant along with a greater part of the whitish zone was removed by aspiration. Swirling the pellet with sucrose buffer facilitated the removal of the whitish zone while the grey pellet remained undisturbed by this procedure. The pellet, which now consisted largely of the sticky dark grey zone was resuspended in a minimum volume of sucrose buffer to obtain crude lysosome preparation.

Percoll density gradient centrifugation was employed for the further fractionation of crude lysosome. Gradient was prepared by, mixing Percoll, 2 M sucrose and 100 mM MOPS/Tris pH 7 in the ratio 14:3:3 by volume. About 5 ml of the crude lysosome (10-15 mg protein/ml) was mixed with 35 ml of the Percoll mixture and centrifuged at 27,000 *g* for 90 min. After centrifugation 5 distinct zones could be

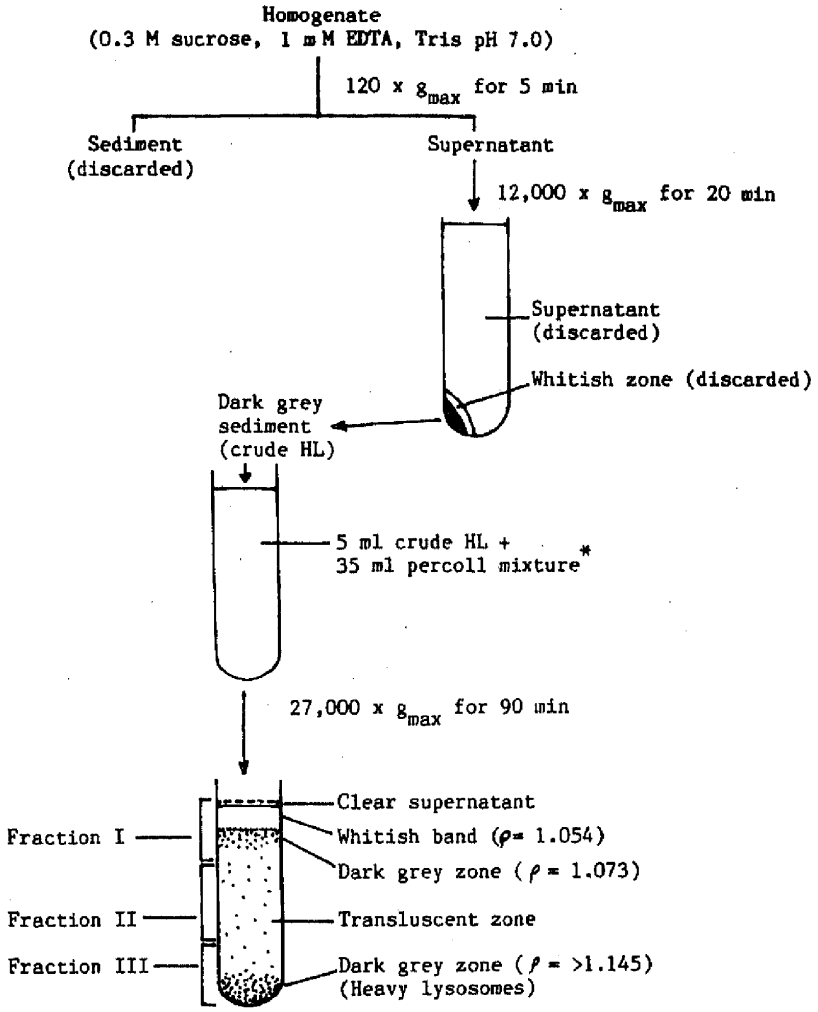


**Figure 1.** Isolation of lysosomes from kidney cortex. All centrifugations were carried out in RC-2 refrigerated centrifuge.
*The Percoll mixture consisted of Percoll, 2 M sucrose and 100 mM MOPS/Tris pH. 7 in the ratio 14:3:3 by volume. Unit of density ($\rho$) is g × $cm^{-3}$.

observed (figure 1) which, from the top to the bottom of the tube were as follows: (i) a clear supernatant; (ii) a whitish band (predominantly mitochondria) equilibrating at $\rho$ = 1·054 g × $cm^{-3}$; (iii) a dark grey band (light lysosome) positioned at $\rho$= 1·073 g × $cm^{-3}$; (iv) a transluscent zone and (v) another dark grey band (heavy lysosome, HL) equilibrating at $\rho$= 1·145 g × $cm^{-3}$ . These zones were carefully separated into 3 fractions (fractions I–III) as outlined in figure 1 by aspiration from the top of the gradient.

Percoll was removed from the fractions by repeated washing with sucrose buffer. Each fraction mixed with about 10-fold excess of sucrose buffer was centrifuged at 12,000 g for 10 min. This procedure enabled the sedimentation of particulate materials while Percoll remained in the supernatant. Repetition of this washing

procedure 3 times was found to be sufficient to remove Percoll completely from the tissue fractions. After the final washing, the pellets were resuspended in a minimum volume of sucrose buffer.

*Electron microscopy*

HL from buffalo kidney cortex were fixed with 1% osmium tetroxide in 100 mM sodium cacodylate buffer, pH 7·4, at 0·4°C for 1 h, washed with 100 mM cacodylate buffer, pH 7·4, twice and then pelletted by centrifuging at 1000 *g* for 10 min, Specimens were dehydrated in ethanol and embedded in araldite. Thin sections were cut on a Sorvall MT 5000 ultramicrotome and stained with uranyl acetate followed by lead citrate. Samples were examined with a Zeiss 105 electron microscope.

*Enzyme assays*

Lysosomal marker enzymes, arylsulphatase (EC 3·1·6·1), N-acetyl-*β*-D-glucosaminidase (EC 3·2·1·30) and cathepsin D (EC 3·4·4·23) were assayed as described by Barrett and Heath (1977). Activities of 5′-nucleotidase, succinate *p*-iodonitroletrazolium violet reductase and glucose-6-phosphatase were determined according to Morre (1971) as markers for plasma membrane, mitochondria and microsomes respectively. Catalase (Baudhuin *et al.,* 1964) was assayed as marker enzyme for peroxisomes.

*Protein*

Protein contents of the samples were assessed by the method of Lowry *et al.* (1951) with bovine serum albumin as Standard.

*Density*

Density distribution in the Percoll gradient was determined by using density marker beads.

## Results

As outlined in figure 1, the procedure for the isolation of kidney lysosomes consisted of obtaining a crude fraction from the tissue homogenate by differential centrifugation which was further fractionated by isopycnic banding on Percoll density gradient. The crude lysosome fraction was 2-4-fold enriched in lysosomal marker enzymes over the homogenate and it accounted for 15-20% of arylsulphatase, 10-14% of succinate-INT-reductase, 1-2% of glucose-6-phosphatase and 2-3% of 5′-nucleotidase (data not shown). The distribution pattern of marker enzymes in the 3 different fractions obtained during further fractionation of the crude lysosome on Percoll density gradient is depicted in figure 2. Major part of the lysosomal enzymes (cathepsin D, arylsulphatase and N-acetyl-*β*-glucosamindase)

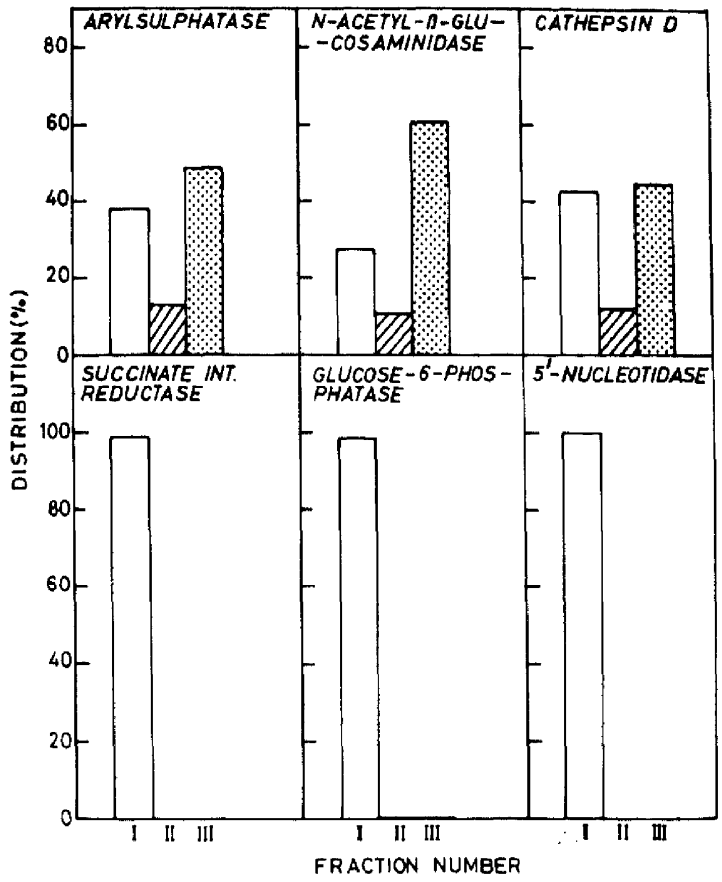


**Figure 2.** Fractionation of crude HL from buffalo kidney cortex on Percoll density gradient. Crude lysosome fractions isolated from buffalo kidney cortex were mixed with the Percoll mixture and centrifuged at 27,000 *g* for 90 min (materials and methods). Fractions I—III were collected from the top of the gradient by aspiration as outlined in figure 1.

was found to be associated with fraction I (28-38%) and fraction III (45-61%). However, fraction I also contained a large proportion (99·5-100%) of nonlysosomal markers such as succinate-INT-reductase, glucose-6-phosphatase and 5′-nucleotidase. Apparently fraction I contained light lysosomes ($\rho$=1·073), heavily contaminated with nonlysosomal constitutents which possess closely similar equilibration densities (figure 1). Fraction III (HL), on the other hand possessed less than 0·003% and 0·06% of succinate-INT-reductase and glucose-6-phosphatase respectively, while 5′-nucleotidase was not detectable. Thus, fraction III apparently consisted only of lysosomes with little contamination from other subcellular components. Data on the purity of lysosomes in this fraction (HL) isolated from buffalo kidney cortex is presented in table 1. Relative specific activities (RSA) for the lysosomal marker enzymes, arylsulphatase, N-acetyl-$\beta$-glucosaminidase and cathepsin D were 29, 45 and 26 respectively while the corresponding values were only 0·02-0·04 for mitochondrial, microsomal and peroxisomal enzymes. Morphological

**Table 1.** Purity of buffalo kidney lysosomes*.

| Marker enzymes | n | Specific activity | RSA | Yield (% of homogenate) |
|---|---|---|---|---|
| Arylsulphatase | 9 | 0·28 ± 0·04 | 29 | 3·2 ± 0·8 |
| N-Acetyl-β-D-glucosaminidase | 5 | 1·20 ± 0·30 | 45 | 4·8 ± 2·2 |
| Cathepsin D | 5 | 0·30 ± 0·09 | 26 | 2·4 ± 1·2 |
| Succinate-INT-reductase | 9 | 0·27 ± 0·27 | 0·02 | 0·007 ± 0·007 |
| Glucose-6-phosphatase | 9 | 0·006 ± 0·003 | 0·04 | 0·05 ± 0·04 |
| 5′-Nucleotidase | 9 | ND | ND | ND |
| Catalase | 5 | 0·03 ± 0·01 | 0·03 | 0·005 ± 0·001 |

*Activities of all the enzymes were determined in the presence of 0·1% Triton X-100, Specific activity of catalase represents Δ absorbance at 410 nm/mg protein/min while the activities of all the other enzymes are expressed as μmol/mg protein/min. The values are averages (±SD) of 'n' separate lysosomal preparations. Relative specific activity is calculated as the ratio of the specific activity in the fraction to that in the homogenate. ND, Not detectable.

observations (figure 3) showed lysosomes in HL, as dark dense granules with diameters ranging from 0·3-1·9 μm. The preparation consisted almost exclusively of electron-dense granules. No mitochondrial profile was noticed in 3 different lysosome preparations.

Data on the relative enrichment of lysosomal marker enzymes in HL fractions

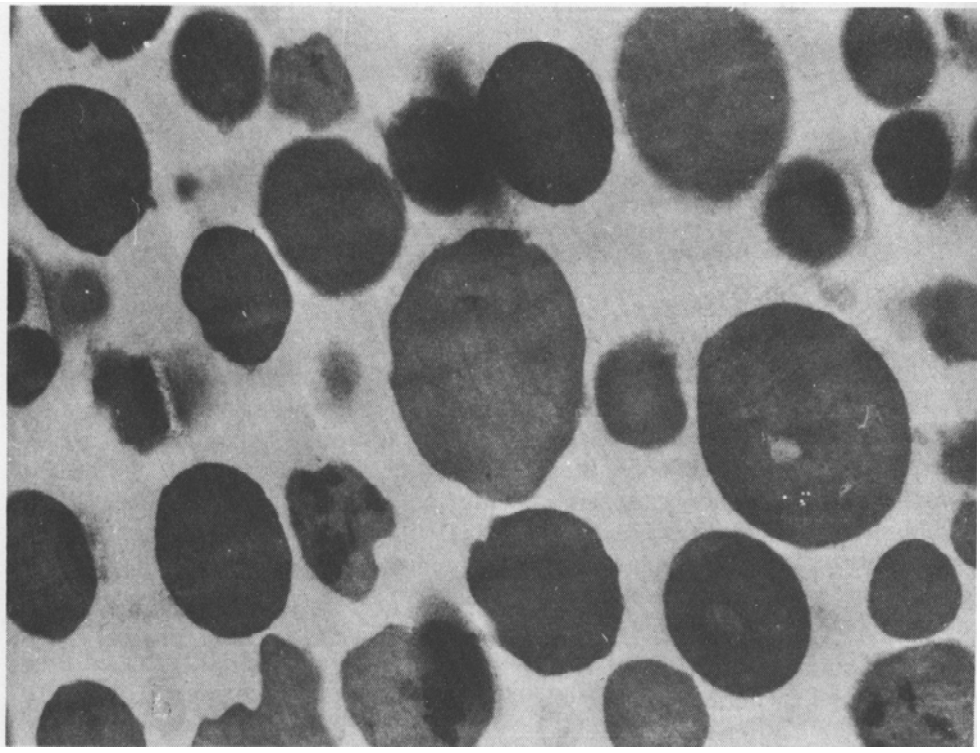

**Figure 3.** Electron micrograph of lysosomes from buffalo kidney cortex (× 32,000).

isolated from beef, lamb and rat kidney cortex homogenates are presented in table 2. Relative specific activities of arylsulphatase were 63, 28 and 31 for lysosomes obtained from rat, beef and lamb respectively. The yield of succinate-INT-reductase was less than 0·009% in these preparations. Activities of glucose-6-phosphatase in rat, lamb and beef lysosomes were 0·18, 0·01 and 0·004% respectively. 5′-Nucleotidase was not detected in lamb lysosomes while its activities in rat and beef respectively were 0·19 and 0·03%.

**Table 2.** Purity of lysosomes isolated from kidney cortex of rat, beef and lamb*.

| | Source | | |
|---|---|---|---|
| | Rat | Beef | Lamb |
| Arylsulphatase | | | |
| Specific activities | 1·06 ± 0·08 | 0·19 ± 0·02 | 0·12 ± 0·01 |
| RSA | 63 | 28 | 31 |
| Yield (%) | 3·7 ± 1·0 | 0·81 ± 0·6 | 1·0 ± 0·6 |
| N-Acetyl-β-glucosaminidase | | | |
| Specific activities | 2·4 ± 0·6 | 0·95 ± 0·3 | 0·5 ± 0·1 |
| RSA | 57 | 22 | 31 |
| Yield (%) | 3·2 ± 1·2 | 0·48 ± 0·2 | 0·7 ± 0·5 |
| Succinate-INT-reductase | | | |
| Specific activities | 0·4 ± 0·2 | 0·11 ± 0·07 | 0·16 ± 0·07 |
| RSA | 0·03 | 0·007 | 0·02 |
| Yield (%) | 0 009 ± 0·004 | 0·0003 ± 0·0002 | 0·001 ± 0·001 |
| Glucose-6-phosphatase | | | |
| Specific activities | 0·06 ± 0·04 | 0·013 ± 0·01 | 0·03 ± 0·006 |
| RSA | 0·7 | 0·06 | 0·3 |
| Yield (%) | 0·18 ± 0·09 | 0·004 ± 0·003 | 0·014 ± 0·004 |
| 5′-Nucleotidase | | | |
| Specific activities | 0 03 ± 0·02 | 0·003 ± 0·001 | ND |
| RSA | 0·4 | 0·3 | — |
| Yield (%) | 0·19 ± 0·03 | 0·03 ± 0·02 | — |

*Specific activities of all the enzymes are expressed as μmol/mg protein/min.
RSA is calculated as the ratio of specific activity in the fraction to that in the homogenate. Yield is computed on the basis of the activity in homogenate which is considered as 100%. ND, Not detected.

## Discussion

Lysosomes from kidney cortex were first isolated as droplets of varying sizes by Straus (1954, 1956) using differential centrifugation. Shibko and Tappel (1965) and Maunsbach (1974) achieved enhanced enrichment of kidney lysosomes by combining the differential centrifugation procedure with sucrose density gradient steps. The method described here employs isoosmotic gradient of Percoll to separate lysosomes free of other subcellular contaminants. The RSA of 26-45-fold observed for arylsulphatase, N-acetyl-β-glucosaminidase and cathepsin D (table 1)

in buffalo kidney lysosomes are comparable to the corresponding values reported for highly purified lysosome preparations from hepatic (Symons and Jonas, 1987; Nakabayashi and Ikezawa, 1988; Rupar and Whitehall, 1988) and renal tissues (Andersen *et al.,* 1987; Kojima *et al.,* 1987). The purity of buffalo kidney lysosomes is apparent from the low activities of mitochondrial, microsomal and peroxisomal marker enzymes in the preparation (table 1). The absence of nonlysosomal structures in the electron micrograph (figure 3) confirms this observation. Values for structure-linked latency of lysosomal enzymes were found to be in the range of 78-90% (data not shown). This, along with the morphological data (figure 3) depicting the particles as electron-dense granules surrounded by intact membranes points to the integrity of lysosomes. Thus, renal lysosomes obtained by this procedure should be suitable to investigate the permeability characteristics of lysosomal membrane. The earlier investigations of Harikumar and Reeves (1983, 1986) showed that rat kidney lysosomes isolated by the Percoll gradient procedure indeed hold ion gradients with high efficiency.

The efficacy of Percoll method for the successful isolation of renal lysosomes from a variety of sources such as rat, beef and lamb is evidenced by the data presented in table 2. Lysosomal preparations obtained from all these sources showed high enrichment (22-63-fold) of acid hydrolases with little contamination from other subcellular organelles. However, the yield of lysosomes from individual sources was found to vary markedly (table 2). Whether this represented a difference in the content of lysosome particles *in vivo* or it was due to differences in the relative amenability of the tissues to the fractionation procedure is not clear. Since homogenization methods have a decisive role in determining lysosomal integrity (de Duve, 1971; Beaufay 1972), it would be reasonable to assume that the higher yield of lysosomes in rat kidney could at least partly be due to the relatively milder homogenization methods with which this tissue could be disrupted. Despite variations in the yield, the relative specific activities (tables 1 and 2) and structural latency (data not shown) of acid hydrolases in lysosomes isolated from buffalo, rat, beef and lamb (tables 1 and 2) were comparable. Thus, it is evident that the procedure outlined in figure 1 could be used as a general method to obtain highly pure lysosomes from kidney cortex tissue. Four major advantages of the procedure are (i) no density altering agents such as Triton WR-1339 which induce nonphysiological alterations in lysosomes (Leighton *et al.,* 1968; Trout *et al.,* 1981) are used, (ii) the use of Percoll gradients precludes osmotic stress that sucrose would exert on lysosomes, (iii) the method does not involve any ultracentrifugation steps, thus minimizing possible constraints imposed on the organelles by the centrifugal force, and (iv) starting with the cortex tissue, the whole isolation procedure could be completed in less than 3 h. The rapidity of the isolation procedure is particularly important in that, it ensures availability of lysosomes with minimum damage which otherwise would be caused by prolonged separation procedures and overnight storage. The method will also be useful in preparative investigations since it is standardized to obtain lysosomes from buffalo, beef and lamb kidneys which are commercially available in large quantities.

**Acknowledgements**

We are grateful to Mrs. S. N. Palange and Dr. A. N. Bhisey, Cell Biology Division,

Cancer Research Institute, Parel, Bombay for the electron micrographs of lysosome preparations.

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