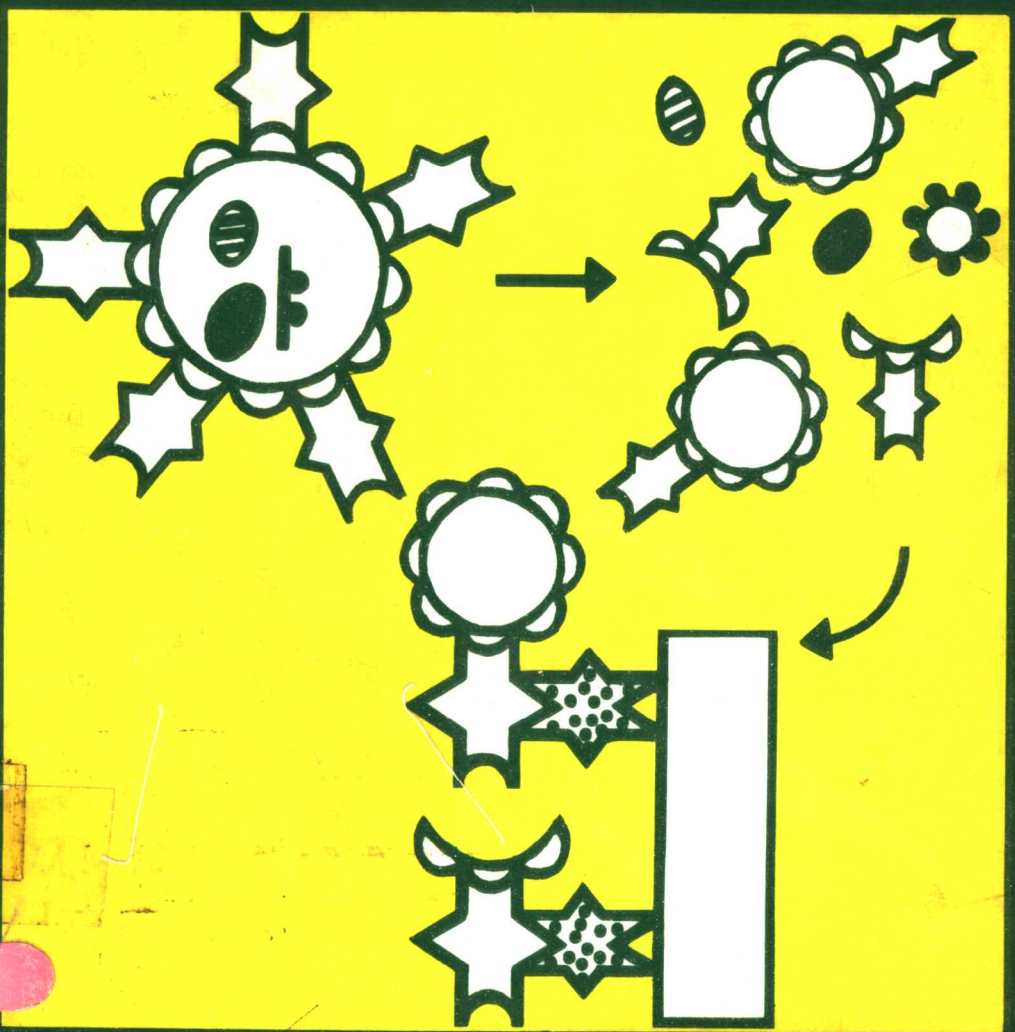
MEMBRANOUS ELEMENTS AND MOVEMENT OF MOLECULES

METHODOLOGICAL SURVEYS IN BIOCHEMISTRY Volume 6

E. REID editor



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Membranous Elements and Movement of Molecules

METHODOLOGICAL SURVEYS Vol. 6
(Cell Biochemistry including Muscle)

Edited by

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Editor's Preface

Our understanding of muscle biochemistry has come largely from carefully conducted studies entailing the isolation of membranous elements. This book should fill a notable gap in the literature, insofar as it begins with accounts of the requisite techniques as developed and applied by eminent investigators in the muscle field. (For a 'guide' to muscle techniques surveyed, see pp. 1-2.)

The theme of isolated membranous elements runs right through the book, latterly in connection with clarifying how molecules reach intracellular destinations or depart from the cell. Whilst energy metabolism is excluded, diverse types of molecule are considered – including membrane precursors, export proteins, hormones and drugs. Good and imaginative methodology is vital to this 'growth area' in cell biochemistry, and will assume increasing importance in the repertoire of biochemical pharmacologists although only a minority are as yet alert to the promise of the subcellular approach. An example of present-day ignorance is the mode of passage of drugs to the endoplasmic reticulum (e.r.) once they have entered the cell. Whilst an endeavour has been made to give a balanced account of methods for studying molecular movement, some amplification will have to be sought elsewhere (with guidance from the relevant NOTES & COMMENTS), e.g. for 'lysosomotropy' and for information transfer from the nucleus. Indeed, the field of molecular movement is still too young to render an integrated survey of methods easy to compile, unlike ion transport (but the 'calcium pump' *is* surveyed).

Amongst topics related to molecular movement are storage organelles, novel centrifugal approaches, and the 'topography' of membranes especially with respect to enzymes. For such topics, some of the methods are so new as to be making their first appearance in the present book. Throughout the book and the 5th Subcellular Methodology Symposium which generated it (July, 1976), attention has been given to the vital matter of reputable 'markers' for characterizing subcellular elements. In this respect the present book builds on the guidance conveyed in earlier volumes of this Series.

Henceforth the Series sub-title is to be *Methodological Surveys* rather than the cumbrous *Methodological Developments in Biochemistry,* with alternation between subcellular biochemistry and bioanalysis (of small molecules) whilst not excluding the possibility of an occasional volume in a quite different biochemical area. Suggestions for possible themes will be welcomed, especially where a survey would be of use to bench workers in company laboratories cognate to the Centre's help-industry activities.

Acknowledgements.— For meticulous typing, tribute is paid to Mrs. R. Sarker. For organizing the Symposium contributions on muscle, credit goes to Dr. Denis R. Headon, who is also thanked for having compiled a guide to topics as an aid to information retrieval. Dr. Paul Tulkens gave advice on the planning of the 'movement' contributions. Much trouble has been taken by the individual authors, some of whom are using this book channel for primary publication of novel methodology. The European Cell Biology Organization helped towards Symposium costs, as co-sponsor.

Where a figure or tabulated information has been reproduced from a previous publication, acknowledgement has been made in the contribution concerned. Sources include *Archives of Biochemistry & Biophysics, Biochimica et Biophysica Acta, Cytobios, Endeavour, Journal of Biological Chemistry, Journal of Cell Biology, Journal of Endocrinology, Journal of Physiology, Life Sciences, and Nature (London)*. The publishers who have kindly given permission include Academic Press and the Rockefeller University Press, and also (for items reproduced from books) Elsevier and North-Holland. Thanks are also expressed to named individuals, including Dr. G. Gregoriadis and Dr. S. Orrenius.

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25 February 1977

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NOTE: For checking whether a particular topic occurs in several places, reliance should be placed on this Contents list and on the Index, rather than on text cross-references. Look in the appropriate NOTES and COMMENTS for possible supplementary material. For topic 'guides' see page 2 (muscle) and final text page (various).

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#D HOMING OF MEMBRANES, DRUGS AND OTHER SUBSTANCES

RECEPTORS AND CARRIERS

PROTEIN MOVEMENT AND MEMBRANE DYNAMICS

DISPOSAL CHANNELS AND DRUG HANDLING

#A Membranes from Muscle

FOREWORD

This Foreword aims to facilitate information retrieval in an area hitherto ill-served by survey literature, concerning aspects of subcellular methodology utilized in the study of muscle membranes and the movements of calcium ions. The subject-matter of the section falls into three groups, namely *preparation and characterization of membranous elements, membrane topography* and *calcium transport*. Various topics considered in the main expositions (#A-1 to #A-10) are set out in Scheme 1. The topic headings selected cover quite a wide range of methods. W.T. Stauber, for example, employs enzymic digestion techniques prior to homogenization of the tissue, while density perturbation procedures and differential fragmentation of skeletal muscle membranes have been used in the characterizations considered in #A-4. Although such information may not be obvious from Scheme 1, there is a reasonable representation of topics, together with cross-referencing.

Scheme 2 is a flow chart showing interconnections amongst authors in respect of subject-matter, including short expositions to be found amongst *Notes & Comments* (#A-NC). Some of the interconnections might be considered rather tenuous, but they are nevertheless worth-while. Thus, different authors may be dealing with membranes isolated from fast and slow muscle, with calcium movements, with calcium uptake, with Ca^{2+},Mg^{2+}-dependent ATPase or with particular aspects of preparation and characterization. Again the Scheme does not disclose, for example, that stop-flow techniques have been employed by G. Inesi (and are touched on by C.C. Ashley & T.J. Lea), or that functional capabilities of sarcoplasmic reticulum, isolated from different muscle types, were assessed by R.J. Solaro. Yet the two Schemes may serve to increase the usefulness of the section as a whole.

DENIS R. HEADON
University College, Cork

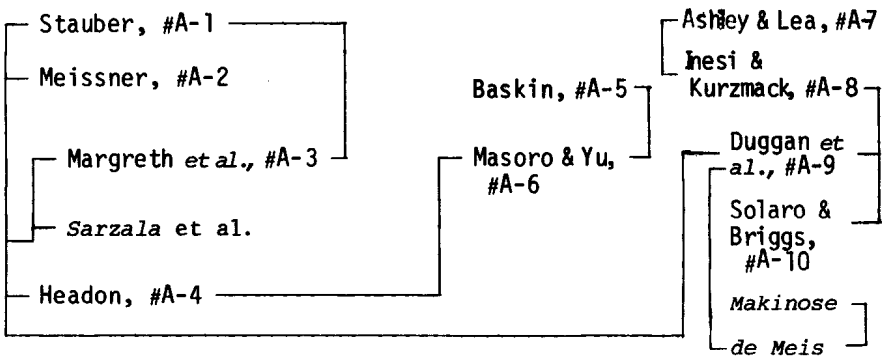
Scheme 1. Topics considered in the main part of the MUSCLE Section.

	Stauber #A-1	Meissner #A-2$^{\phi}$	Margreth *et al.* #A-3$^{\phi}$	Headon #A-4$^{\phi}$	Baskin #A-5$^{\phi}$	Masoro & Yu #A-6$^{\phi}$	Ashley & Lea #A-7$^{\phi}$	Inesi & Kurzmack #A-8$^{\phi}$	Duggan *et al.* #A-9	Solaro & Briggs #A-10$^{\phi}$
Homogenization	✓		✓	✓					✓	
Density gradient centrifugation	✓	✓		✓					✓	
Marker constituents	✓	✓	✓	✓					✓	
Electrophoresis		✓	✓	✓	✓	✓				
Morphological studies (e.m.)					✓					
Protein labelling*		✓		✓		✓		✓		
Enzymic digestion of proteins			✓		✓	✓				
Calcium movements†							✓	✓	✓	✓
Ca^{2+}, Mg^{2+}-ATPase (pump)			✓		✓	✓		✓	✓	

** including use of ^{32}P in phosphoprotein formation, besides other techniques for membrane protein localization*

† including calcium uptake activities $^{\phi}$*see also NOTE & COMMENTS*

Scheme 2. Inter-connecting topics (in italics if in the Notes and Comments which follow on from #A-10).



#A-1

ORGANELLE SEPARATION FROM RED AND WHITE SKELETAL MUSCLE

WILLIAM T. STAUBER
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University of Iowa, Iowa City, U.S.A.

On applying enzymatic techniques to anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) avian muscles, the yield of sedimentable organelles was five times better than with mechanical homogenization. Differential centrifugation established that mitochondria can be partially separated from lysosomes, sarcoplasmic reticulum (s.r.), and plasma-membrane (p.m.) fragments as evidenced by the distribution patterns of cytochrome oxidase activity from both ALD and PLD. With the PLD muscle the light-mitochondrial fraction was enriched in 5'-nucleotidase and β-naphthylamidase (p.m. markers), cathepsin D, N-acetyl-β-glucosaminidase and other lysosomal enzymes, catalase, and calcium-accumulating activity (s.r. fragments). The ALD muscle was similar except that the peak calcium-accumulating activity occurred in the microsmal fraction. The enzyme distributions were also compared by isopycnic-zonal centrifugation for both muscles and illustrate the difficulty in obtaining pure organelle preparations.

This study was designed to isolate by differential and zonal centrifugation methods subcellular organelles from avian anterior (red) and posterior (white) latissimus dorsi muscles and to compare the products with those from rat gastrocnemius muscle. The term differential centrifugation connotes sedimentation of material in tubes without a gradient, and zonal centrifugation connotes sedimentation in sucrose gradients in various zonal rotors*.

**EDITORIAL COMMENTS: Nomenclature varies; 'differential pelleting' is a recommended term, and 'zonal centrifugation' can encompass gradient runs in tubes unless more narrowly defined, as here.*

PREPARATION OF HOMOGENATES AND EVALUATION OF PARTICLE INTEGRITY

The problem of tissue homogenization has received much attention over the last 20 years, and major improvements have been made for almost all tissues except skeletal muscle. Indeed, simple mechanical blending is still the standard disruptive technique used for the preparation of sarcoplasmic reticular (s.r.) fragments. These fragments should be considered as membrane 'ghosts', since much of their content is lost [1]. The greatest obstacle to 'gentle' homogenization of skeletal muscle is its tough connective tissue framework. Enzymatic procedures similar to those employed for smooth muscle [2] have been developed in our laboratory which allow homogenization of skeletal muscle with the Potter-Elvehjem homogenizer [3]. Quite large amounts of s.r. vesicles can thereby be isolated that still contain much of their *in vivo* non-ionic constituents.

Either anterior (ALD) or posterior (PLD) latissimus dorsi muscles from young Cornish-cross chickens were pooled to yield 100-300 mg samples for differential centrifugation and 10 g samples for zonal centrifugation. These muscles were incubated for 1 h at room temperature in Hank's solution to which was added 1.2 mg/ml of both collagenase (Type 1) and lysozyme (enzymes from Sigma Chemical Co.). For differential centrifugation, 1 ml of this enzyme-fortified solution was used for each 10 mg of ALD or PLD muscle. For zonal centrifugation, 500 ml of the enzyme-fortified solution was used for each 10 g of either muscle. In both cases, the muscle fragments were collected by low speed centrifugation (1,500 *g* × 10 min), re-suspended in 0.25 M sucrose (pH 7.2), and re-centrifuged at the same speed.

Following re-suspension of the muscle fragments in 0.25 M sucrose (pH 7.2) containing 50 units/ml of heparin*, homogenization was effected by either two strokes of a Potter-Elvehjem homogenizer (clearance 4-6 μm; differential centrifugation) or five strokes of a Dounce homogenizer fitted with the B pestle (zonal centrifugation). These conditions consistently yielded latency values for cathepsin D of 50% and 59% for the ALD and PLD homogenates respectively, where 100% latency indicates complete integrity of lysosomes. Thus, these values represent the proportion of intact lysosomes. In contrast, only 45% latent activity was detectable for cathepsin D following mechanical blending in a VirTis 45 blender. Measurement of bound and free marker enzyme activity can be used to evaluate the integrity of nuclei, mitochondria, lysosomes and peroxisomes. Our adjustment of homogenization conditions was governed by

* *The heparin addition [cf. article by D.H. Headon in Vol. 4,* Subcellular Studies*] serves to stabilize membrane aggregates resulting from homogenization in such a configuration that facilitates their separation. Heparin can be replaced by any polyanion.*

Table 1. Differential centrifugation scheme (de Duve *et al.*[8])

Fraction	s_m in S units	Centrifuge and rotor	$\omega^2 t$ rad^2/nsec
N Nuclear-myofibrillar	210,000	Lourdes LRA, rotor 9RA	0.02
M Heavy-mitochondrial	17,300	Beckman L, rotor 40	0.30
L Light-mitochondrial	2,300	Beckman L, rotor 40	2.30
P Microsome	190	Beckman L, rotor 40	28.00
S Soluble	-	-	-

lysosome integrity because we felt this was the most fragile membrane-bound organelle in skeletal muscle tissue. Also, it was our belief that lysosomal enzymes were located inside portions of the s.r. [3, 4]; consequently, we had an indication of the degree of fragmentation of the s.r.

TISSUE FRACTIONATION: DIFFERENTIAL CENTRIFUGATION

Our studies [5-7] showed that the classical five-fraction differential centrifugation scheme of de Duve *et al.* [8] (Table 1) could partially separate myofibrillar protein (N fraction), and mitochondria (M fraction) from the other organelles and membrane fragments, the latter being located primarily in the L fraction (Fig. 1). It could not, judged by appropriate markers, resolve p.m. (5'-nucleotidase and leucyl-β-naphthylamidase), s.r. (Ca uptake), lysosomes (cathepsin D, α-glucosidase, *N*-acetyl-β-glucosaminidase and β-galactosidase) or catalase-containing organelles. There was, however, a definite improvement in the yield of organelles when compared to simple mechanical blending of rat gastrocnemius muscle (Fig. 2). The L fraction now represented 5% of the total cellular protein and about 20% of the total activity of those enzymes enriched therein [3].

The unexpected distribution of a portion of aldolase and lactic dehydrogenase (LDH) in a sedimentable form, although consistent with histochemical observations [9, 10], could have occurred if the membrane vesicles had entrapped some soluble

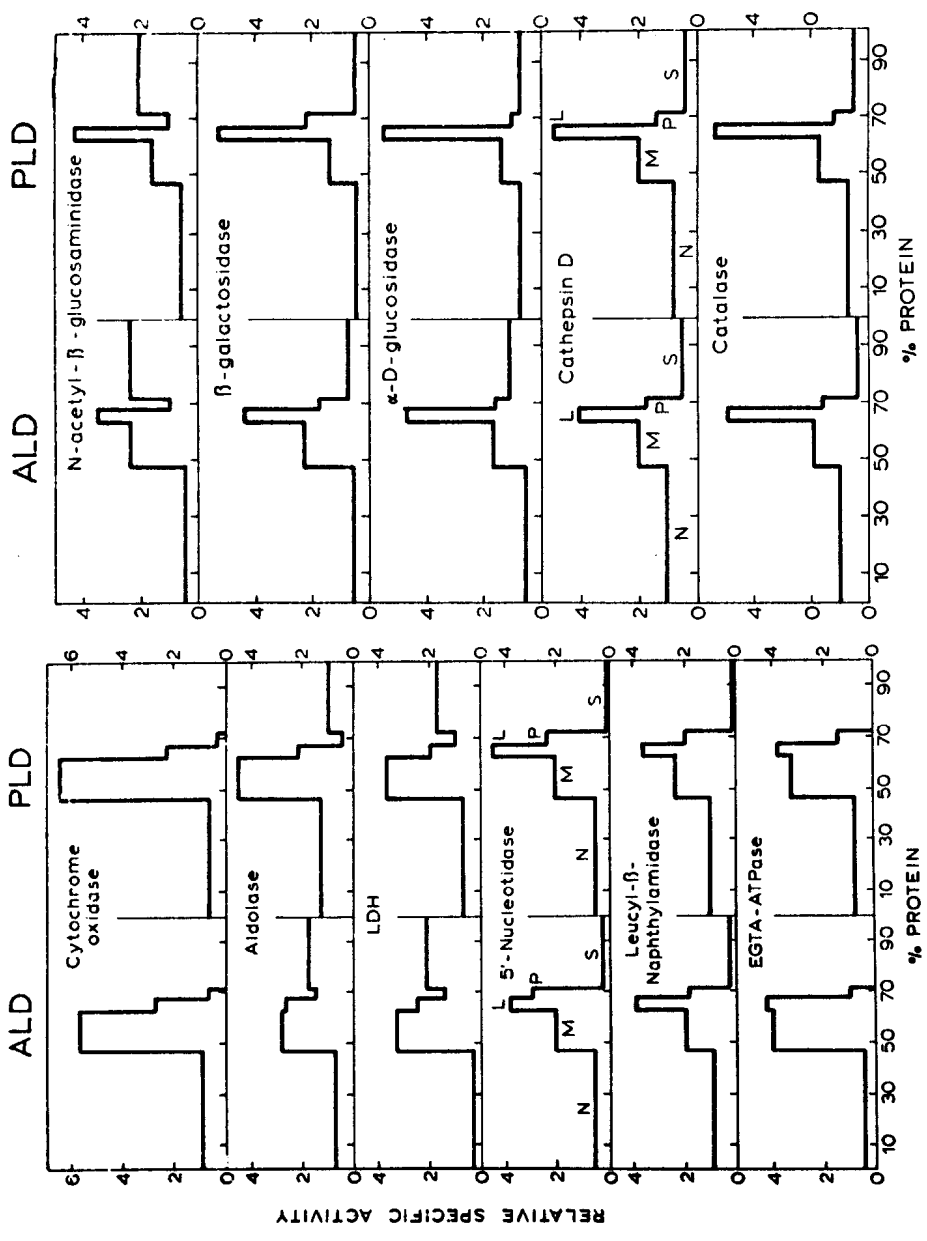

Fig. 1 *(continued opposite, with Legend)*