THE UNIVERSITY OF MANITOBA

CENTRIFUGATION OF MITOCHONDRIA ON DENSITY GRADIENTS AND ITS APPLICATION TO SEPARATE DIFFERENT MITO-CHONDRIAL POPULATIONS FROM DYSTROPHIC HAMSTER SKELETAL MUSCLE

by

SERGE PICARD

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SERGE PICARD

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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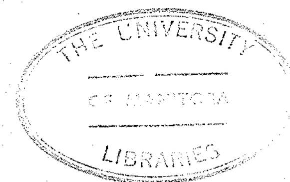

L'homme est né libre et partout il
est dans les fers.

J.-J. Rousseau.

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ABSTRACT

A new technique was developed to isolate intact mitochondria from a homogenate, making it possible to omit the multiple differential centrifugation steps used in conventional procedures.

Rate zonal centrifugation was performed to determine the sedimentation pattern of the mitochondrial fraction, from a pigeon heart homogenate, in a linear iso-osmotic Ficoll-sucrose density gradient, using a swinging-bucket rotor. The mitochondria isolated by this single step density gradient centrifugation were studied polarographically. The oxidative phosphorylation in the mitochondria isolated by this method were very good, having a respiratory control ratio over 9, thus showing the functional intactness of these organelles. Electron micrographs showed that the mitochondrial fractions were relatively free of contamination. This technique was then adapted to a reorienting density gradient zonal rotor to show the possibility of large scale isolation of functionally intact mitochondria by a single step density gradient centrifugation.

The effects of linear sucrose density gradients and linear Ficoll-sucrose density gradients on mitochondrial oxidative phosphorylation were compared using previously isolated rat liver mitochondria, hamster heart mitochondria and hamster

skeletal muscle mitochondria. Rate zonal centrifugation was performed with each type of mitochondria and a quasi-isopycnic centrifugation was also used with the skeletal muscle mitochondria. The Ficoll-sucrose density gradient yielded a better quality of rat liver mitochondria, as shown by a higher respiratory control ratio, than the sucrose density gradient. Both types of density gradients allowed the recovery, in the presence and in the absence of albumin, of a comparable quality of heart mitochondria. The Ficoll-sucrose density gradient also allowed the recovery of a better quality of skeletal muscle mitochondria than the sucrose density gradient when either quasi-isopycnic centrifugation or rate zonal centrifugation were performed.

The density gradient centrifugation method at high centrifugal forces was applied to the separation of previously isolated skeletal muscle mitochondria from dystrophic hamsters, strain BIO-14.6. Using discontinuous Ficoll-sucrose density gradients and continuous sucrose density gradients, it was possible to separate one or more mitochondrial fractions, with high Ca^{++} levels, which were not present in the normal skeletal muscle mitochondria. Density gradient centrifugation allowed the identification in dystrophic skeletal muscle of abnormal mitochondrial fraction(s), which might be related to the development of the disease.

LIST OF FIGURES

FIGURE PAGE

1. Zonal centrifugation performed in a density gradient 7

2. Dilution volume vs dilution time for the slow dilution of the mitochondrial suspension recovered from hyperosmotic sucrose solutions 49

3. SZ-14 reorienting density gradient zonal rotor and accessories 52

4. Schematical representation of the Oxygraph cuvette fitted with an oxygen electrode 54

5. Example of a typical polarographic experiment..... 56

6. Effects of different concentrations of Ficoll-sucrose gradient solutions on the protein determination at 280 nm and on the protein determination by the Lowry method 59

7. Sedimentation pattern of mitochondria isolated in sucrose-mannitol Stock Medium and subjected to rate zonal centrifugation in a Ficoll-sucrose-mannitol density gradient 70

8. Cytochrome oxidase activity and total protein determination after rate zonal centrifugation of pigeon heart homogenate, at two centrifugal forces.

   8A: 762 x G 96
   8B: 2,705 x G 97

9. Electron micrographs of mitochondria isolated from a pigeon heart homogenate using a single step density gradient centrifugation in a Ficoll-sucrose density gradient ....

   9A: Upper fraction 100
   9B: Upper fraction 101
   9C: Lower fraction 102

FIGURE PAGE

10. Sedimentation pattern of a pigeon heart homogenate after a rate zonal centrifugation in a Ficoll-sucrose density gradient, using an SZ-14 reorienting density gradient zonal rotor 104

11. Polarographic tracings of respiration by freshly isolated normal and dystrophic mitochondria and by dystrophic mitochondria recovered from the "28%" Ficoll band 134 135

12. Hypothetical sequence of biochemical disorders occurring in the progress of muscular dystrophy in the BIO-14.6 hamsters 178

LIST OF TABLES

TABLE PAGE

1. Comparison of oxidative phosphorylation measurements of pigeon heart mitochondria isolated in sucrose Stock Medium and in sucrose-mannitol Stock Medium ... 67

2. Comparison of oxidative phosphorylation by mitochondria isolated in sucrose Stock Medium and in sucrose-mannitol Stock Medium and subjected to rate zonal centrifugation in different density gradient solutions 71

3. Oxidative phosphorylation by mitochondria recovered, from heart homogenate subjected to rate zonal centrifugation, in different density gradient solutions 76

4. Oxidative phosphorylation by previously isolated pigeon heart mitochondria and by mitochondria isolated from pigeon heart homogenate on a Ficoll-sucrose - KCl density gradient 78

5. Oxidative phosphorylation by mitochondria isolated from heart homogenate by rate zonal centrifugation in a Ca^{++}-free Ficoll-sucrose density gradient 81

6. Oxidative phosphorylation by mitochondria isolated from pigeon heart homogenate in a single step density gradient centrifugation 83

7. Cytochrome oxidase activity measured in the presence of different concentrations of Ficoll 85

8. Cytochrome oxidase activity of pigeon heart mitochondria measured in the presence of two different concentrations of Ficoll and varying concentrations of Triton X-100 87

9. Cytochrome oxidase activity of hamster heart mitochondria measured in the presence of two different concentrations of Ficoll and varying concentrations of Triton X-100 89

TABLE PAGE

10. Cytochrome oxidase activity of hamster skeletal muscle mitochondria measured in the presence of two different concentrations of Ficoll and varying concentrations of Triton X-100 90

11. Cytochrome oxidase activity of pigeon heart mitochondria isolated from a homogenate using rate zonal centrifugation 92

12. Cytochrome oxidase activity and total protein determination after a rate zonal centrifugation of pigeon heart homogenate, at two centrifugal forces. 94

13. Oxidative phosphorylation in the two fractions, from a pigeon heart homogenate, recovered after rate zonal centrifugation on a Ficoll-sucrose density gradient 98

14. Respiration and oxidative phosphorylation by mitochondria isolated from a pigeon heart homogenate in an SZ-14 reorienting density gradient zonal rotor.. 106

15. Previously isolated rat liver mitochondria subjected to rate zonal centrifugation on sucrose and Ficoll-sucrose density gradients 114

16. Previously isolated hamster heart mitochondria subjected to rate zonal centrifugation on Ficoll-sucrose density gradients, in the presence and in the absence of 0.1% bovine plasma albumin 116

17. Previously isolated hamster heart mitochondria subjected to rate zonal centrifugation on sucrose density gradients, in the presence and in the absence of 0.1% bovine plasma albumin......................... 117

18. Previously isolated hamster skeletal muscle mitochondria subjected to rate zonal centrifugation on sucrose and on Ficoll-sucrose density gradients, in the presence of 0.1% bovine plasma albumin 122

19. Previously isolated hamster skeletal muscle mitochondria subjected to isopycnic centrifugation on sucrose and on Ficoll-sucrose density gradients, in the presence of 0.1% bovine plasma albumin 123

TABLE PAGE

20. Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band 131

21. Oxidative phosphorylation and cytochrome oxidase activity of normal and dystrophic mitochondria before and after centrifugation on a discontinuous Ficoll-sucrose density gradient 133

22. Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band; 32 to 133 day old dystrophic hamsters 138

23. Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band; 32-36 day old dystrophic hamsters 144

24. Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band; 71-74 day old dystrophic hamsters 146

25. Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band; 130-133 day old dystrophic hamsters 148

26. Oxidative phosphorylation by normal and dystrophic mitochondria before and after recovery from discontinuous Ficoll-sucrose density gradients 150

27. Densities and viscosities of aqueous Ficoll and sucrose solutions, at different concentrations 151

28. Calcium content of freshly isolated dystrophic mitochondria and number of dystrophic mitochondrial populations separated on discontinuous Ficoll-sucrose density gradients 154

29. Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered in a sucrose density gradient; 67-82 day old dystrophic hamsters 157

TABLE PAGE

30. Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered in a sucrose density gradient; 18-20 day old dystrophic hamsters 161

31. Mitochondrial calcium content and skeletal muscle calcium content at different ages of normal hamsters 163

GLOSSARY

ADP	- adenosine-5'-diphosphate.
ATP	- adenosine-5'-triphosphate.
ADP/O (P/O)	- ratio of molecules of ADP consumed per atom of oxygen utilized.
differential centrifugation	- the particles with different sedimentation rates are separated by a sequence of centrifugations at different speeds.
2,4-DNP	- 2,4-dinitrophenol.
EDTA	- ethylenediaminetetra-acetic acid.
EGTA	- ethyleneglycol-bis-(β -aminoethyl ether) N,N'-tetra-acetic acid.
Ficoll	- trade name for a polymer of sucrose of a molecular weight of 400,000.
g	- gram.
G	- force of gravitation.
iso-osmotic density gradient	- density gradient in which the osmolarity of the solution is equal to that of the organelles to be separated.
isopycnic centrifugation	- the particles are separated by sedimenting them through a density gradient until each species attains an equilibrium position at a level corresponding to its own density.
muscular dystrophy	- (Greek roots: dys-:difficult, + trophein:to nourish) progressive atrophy of the muscles with no discernable lesion of the spinal cord (74).
Nagarse	- bacterial proteinase.
NAD^+	- nicotinamide adenine dinucleotide.

O.D.	- optical density.
Pi	- inorganic phosphate.
rate zonal centrifugation	- the particles are separated according to their sedimentation rate by centrifuging them through a density gradient (3).
respiration rate (O_2 rate)	- consumption of oxygen expressed as µmoles O_2/min/g mitochondrial protein.
state 3 respiration rate	- respiration rate in the presence of ADP.
state 4 respiration rate	- respiration rate in the absence of ADP.
respiratory control ratio or index (RCR or RCI)	- state 3 respiration rate/state 4 respiration rate.
TMPD	- N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride.
Tris	- tris (hydroxymethyl) aminomethane.
p	- probability that the means of the two sample populations under comparison are equal.
α	- level of statistical significance.
ANOVA	- analysis of variance. Test for a difference in the mean measurements of two or more groups; the parts of the sample variance are analyzed for this purpose.
r	- correlation value; shows the relationship of interdependence between two or more variables.
N.S.	- not significant ($p > 0.05$).
S.E.	- standard error of estimate.

TABLE OF CONTENTS

PAGE

ACKNOWLEDGEMENTS iii

ABSTRACTS iv

LIST OF FIGURES vi

LIST OF TABLES viii

GLOSSARY xii

TABLE OF CONTENTS xiv

I. INTRODUCTION 1

II. LITERATURE REVIEW 6

I. Density gradient centrifugation 6

1. Definition of density gradient centrifugation 6
2. Factors influencing the sedimentation rate .. 8
3. Rotors used in density gradient centrifugation 11
(a) Swinging-bucket rotor 11
(b) Zonal rotor 14
4. Density gradient solutions 16

II. Muscular dystrophy 24

III. Respiration and oxidative phosphorylation of dystrophic skeletal muscle and heart mitochondria 28

III. MATERIALS AND METHODS 38

A. MATERIALS 38

I. Chemicals 38
II. Animals 40

B. METHODS 41

I. Procedures for isolation of mitochondria 41

1. Reagents 41
2. Preparation of rat liver mitochondria 42

PAGE

3. Preparation of hamster skeletal muscle mitochondria 42
4. Preparation of hamster heart mitochondria.. 43
5. Preparation of pigeon heart mitochondria... 44
6. Preparation of pigeon heart homogenate for: 44
(a) experiments with the SZ-14 reorienting density gradient zonal rotor.......... 44
(b) experiments on the isolation of mitochondria in a single step density gradient centrifugation 45

II. Density gradient centrifugation 45

1. Composition of the gradient solutions 45
(a) Ficoll-sucrose density gradients 45
(b) Sucrose density gradients 46
2. Density gradient ranges 46
3. Rotor used 46
(a) Swinging-bucket rotors 46
(i) Beckman SW-41 swinging-bucket rotor 46
(ii) Sorvall HB-4 swinging-bucket rotor 48
(b) Sorvall SZ-14 reorienting density gradient zonal rotor 50

III. Polarographic measurements.................. 53

1. Instrument 53
2. Reaction Medium 53
3. Example of a typical polarographic test... 55
4. Calculations.............................. 57

IV. Assays 57

1. ADP assay 57
2. Protein estimation 58
(a) direct measurement at 280 nm 58
(b) colorimetric method 58
(i) Reagents......................... 58
(ii) Procedure 60
3. Cytochrome oxidase activity 61
(i) Reagents 61
(ii) Procedure 61
4. Calcium determination 62

V. Electron micrographs 62

VI. Statistical treatment 63

PAGE

IV. RESULTS 64

I. Mitochondria isolated from pigeon heart homogenate in a single step density gradient centrifugation .. 64

1. Introduction 64

2. Comparison of the effectiveness of sucrose Stock Medium and sucrose-mannitol Stock Medium used for the conventional Nagarse procedure of isolating mitochondria 66

3. Sedimentation pattern of previously isolated mitochondria subjected to rate zonal centrifugation 68

4. Mitochondria obtained directly from a whole homogenate layered on density gradient and subjected to rate zonal centrifugation 72

(a) density gradient solutions 74
(b) cytochrome oxidase activity studies 82
(c) centrifugation speed study.................. 93
(d) SZ-14 reorienting density gradient zonal rotor experiments 103

5. Conclusion 107

II. Comparison of oxidative phosphorylation by previously isolated mitochondria after having been subjected to rate zonal centrifugation either in a sucrose density gradient or in a Ficoll-sucrose density gradient.... 110

1. Introduction 110

2. Isolated rat liver mitochondria subjected to rate zonal centrifugation 113

3. Isolated hamster heart mitochondria subjected to rate zonal centrifugation 115

4. Isolated hamster skeletal muscle mitochondria subjected to two different centrifugal forces ... 119

5. Conclusion 124

PAGE

III. Differently sedimenting populations of mitochondria, previously isolated from skeletal muscle of normal and dystrophic hamsters, in sucrose and Ficoll-sucrose density gradients 127

1. Introduction 127

2. Determination of the Ficoll-sucrose density gradient shape and its application 128

3. Comparison of the sedimentation pattern of previously isolated normal and dystrophic skeletal muscle mitochondria subjected to discontinuous Ficoll-sucrose density gradient centrifugation 136

(a) Comparative study of the sedimentation pattern of isolated normal and dystrophic mitochondria, without considering the age of the animals 137

(b) Comparative study of the sedimentation pattern of normal and dystrophic mitochondria, considering the age of the animals 143

(c) Relation between the Ca^{++} content of the freshly isolated mitochondria and the number of populations obtained after discontinuous Ficoll-sucrose density gradient centrifugation. 153

(d) Discussion 155

4. Comparison of the sedimentation pattern of previously isolated normal and dystrophic skeletal muscle mitochondria subjected to continuous sucrose density gradient centrifugation 156

5. Discussion 165

V. GENERAL DISCUSSION 168

I. Density gradient centrifugation 168

II. Muscular dystrophy 169

1. Discussion of and conclusion on the results presented above in Results III 169

2. Abnormalities of energy levels in the cell in relation to Ca^{++} accumulation by mitochondria...... 172

3. Membrane defect in muscular dystrophy.............. 173

4. Hypothetical sequence of biochemical disorders occurring in the progress of muscular dystrophy ... 176

PAGE

VI. BIBLIOGRAPHY 180

I. INTRODUCTION

The Duchenne type of muscular dystrophy is the most severe type of all the human muscular dystrophies. It is a genetic defect expressed by muscular weakness and wasting. This X-linked recessive disease occurs only in boys, but some cases of severe dystrophy are reported in young girls as autosomal recessive (98,100, 101). The muscle weakness appears during the childhood. The disease progresses rapidly, the patients soon are unable to walk and they die at the end of the second decade from cardiac failure or from respiratory infections (101). It is generally not possible to study human muscular dystrophies due to the difficulty of obtaining muscle samples. There is, however, the possibility of studying the muscle in dystrophic animals such as mice and hamsters in which the disease is similar to the Duchenne muscular dystrophy in man (103). The muscle diseases in mice (136) and in hamsters (137-146, 150, 151, 172, 198, 204) have been studied in our laboratory.

A mitochondrial defect of oxidative phosphorylation, associated with high Ca^{++} level, was reported in the skeletal muscle of dystrophic hamsters, strain BIO-14.6 (140, 141, 142, 145). This abnormality could be detected with NAD^{+}-linked substrates only. It was shown that the dystrophic mitochondria, *in vivo*, had a higher Ca^{++} level than the normal mitochondria

(144). By histological studies, the dystrophic muscle presents some normal-appearing areas, with normal Ca^{++} level, and some areas of muscle necrosis observed as white streaks in the muscle mass in which the Ca^{++} content was 13 times higher than normal (145). Therefore, the presence of normal and abnormal mitochondria, the latter with a high Ca^{++} content, was expected, according to the histology of the muscle.

A method to separate the normal mitochondria from the abnormal ones has to be developed. A density gradient centrifugation technique was used in order to achieve this separation, Greenawalt et al (147) having shown that artificially Ca^{++}-loaded mitochondria were more dense than the normal mitochondria. Density gradient centrifugation (also called zonal centrifugation) technique allows the separation of particles according to their densities, or their sedimentation rates, which depend on the size and shape of the particles; the fractions are collected from the gradient in different bands or zones. Therefore, density gradient centrifugation techniques can be divided into two types: rate zonal centrifugation and isopycnic centrifugation.

The rate zonal centrifugation technique involves a steep density gradient. The separation of the particles having different sedimentation rates is achieved according to their size and shape.

The isopycnic gradient centrifugation technique achieves the separation according to particle densities. The density gradient used must overlap the whole range of densities of the particles studied. The sedimentation is carried out until the particles reach positions in the gradient which correspond to their own density.

Sucrose is the most frequently used of all density gradient-forming materials, for either rate zonal or isopycnic centrifugation experiments. The main problem with a sucrose density gradient is believed to be the high osmotic pressure of hypertonic sucrose solutions which damage the mitochondria due to osmotic stress (53). Mitochondria, after sucrose density gradient centrifugation, have been reported either coupled (10, 68, 89), poorly or completely uncoupled (10, 83, 84) or damaged as judged from enzyme solubilization (55, 56).

Therefore, the aim of this project was to develop a method which would yield intact mitochondria after density gradient centrifugation.

Ficoll, a polymer of sucrose, can be used to make dense solutions with a low osmotic pressure because of the high molecular weight (400,000) of this polymer. Ficoll was therefore used in this study to make a density gradient by increasing its concentration linearly with 0.25 M sucrose being added throughout the gradient to give an iso-osmotic medium. Such a density gradient should avoid the osmotic pressure problem observed with a simple sucrose density gradient.

Ficoll-sucrose density gradients have been reported to inhibit the ability of mitochondria to couple phosphorylation to respiration (88, 89, 92). In contrast, Ficoll-sucrose density gradients have been reported to yield coupled mitochondria in the presence of albumin (92) and in the absence of albumin (83, 84, 93).

The object of the first part of the project was to separate, by a single step density gradient centrifugation, the mitochondrial fraction from a pigeon heart homogenate layered on a continuous Ficoll-sucrose density gradient and to study oxidative phosphorylation in these mitochondria.

The objective of the second part of this project was to compare oxidative phosphorylation by previously isolated mitochondria when subjected to rate zonal centrifugation in a sucrose density gradient and in a Ficoll-sucrose density gradient. From the literature, it seemed that the mitochondrial isolation procedure, the composition of the density gradient, the centrifugal forces, the recovery from the gradient solutions are different factors which might influence the quality of mitochondria subjected to density gradient centrifugation. Therefore a study was done to compare the effect of both types of density gradients on oxidative phosphorylation by mitochondria previously isolated from different tissues using the same isolation procedure.

The third part of the project was aimed at applying the density gradient centrifugation technique to the study of skeletal muscle mitochondrial populations in the disease model of dystrophic hamsters.

II. LITERATURE REVIEW

This literature review is limited to the information immediately relevant to the study. The literature review is divided into three major parts:

I. Density gradient centrifugation.

II. Muscular dystrophy.

III. Oxidative phosphorylation and respiration of dystrophic skeletal muscle and heart mitochondria.

I. Density gradient centrifugation.

1. Definition of density gradient centrifugation.

Density gradients, made by increasing the concentration of one component of the medium continuously from the top to the bottom of the tube or the rotor chamber, are used to stabilize, against convection currents and other anomalous effects (3), the medium through which the particles sediment, forming zones or bands, according to their sedimentation rates or densities. This technique is also called zonal centrifugation. The concept of density gradient centrifugation can be approached in two ways, depending on what one wants to achieve; separation according either to the sedimentation rates or to the densities of the particles (Figure 1).

Figure 1: Zonal centrifugation performed in a density gradient.

A: The sample, containing a mixture of particles of different size, shape and density, is layered on the top of the density gradient.

B: After rate zonal centrifugation performed at low centrifugal force for a short time, the particles are separated according to their sedimentation rates. Therefore, the particles in the lower band sediment at a faster rate than the ones in the upper band.

C: After isopycnic gradient centrifugation, the particles are separated according to their densities. The centrifugation is performed until they reach the zone, in the gradient, which corresponds to their own density.

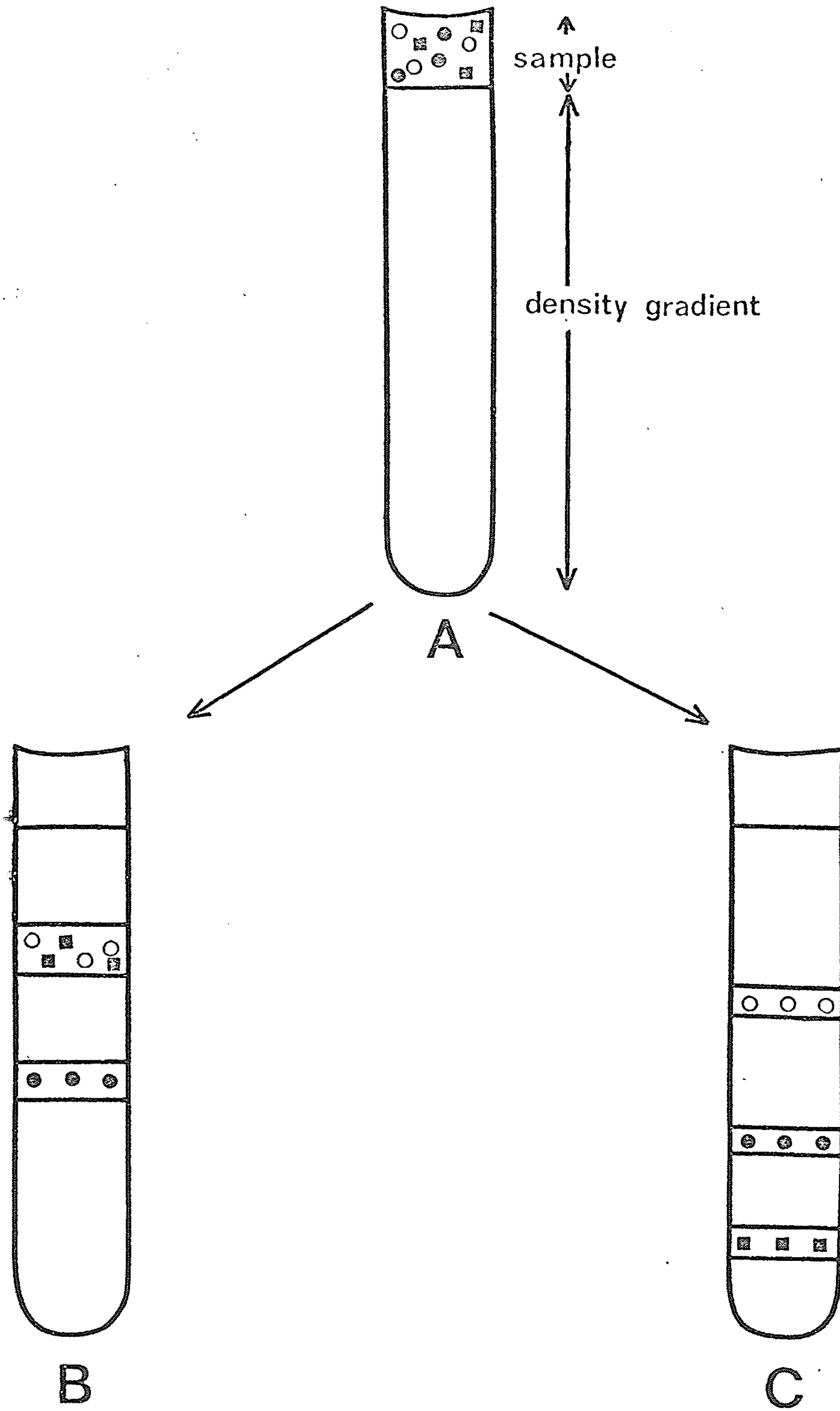


Figure 1

Isopycnic gradient centrifugation (1, 2, 3), also called equilibrium zonal centrifugation (7), can be done if the gradient extends over the whole range of densities of the particles that are to be separated. The particles in the sample layered on the gradient sediment and stay in equilibrium in the layer of medium corresponding to their densities. The separation of the sample components is accomplished, at least theoretically, according to the difference in their respective densities (1).

The rate zonal centrifugation (7), also called gradient differential centrifugation (2, 3), is done with a gradient of a narrower range. The sample is also layered on the gradient prior to centrifugation. Then all the components sediment through the gradient under the influence of the centrifugal forces. As they migrate, the particles separate into zones according to their sedimentation rates. However, if one interrupts the centrifugation while the particles are still migrating, they will be recovered in different zones of the gradient depending on their sedimentation rates. The sedimentation rate of the particles depends on their size, shape and densities (1), as discussed below.

2. Factors influencing the sedimentation rate.

The sedimentation rate, expressed in terms of radial velocity of a sedimenting particle, is influenced by several factors as will be shown later. The

equation which gives the rate at which a particle sediment in a centrifugal field, can be written as follows:

$$V = \frac{dx}{dt} \qquad \text{(I)}$$

where V = radial velocity of a sedimenting particle, in cm sec^{-1}.

dx = distance travelled (cm) radially in a very small interval of time dt (sec).

However, to study the sedimentation rate of a particle, the sedimentation constant (S) is used conventionally in the literature, and is defined as the velocity of sedimentation per unit centrifugal field (4):

$$S = \frac{V}{\omega^2 x} = \frac{2r_o^2 (\rho_p - \rho_m)}{9\eta} \qquad \text{(II)}$$

for a spherical particle, and by

$$S = \frac{2r_o^2 (\rho_p - \rho_m)}{9\eta\theta} \qquad \text{(III)}$$

for a non-spherical particle, where:

ω = angular velocity, in radian sec^{-1}.

x = radial distance from the axis of rotation, in cm.

r_o = radius of the particle, in cm.

ρ_p = density of the particle, in g cm^{-3}.

ρ_m = density of the medium, in g cm^{-3}.

η = viscosity of the medium, in poises (g cm^{-1} sec^{-1}).

θ = ratio of the frictional coefficient of the particle to that of a spherical particle of equal volume.

The mathematical development of these equations II and III are discussed in de Duve's (4) and Anderson's articles (3).

The composition of the media, through which the particles sediment, enters directly into the equations in the form of density (ρ_m) and viscosity (η). The sedimentation constant is proportional to the difference between the density of the particles and the density of the medium, and inversely proportional to the viscosity of the media. Therefore, it is important to have a compound which yields a low viscosity gradient solution, otherwise the sedimentation constant is decreased. Also, the composition of the media enters indirectly in equations II and III, if one deals with organelles which act like osmometers. If the membranes are permeable to the gradient components, then r_o, ρ_p, which are characteristics of the particles, will be changed and those equations then have to be corrected for such phenomena (4).

Another factor which influences the sedimentation constant is the temperature at which the centrifugation is carried out. For example, the viscosity of a sucrose solution will change by 15% to more than 30%, between 0^oC and 5^oC, depending on the concentration of the sucrose solutions (4).

3. Rotors used in density gradient centrifugation.

(a) Swinging-bucket rotor.

Swinging-bucket rotors have been used very successfully by workers in different biological areas. High speed swinging-bucket rotors have been used for different types of studies including separation of ribosomal and mitochondrial RNA from yeast (8), isolation of mitochondria (9), purification of previously isolated mitochondria (10), demonstration of the heterogeneity of mitochondria (11). Many other applications of density gradient centrifugation in swinging-bucket rotors can be found in review articles (3-7).

There are several inherent problems in using swinging-bucket rotors. These are mentioned briefly in the following paragraphs.

Preparing the gradient is time consuming, the gradient range may differ, by experimental variations, from one tube to the other, the amount of material that can be layered on the gradient is quite limited due to the limited gradient volume (4 to 50 ml) and care must be taken in handling the gradient tubes. Also, artifacts due to centrifugation itself must be avoided (3), as discussed below.

Changes in angular velocity which generates rotational movement of the fluid in a horizontal plane causes what is known as "swirling". This can be avoided by having

a slow acceleration and a slow deceleration. A stabilizer can be fixed on the rotor to prevent any wobbling of the rotor at low speeds (4).

Due to a non-uniform temperature throughout the tube, thermal convection may result. However, the density gradient by itself largely counteracts any convection and by working at low temperature and under vacuum, this problem can be minimized (6).

Due to aerodynamic effects, a movement of the upper part of the liquid may be created. This can be prevented by capping the tube (6).

The particles in a centrifugal field do not sediment in a parallel fashion, but rather "fan out" from the axis of rotation, causing wall effects. When portions of the sedimenting particles hit the wall, the particles have a tendency to agglutinate and either adhere or slide as a mass to the bottom. Schumaker (6) has reviewed the wall effects and concluded that the sedimentation into the walls seem to have little or no effect on the observed sedimentation velocities for a wide variety of biological materials including proteins, viruses, and cell particulates. To prevent the wall effect, Anderson (3) proposed the use of a sector shaped centrifuge tube, restricted to relatively low speed, for fractionation of large cell particulates.

Hydrodynamic effects (3, 6, 13) can be divided into two parts: (i) streaming effect or droplet sedimentation (7), and (ii) the turnover effect:

(i) The streaming effect, as defined by Anderson (3), is seen when a 10% liver brei is layered over a sucrose solution having a slightly higher density. Material from the layered brei soon begins to fall in streams through the denser layer without any centrifugation. This effect can be overcome by diluting the brei layer, or by increasing the density difference between the two layers. Centrifugal force increases the magnitude of the streaming effect.

(ii) The turnover effect may be responsible for a poor resolution of the layered system. If one layers a suspension over a denser solution and centrifuges, as the particles pass the interface, the upper area then becomes more dense than the lower area due to the particles and they move as a body through the gradient solution. This type of artifact is amplified with a discontinuous gradient where the same phenomenon happens at each layer. It would therefore be less serious in a continuous density gradient (3).

According to Anderson (12), the ideal rotor should allow 1) sedimentation and resolution in a sector-shaped compartment, 2) rapid gradient formation in the rotor with minimal stirring or convection, 3) sharply defined starting

or sample zones, 4) high rotational speed, 5) large capacity and 6) rapid recovery of the gradient after centrifugation without loss of resolution.

(b) Zonal rotor.

Most of the artifacts of gradient centrifugation using swinging-bucket rotor can be avoided by using a zonal rotor. This type of rotor was developed by Anderson at the Oak Ridge National Laboratories (14). A zonal rotor consists of a large cylindrical chamber divided into a number of sector-shaped compartments by vertical septa that radiate from an axial core to the rotor wall (15). Following are the characteristics of the two main series of zonal rotors, A and B.

The A-series are low-speed zonal centrifuge rotors. They are loaded and unloaded dynamically through a rotating, fluid line seal assembly (14) and used for rate zonal centrifugation or isopycnic centrifugation. The transparent plastic end caps allow direct observation of the separation (16). The B-series are essentially the same rotors except that these rotors are designed for high-speed centrifugation (17). Sheeler (15) describes technical details of both types of rotors with their possibilities and the disadvantage of the rotating-seal assemblies.

The reorienting gradient zonal rotor was also introduced by Anderson but the first rotor of this type

commercially available was the SZ-14 reorienting gradient zonal rotor, manufactured by Sorvall Inc., Newtown, Con., U.S.A. This rotor can be loaded dynamically, when spinning at 2,500 r.p.m. or statically, at rest. The gradient reorients during the acceleration from a vertical to a radial position. This rotor is emptied at rest preventing further sedimentation during the unloading process which could be a problem with some model A rotors, which require dynamic unloading after rate zonal centrifugation experiments. The Sorvall company reviewed the possibilities of this rotor (18) and concluded that the same type of experiments as with the A- and B-series can be done using this SZ-14 reorienting density gradient zonal rotor, however without the disadvantages and complications associated with rotating seals of regular zonal rotors.

Separation of subcellular fractions (21, 23, 24, 25) have been achieved with some rotors of the A-series. Studies on ribonucleases (19, 20), on DNA (22) have been accomplished using also the rotors of the A-series.

Viruses (26), coliphages (27), glycogen (32, 33), nuclear fractions (30), microsomes (31), ribosomes (36), lysosomes (28, 29), mitochondrial fractions (29, 35) have been isolated or purified with the rotors of the B-series. Also the distribution of acid phenyl phosphatase activities (34) and cytochrome oxidase activities (35), which are marker enzymes for microsomes and mitochondria respectively, has been studied after zonal centrifugation experiments.

4. Density gradient solutions.

The ideal material for density gradient preparation should be a chemically inert, readily available, high molecular weight compound that will give solutions of high density at low viscosity (37).

A large variety of chemicals have been used to make gradient solutions. Often used are deuterium oxide (D_2O), salts, sucrose and other sugars as well as synthetic polymers.

Advantages of using D_2O-sucrose density gradients are as follows: 1) it gives solutions with high specific gravities and low viscosities; 2) D_2O and H_2O react to the same extent with the particles being studied; 3) it reduces the osmotic pressure of isopycnic sucrose solutions (4). D_2O-sucrose gradients had been used by Beaufay to study the distribution of isolated rat liver mitochondria (38). H_2O-D_2O sucrose density gradients were used by Thomson *et al* (154), for sedimentation studies of mouse liver mitochondria.

Salts like potassium citrate, potassium tartrate, cesium chloride, sodium bromide, potassium bromide, cesium sulfate, rubidium chloride were also used to make gradients. They allow high density with low viscosity solutions. Cesium compounds have been used in preparing density gradients for isolating viruses (40), subcellular fractions and nucleic acids in zonal centrifugation experiments (39). Glycogen has been isolated from rat liver homogenate on cesium chloride gradients (41).

Solutions of glycerol (46, 47), sucrose and other sugars like sorbitol (48), polyglucose (49) and Ficoll, a polymer of sucrose, are being used to make gradients; they are uncharged, chemically inert and do not absorb ultraviolet light. The last characteristic is important because the sedimentation pattern of the particles in the density gradient is often determined by monitoring at 260 or 280 nm.

Since centrifugation of mitochondria on Ficoll-sucrose and sucrose density gradients will be compared in the Results, II, more emphasis will be placed in the following section on the properties of these two types of density gradients and their influences on mitochondrial structure and function.

Sucrose is the most frequently used of all density gradient-forming materials for zonal centrifugation experiments. A principal disadvantage of sucrose solution is its high viscosity when the solution is very concentrated. Then the sedimentation rates are quite low, and the problem can only be overcome by increasing the time and the speed of centrifugation.

The sucrose density gradients are not ideal especially for isopycnic sedimentation which requires highly hypertonic solutions. A sucrose density gradient therefore always superimposes not only a viscosity gradient but also an undesirable osmotic pressure gradient.

Mitochondria contain two compartments, one permeable to sucrose and one impermeable to sucrose (50). The first is the space between the outer and inner mitochondrial membranes (43). The mitochondrial volume adjusts itself by water movement to changes in the external osmotic pressure, acting like an osmometer (51). As the concentration of sucrose is increased, it diffuses through the permeable outer membrane into the sucrose accessible space, the water diffuses out of the sucrose inaccessible space, decreasing linearly, and the inner compartment shrinks (50).

It was shown that a sucrose concentration above 0.3 M inhibits respiration of intact liver mitochondria (52). Sucrose is also an inhibitor of ATP-induced mitochondrial contraction (53), and of the ATP-Pi exchange reaction (54). High sucrose concentrations suppress the transport of inorganic phosphate ions across the mitochondrial membranes (83). Even if large-amplitude swelling is reversible by ATP, enzymatic changes in the mitochondria might take place which lead to "loose" coupling of oxidative phosphorylation, decline in phosphorylation and loss of pyridine nucleotides (53). High sucrose concentrations decrease the mitochondrial respiratory control (RCR), probably due to the incidental osmotic stress (53).

After zonal centrifugation on sucrose density gradients, electron micrographs show damage to the mitochondrial structure and such damage is also shown by solubilization and loss of mitochondrial enzymes (55, 56).

In spite of the above osmotic disadvantages, sucrose density gradients have been used successfully by several workers (a) to fractionate a whole homogenate by rate zonal centrifugation, each subcellular fraction being identified by their specific marker enzymes (57-62), (b) to separate different populations of mitochondria (9, 45, 63-73, 143) and (c) for a further purification of mitochondrial preparation (10, 21, 38, 75).

Wattiaux *et al* (56, 76-80) also studied the effect of hydrostatic pressure on the mitochondrial enzymatic sedimentation pattern. Rat liver mitochondria deteriorate when centrifuged at high speed in a sucrose density gradient. Recently, these authors (81) reported that a higher centrifugation temperature can overcome the deleterious effect of high speed centrifugation on mitochondrial membranes.

Pollak and Morton (82) reported also that the mitochondrial recovery after density gradient centrifugation is dependent on the centrifugation temperature, suggesting that a temporary temperature drop slightly below $0^{o}C$ is a possible source of artifactual separation of mitochondria.

"Poorly" coupled mitochondria obtained by zonal centrifugation on a sucrose density gradient had been reported by Dimino and Hoch (83), and Grimwood and Wagner (84) using rat liver mitochondria. Bullock (68, 165) on the other hand reported very well coupled rat skeletal muscle mitochondria purified on a discontinuous sucrose density gradient. Douce et al (10) reported coupled mitochondria, isolated from a large variety of plants, after purification on a discontinuous sucrose density gradient; however, rat liver and pigeon heart mitochondria did not survive the purification procedure (10). These authors also reported a slow dilution technique to avoid osmotic shock and membrane damage which may occur when the mitochondria are rapidly changed from the hyperosmotic sucrose solution of the gradient to a normotonic sucrose solution. Their normotonic sucrose solution is a suspending medium, containing bovine serum albumin, which they used to recover the individual mitochondrial fractions from the gradient.

To prevent the osmotic effect of high sucrose concentrations, other substances of high molecular weight have been used to make density gradient close to iso-osmoticity: diidon (6), glycogen (42-44), heptahexocide (4), polyvinylpyrolidine (45), Ficoll (85).

Ficoll has been used by Holter (85) to make aqueous density gradients. The following are the characteristics of Ficoll, synthetized by Ingelman and Flodin, as reported

by Holter: "Ficoll is a highly water-soluble, polymolecular, neutral colloid with properties similar to polysaccharides. Its molecules are spherical. The colloid is stable in neutral and alkaline, non-oxidative solutions. Its aqueous solutions have a relatively low viscosity. The average molecular weight of the unfractionated substance is about 50,000". The substance available now has a molecular weight of 400,000. Because of its high molecular weight, high concentrations of Ficoll solutions have a low osmotic effect. However, Shortman (97) reported markedly increased osmotic activity of high Ficoll concentrations, due to some water binding effect. At the same concentration, a Ficoll solution and a sucrose solution have similar densities (87).

Pharmacia Canada Ltd. has published several pamphlets with references for the use of Ficoll in density gradient centrifugation experiments (86, 96).

An iso-osmotic Ficoll-sucrose density gradient can be made by increasing the Ficoll concentration and by adding throughout an iso-osmotic concentration of sucrose. Ficoll itself in such a gradient will contribute very little to the overall osmotic pressure. Consequently, the organelles can be centrifuged in a solution corresponding to their own osmoticity, with minimal damage due to osmotic effects. Following are some applications of an iso-osmotic Ficoll density gradient with respect to mitochondria.

Johnson (87) reported purification of a crude pellet of rat brain mitochondria. Beaufay (43) reported that the rat liver mitochondrial fractions were much denser in the presence of high concentration of Ficoll than they were in the presence of glycogen and became concentrated at the bottom of the tube. Stahl et al (88) reported that purification of bovine brain mitochondria in a Ficoll concentration higher than 8% yielded mitochondrial fractions which exhibited greatly diminished P/O ratios. Buetow et al (89) reported that purification of mitochondria isolated from Euglena Gracilis on a discontinuous Ficoll density gradient (4, 8 and 12%) resulted in loss of phosphorylating ability. On the other hand their purification on a sucrose density gradient improved phosphorylation and respiration. Lusena and Depocas (91) reported centrifugation, in Ficoll density gradients of rat liver mitochondria to separate light and heavy fractions; however, they observed that Ficoll depressed glutamate dehydrogenase activity in rat liver mitochondria. Basford (92) used 8% Ficoll to increase the density of the isolation medium to prepare a relatively pure brain mitochondrial fraction. In the presence of bovine serum albumin, such mitochondria exhibit good P/O ratios and respiratory control ratios. Brown (29) reported the separation of mitochondria, peroxisomes and lysosomes by zonal centrifugation in a Ficoll density gradient. Clark et al (93) used a 6% Ficoll medium to purify rat brain mitochondria and the pellet studied

polarographically showed high RCR's and O_2 rates, even in the absence of bovine serum albumin. Day et al (94) by substituting an iso-osmotic Ficoll-sucrose density gradient for a hyperosmotic sucrose density gradient showed that it was possible to reproduce consistently zonal centrifuge absorbancy profiles of adult rat brain homogenates. Wilson (95) reported heterogeneity of rat liver mitochondria, based on cytochrome and enzymatic differences, separated by rate zonal centrifugation in a Ficoll density gradient. Finally, Dimino and Hoch (83), Grimwood and Wagner (84) reported that tightly coupled liver mitochondria could be recovered from a linear Ficoll-sucrose density gradient.

From the above mentioned studies and from theoretical considerations, it appears that Ficoll-sucrose density gradient may be a useful tool to study mitochondria subjected to zonal centrifugation. This type of density gradient minimizes the osmotic pressure problem encountered with a sucrose density gradient.

Opinions in the literature regarding the effect of sucrose density gradient and Ficoll-sucrose density gradient on the mitochondrial oxidative phosphorylation are not unanimous. However, except for the observations of Bullock (68, 165), it seems that mammalian mitochondria are not coupled after sucrose density gradient centrifugation. A sucrose density gradient subjects the mitochondria to hyperosmotic

conditions, while Ficoll-sucrose density gradient probably minimizes this problem. The effect of these two types of density gradient solutions on oxidative phosphorylation is more ambiguous. The source of the mitochondria, the isolation procedure, the gradient type (continuous or discontinuous), the speed of centrifugation and the dilution procedure might be involved in the quality of the mitochondria recovered from a density gradient. Thus, while Ficoll-sucrose density gradients theoretically offer some advantages over plain sucrose density gradients, some additional factors, just mentioned above, would have to be carefully controlled to make a valid comparison between these two types of density gradient solutions.

II. Muscular dystrophy.

Charles Bell, in 1830, probably gave the first recognizable clinical description of this muscle disease (99). In 1850, Aran drew attention to a group of conditions which gave rise to progressive muscular weakness and wasting, and in 1852, Meryon described a form of granular degeneration of the voluntary muscles (100). In 1868, Duchenne (104) gave a description of a pseudohypertrophic muscular paralysis of children. Some other cases of muscular weakness have been described but Wilhelm Erb in 1884 combined the conflicting case reports and established in 1890 the term progressive

muscular dystrophy which was a primary degenerative disorder of the muscle fibers. His classification of dystrophy had four subvarieties: one beginning in the adult life and three in childhood, the first being an infantile variety with pseudohypertrophy as described by Duchenne, the second being a facioscapulohumoral form (Landouzy and Dejerine) and a third being a juvenile muscular atrophy (Erb) (99).

As defined by Rowland (101), a myopathy is a disorder of skeletal muscle in which there is no clinical, histologic or electromyographic evidence of denervation or emotional disorder. Muscular dystrophies are forms of myopathies. They may be a nutritional myopathy due to a dietary deficiency of vitamin E (108, 109) (animal muscular dystrophy) or a genetically determined abnormality (98, 100, 101) (Duchenne muscular dystrophy and animal muscular dystrophy resembling Duchenne muscular dystrophy), both of them being characterized by progressive weakness and wasting of skeletal muscle. A condition resembling muscular dystrophy may also be generated experimentally by denervation of skeletal muscle (108).

The Duchenne muscular dystrophy is the most commonly found human type of dystrophy. It is expressed in the male, rarely or not at all in the female. This type is referred as an X-linked recessive disease but some cases of severe

dystrophy have been reported in young girls as autosomal recessive (98, 100, 101). The prevalence of Duchenne muscular dystrophy is about 50 per million population with an incidence rate of 160-250 per million live births (101, 118). The muscular weakness is generally evident at about 3 years of age, by symmetrical involvement of pelvic girdle musculature and later of the shoulder girdle. Between 10 and 15 years of age, the child is unable to walk. Most of the patients die from sudden cardiac failure or from respiratory infections towards the end of the second decade (100, 102). Some studies have indicated a generally lowered intelligence quotient in boys with Duchenne muscular dystrophy and a high frequency of frank mental retardation (100, 101). Electrocardiographic abnormalities occur commonly in Duchenne muscular dystrophy but the electrocardiogram changes do not seem to be progressive. Some investigators attribute the myocardial disorder to a separate genetic defect (101, 102).

According to presently discussed theories, the muscular dystrophy may be myopathic, i.e. the disease process is assumed to involve the muscle directly, without damaging its nerve supply; it may be neuropathic (110-112, 117), i.e. the motor nerve cells or their processes are first affected, resulting secondarily in atrophy of muscle, or it may have a vascular cause (113), i.e. an insufficiency of the capillary circulation is responsible for a muscular anoxia which influences the cell membrane permeability and allows an enzymatic leakage.

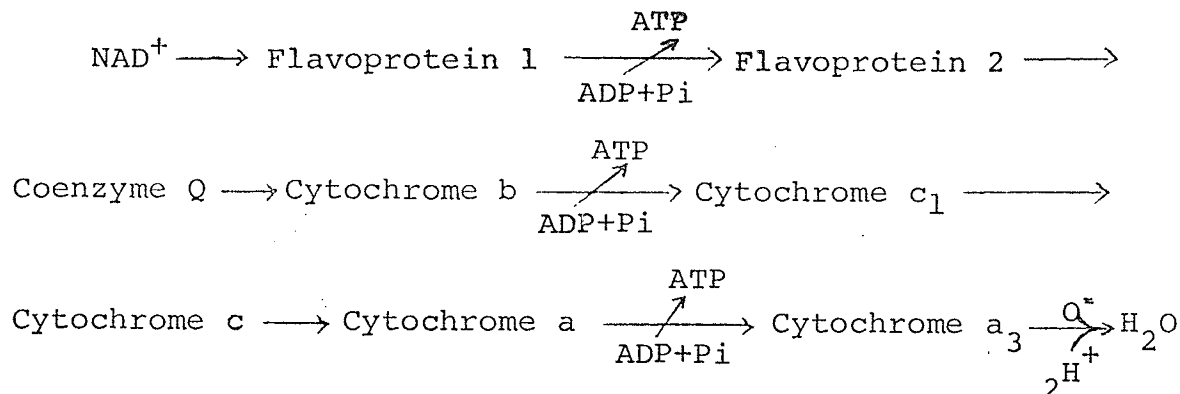
For ethical reasons, the amount of sample that can be taken from a patient who has already a decreased muscle mass, has to be small. Therefore, animal myopathies are studied in the hope that an understanding of these will eventually lead to an understanding of the process of the human dystrophy, even if animal myopathies are not identical to the human disorders (109). There is quite a variety of genetic myopathies in animal species: muscular dystrophies in mouse (114), Syrian hamster (103), chicken (115), duck (116), goat, turkey and lamb have been reported.

No treatment is at present known which has any definite beneficial influence on muscular dystrophy (98, 102). Therefore, the only method to control the incidence of that disease is to detect the carriers of Duchenne muscular dystrophy. Serum creatine phosphokinase (CPK) determination is considered to be the best test for detection of the carrier state (105, 107), but only 70% of definite carriers have abnormal values (101, 119). Ionăsescu reported an increase of protein synthesis by muscle polyribosomes from carriers of Duchenne muscular dystrophy. According to these workers, an assay for amino acid incorporation by polyribosomes from muscles together with a CPK determination could identify more than 90% of Duchenne carriers (106).

III. Respiration and oxidative phosphorylation of dystrophic skeletal muscle and heart mitochondria.

The metabolism of lipids, proteins and carbohydrates is accompanied by conservation of some of the energy of these large organic molecules in the physiologically utilizable form of the phosphate-bond energy of ATP. The Krebs cycle, localized in the mitochondria, is the final common pathway into which all the fuel molecules of the cell (carbohydrates, fatty acids, and amino acids) are ultimately oxidized to CO_2 and water. During the oxidations, the electrons removed from the substrates and/or the hydrogen atoms liberated are transported via a chain of electron carriers, called the respiratory chain, to molecular oxygen, the ultimate electron acceptor in respiration. This process is accompanied by conservation of the free energy of these electrons in the form of phosphate-bond energy of ATP, a process defined as oxidative phosphorylation. Oxidation of NAD^+-linked substrates in mitochondria occurs with formation of three molecules of ATP per atom of oxygen consumed, i.e. an ADP/O ratio of 3 ($_3ADP + {}_3Pi + {}_2H^+ + O^= \longrightarrow {}_3ATP + H_2O$). Oxidation of FAD-linked substrates occurs with formation of two molecules of ATP per atom of oxygen consumed, i.e. an ADP/O ratio of 2 is obtained.

The following diagram shows the probable sites of the phosphorylation in the respiratory chain:



Chance and Williams have shown that the rate of respiration can be controlled by the concentration of ADP (177). When oxidation and phosphorylation are tightly coupled, the ADP is an essential constituent of the phosphorylation process. In the absence of ADP, there is a slow oxygen consumption, which they called State 4. In the presence of ADP, there is a fast oxygen consumption coupled to the phosphorylation of ADP (State 3). The respiratory control ratio (RCR) or also called respiratory control index (RCI) is the ratio of the respiration rate in State 3 over that in State 4 (177). The RCR is used as a criterion of functional intactness of mitochondria (177, 180).

Vignos et al (120) reported that the ATP/ADP ratio is lower in skeletal muscle from patients with Duchenne muscular dystrophy than in normal skeletal muscle. In contrast, this ratio in the adult muscular dystrophy (or limb-girdle dystrophy) is normal (120). One explanation for this inability to maintain a normal level of ATP in Duchenne

muscular dystrophy might be a defect in the process of oxidative phosphorylation. Zymaris *et al* (121) have shown that the concentration of ATP in hind leg muscles of dystrophic mice was significantly lower than that found in normal mouse muscle.

Mitochondria that oxidize glutamate at normal rates and with normal P/O values have been isolated from muscle of patients with Duchenne muscular dystrophy (122). But as the disease progressed, the glutamate oxidation rate decreased until no discernable oxygen consumption could be detected. However, the P/O ratio remain normal with dystrophic mitochondria as long as there was a demonstrable rate of oxidation (122). Olson also reported in the same paper (122) that mitochondria of subjects with limb-girdle dystrophy, having a normal P/O ratio, had a rate of oxidation below control values. Peter *et al* (123, 124, 129)reported similar observations. In less-affected muscle, the mitochondrial measurements were normal but the muscle from patients with far-advanced Duchenne muscular dystrophy gave a low yield of mitochondria with low respiration rates, poor RCR's and low ADP/O ratios. They also reported normal respiration rates, normal RCR's and normal ADP/O ratios from dystrophic muscles of a patient with dystrophia myotonica. Ionăsescu *et al* (125) reported studies done on muscle homogenate; in malignant Duchenne muscular dystrophy, a depressed RCR and normal P/O ratio were found,

characteristic of a loose coupling of oxidative phosphorylation. In facioscapulo-humoral and limb-girdle form dystrophy, normal RCR and partial uncoupling of phosphorylation from respiration were reported, due to a diminution in the phosphorus uptake. In 1970, they confirmed (126) their findings about Duchenne muscular dystrophy by showing significantly lower RCR and normal P/O ratio in human muscle homogenate, again indicative of loosely coupled oxidative phosphorylation. Hudgson _et al_ (127) reported poorly coupled oxidative phosphorylation in muscles of 6 members of a family having a form of progressive muscle disease. Hülsmann _et al_ (128) reported five cases of loosely coupled mitochondria from human skeletal muscle diseases, these cases not being closely related clinically; in contrast to the RCI, no abnormality in the P/O ratio was observed.

Experiments carried out with laboratory animals with muscular dystrophy showed similar findings. Lochner and Brink (130), using mitochondria from muscle of Syrian hamsters with hereditary muscular dystrophy, reported normal oxygen uptake but depressed P/O ratios and ATP formation. The Pi content was significantly higher in the dystrophic muscles, the ATP level being normal. March _et al_ (131), using genetically dystrophic chickens, reported an elevated rate of oxygen uptake with the isolated muscle mitochondria; also accelerated

loss of ATP from mitochondria of dystrophic muscle was observed. However, Ashmore and Doerr (132) reported no significant abnormality of ADP/O ratio of muscle mitochondria from dystrophic chickens; low rate of pyruvate-malate and α-glycerophosphate oxidation was observed.

Lin *et al* reported (133) an accumulation of fat in the skeletal muscle of the dystrophic mice. A decrease in both palmitate and pyruvate oxidation in the muscle mitochondria of dystrophic mice was observed using either malate or succinate as substrate. Later (134), they confirmed their results by studying the oxidation of palmitic acid by the 600 x G supernatant fraction of mouse skeletal muscle homogenate and by skeletal muscle mitochondria. The oxidation was significantly decreased compared with that of the normal controls. Finally (135), they published a study with patients having Duchenne muscular dystrophy. Palmitate oxidation was markedly reduced when compared with control cases. Reduced palmitate oxidation was also observed in definite female carriers of the sex-linked dystrophic gene (135). They concluded (134) that the decrease in the pyruvate and palmitate oxidation by dystrophic muscle mitochondria is most likely due to a defect of the Krebs cycle.

Myocardial degeneration occurs spontaneously in all animals of strain BIO-14.6 of Syrian hamsters. This disorder is transmitted by an autosomal recessive gene (168). The

progressive cardiac condition leads in several stages to congestive heart failure: prenecrosis, hypertrophy and terminal failure (151, 168). Oxidative phosphorylation of heart mitochondria from cardiomyopathic hamsters has been studied. Opie *et al* (166), using a heart homogenate, reported a lowered P/O ratio, suggesting uncoupling of oxidation from phosphorylation. Schwartz *et al* (167) using hamsters with frank congestive heart failure, reported a decrease in the respiratory control ratio and in the oxygen uptake in the heart mitochondria. Lochner *et al* (169) reported loosely coupled oxidative phosphorylation in mitochondria from cardiomyopathic hamsters with heart hypertrophy and/or failure. However, Lindenmayer *et al* (170) reported normal oxidative phosphorylation in heart hypertrophy but decreased oxidative phosphorylation in terminal heart failure. Fedelesova and Dhalla (171) reported a decreased level of creatine phosphate and ATP in the heart of BIO-14.6 hamsters, and also suggested a defect in the process of energy generation in failing hearts.

In our laboratory, normal oxidative phosphorylation was reported in the heart of myocardiopathic hamsters, regardless of the disease stage (137, 138, 151). In the later stages, the mitochondrial content of the myocardium was decreased and the organelles contained less endogenous substrate (172). The low endogenous substrate content in the

mitochondria might explain the abnormalities observed by Lindenmayer and Schwartz, as they used glutamate as substrate without any exogenous source of oxaloacetate, which is needed for transamination of glutamate, such as malate.

Also an extensive study has been done in this laboratory to give a more comprehensive view of oxidative phosphorylation by mitochondria isolated from the dystrophic hamster skeletal muscle, strain BIO-14.6. Wrogemann and Blanchaer (136) reported that the RCR, the respiration rate and the ADP/O ratio were not significantly different in the mitochondria isolated from skeletal muscle of control and dystrophic mice, strain 129/Re. In 97-124 day old dystrophic hamsters, there was no difference reported between oxidative phosphorylation of the dystrophic skeletal muscle if compared with the normal skeletal muscle (137). The P/O ratio and the respiratory rate was depressed in mitochondria isolated from skeletal muscle of dystrophic hamsters, around 210 days old, using pyruvate-malate and palmitate as substrates (138). It suggests a defect in the degradative pathway common to these substrates, i.e. the Krebs cycle. However, the oxidative phosphorylation was normal in young dystrophic hamsters (138). In 265 day old dystrophic hamsters, the mitochondrial respiration rates, the RCR's and the ADP/O ratios were decreased, these animals being in the late stage of hereditary

muscular dystrophy (139). In 30-80 day old dystrophic hamsters which were selected for having extensive muscle streaking, the respiration rate was decreased by 30% - 50% of normal and the stimulation of oxygen uptake usually seen after ADP addition was minimal or absent (140). Such mitochondria had a Ca^{++} content 13 times higher than the mitochondria isolated from the normal hamsters. The mitochondrial defect is largely corrected by adding Mg^{++} to the polarographic test system (140). This oxidative phosphorylation abnormality was observable when NAD^{+}-linked substrates were used, but when the substrate was succinate (+ rotenone) or NADH, the respiratory rate was normal, showing that the electron transport capacity is normal (141, 142). Thakar _et al_ (144) showed that mitochondria of dystrophic hamster skeletal muscle, have higher Ca^{++} levels _in vivo_ than normal skeletal muscle mitochondria. Wrogemann _et al_ (145) reported that the beneficial effect of Mg^{++} on the oxidation of NAD^{+}-linked substrates by dystrophic mitochondria is largely caused by its ability to minimize the loss of NAD^{+} and to facilitate re-entry of lost NAD^{+} into the organelles; but NAD^{+} alone could not restore coupling unless Mg^{++} was also added. Also the existence of a normal and a defective mitochondrial populations in dystrophic muscle has been suggested (145). Mezon _et al_ (143, 146) using sucrose density gradient centrifugation, separated

a distinct mitochondrial population, with very high Ca^{++} level, from dystrophic mitochondria. This population has always been observed even with mitochondria exhibiting normal oxidative phosphorylation.

From this review, it appears that there are some discrepencies in the literature about the mitochondrial function in muscular dystrophy. These discrepencies might be due to (a) artifacts in mitochondrial isolation and different techniques used to determine the oxidative phosphorylation measurements; also the use of a whole homogenate to determine such measurements may give erroneous results, (b) the fact that the disease was studied at different stages of development, (c) the use of different disease models, especially in the case of Duchenne muscular dystrophy where the clinical diagnosis may be difficult.

Nevertheless, from all the studies, it appears that there is a defect of oxidative phosphorylation in the skeletal muscle mitochondria at the late stages of Duchenne muscular dystrophy. In this laboratory, skeletal muscle mitochondria studies have been done with dystrophic hamsters of all ages. In young hamsters (140) with observable macroscopically massive muscle necrosis, at approximately 60 days of age, a mitochondrial defect of oxidative phosphorylation can be observed with a parallel increase in mitochondrial Ca^{++} content. Mg^{++} improved the coupling of

oxidative phosphorylation although the mitochondrial magnesium content was normal. In dystrophic hamsters approximately 60 days old, having few visible signs of muscle disease, the polarographic studies show normal mitochondria (146). However, even at that stage of the disease, it was possible to separate, by density gradient centrifugation, two distinct mitochondrial populations, one having a high Ca^{++} content while the other population sedimented in the same zone of the density gradient as normal mitochondria (146). The presence of the Ca^{++}-defect of the mitochondria even at early stages of hamster muscular dystrophy suggests that the mitochondrial defect is closely linked to the genetic defect and may be responsible for the occurrence of muscle necrosis.

III. MATERIALS AND METHODS

A. MATERIALS

I. Chemicals

The chemicals used were of analytical grade, except when otherwise indicated.

- Obtained from the British Drugs Houses, Ltd.:

  Calcium standard solution

  Cupric sulfate

  Ethylenediaminetetra-acetic acid, disodium salt

  Magnesium chloride

  Sodium D-Tartrate

  Sucrose - "Aristar"

- Obtained from Sigma Chemical Company:

  Adenosine 5'-diphosphate from equine muscle, grade 1, disodium salt

  L- ascorbic acid

  Cytochrome C from Horse Heart, Type III

  Ethyleneglycol - bis - (β-aminoethyl ether) N,N'-tetra-acetic acid

  Ficoll

  L-glutamic acid

  Heparin from hog intestinal mucosa, grade 1, sodium salt

  L-malic acid

  D-mannitol

Pyruvic acid, Type II, sodium salt

Tris(hydroxymethyl)aminomethane, Trizma Base (Reagent grade).

- Obtained from Mallinckrodt Chemical Works Ltd.:

  Potassium phosphate, mono and dibasic

  Sodium carbonate

  Sodium phosphate monobasic

- Obtained from Eastman Organic Chemicals:

  Succinic acid, disodium salt

  N,N,N',N'-tetramethyl-p-phenylene-diamine dihydrochloride.

- Obtained from J.T. Baker Chemical Co.:

  Calcium chloride

  Hydrochloric acid

  Sodium hydroxide

  Sodium phosphate dibasic

- The following chemicals were obtained from the companies indicated.

  Bovine Plasma Albumin, fraction V, from Armour Pharmaceutical Co.

  Crystallized Bovine Plasma Albumin from Armour Pharmaceutical Co.

  Chelex 100, 100-200, sodium form, from Calbiochem.

  2,4-Dinitrophenol from Matheson Coleman and Bell.

  Lanthanum oxide from Rare Earth Division of American Potash and Chemical Corporation.

Nagarse, crystallized lyophilized bacterial proteinase from Nagase et Co. Ltd.

Ortho phosphoric acid from Nichols Chemical Company, Ltd.

Phenol Reagent from Ingram and Bell Ltd.

Potassium chloride from Fisher Scientific Co.

Triton X -100 from Harleco Co.

Rotenone from K+K Laboratories Co.

Deionized, glass redistilled water was used to prepare all the solutions.

II. Animals

- Hamsters from three different strains were used. Control animals of the Lakeview strain were obtained from Lakeview Hamster Colony, Newfield, New-Jersey. Other control animals of the RB strain, were purchased from Trenton Experimental Laboratory Animal Co., Bar Harbor, Maine.

  Dystrophic hamsters of the BIO-14.6 strain were purchased from BIO-Research Institute, Cambridge, Mass.

  Additional animals of the above strains were also obtained locally by random breeding. The animals were fed Purina Lab Chow, lettuce and water ad libitum.
- Holtzman rats were obtained from Dr. K. Dakshinamurti.
- Pigeons were obtained locally.

B. METHODS

I. Procedure for isolation of mitochondria using modifications of the procedure developed by Chance and Hagihara (173).

1. Reagents:

Stock Medium: 0.21 M mannitol

0.07 M sucrose

0.1 mM EDTA

pH 7.4

Homogenizing Medium:

(i) For preparation of pigeon heart, hamster heart, hamster skeletal muscle and pigeon heart mitochondria:

Stock Medium containing 0.01 M Tris-Pi, pH 7.6 and 0.5 mg/ml of Nagarse proteinase

(ii) For preparation of rat liver mitochondria:

Stock Medium containing 0.01 M Tris-Pi, pH 7.6.

Suspending Medium:

Stock Medium containing 0.01 M Tris-Cl, pH 7.4.

All equipment was kept ice-cold and procedures were carried out at 0-4°C.

2. Preparation of rat liver mitochondria:

Holtzman rats (300-350 grams) were decapitated, the liver removed and placed in ice-cold Stock Medium for five minutes. The liver was rinsed three times using fresh Stock Medium, minced with scissors and incubated in five volumes of Homogenizing Medium. The suspension was gently homogenized at 100 r.p.m. with a loose Teflon pestle of 0.66 mm clearance (clearance = difference in diameter between inside tube diameter and pestle) in an A.H. Thomas, size C, homogenizer. The homogenate was diluted with an equal amount of Stock Medium and rehomogenized with a tight Teflon pestle (0.20 mm clearance) in an A.H. Thomas, size C, homogenizer. The homogenate was centrifuged at 400 x G for 5 minutes; the supernatant removed and centrifuged at 12,000 x G for 10 minutes. The resulting pellet was washed with Stock Medium, and suspended in 10 ml Suspending Medium. The suspension was then centrifuged at 8,000 x G for 5 minutes. The pellet was finally suspended to yield a protein concentration of approximately 60 mg/ml.

3. Preparation of hamster skeletal muscle mitochondria:

The hamsters were decapitated, the hind part of the animal skinned and immersed into ice-cold Stock Medium for 5 minutes. The back muscles and all muscles from the hind legs and pelvic girdle were removed and placed into ice-cold Stock Medium. The muscles were freed from extraneous tissues, such

as fat and connective tissue, and then minced very finely using scalpels. Muscle mince, 2.5-3 grams, was incubated in 50 ml Homogenizing Medium for 5 minutes, with frequent stirring. The suspension was homogenized with a loose Teflon pestle in an A.H. Thomas, size C, homogenizer. The suspension was incubated another 5 minutes and then diluted with an equal volume of Stock Medium. The suspension was rehomogenized with a tight (0.30 mm clearance) glass-reinforced Teflon pestle in a Tri-R homogenizer. Differential centrifugations were performed as described for the preparation of rat liver mitochondria (see Methods, I, 2). The final suspension gave a protein concentration of approximately 20 mg/ml.

4. Preparation of hamster heart mitochondria:

This was the same procedure as that described for the preparation of skeletal muscle mitochondria (see Methods, I, 3) except for the following modifications.

The heart was thoroughly rinsed, trimmed and minced. This mince was incubated in Homogenizing Medium in a ratio of 300 mg tissue/20 ml of medium. The total incubation time was 16 minutes. The first homogenization was carried out after 8 minutes in an A.H. Thomas, size C, homogenizer with the loose (0.66 mm clearance) Teflon pestle. At the end of the incubation period, the suspension was diluted with an equal volume of Stock Medium, and rehomogenized in an

A.H. Thomas, size C, homogenizer with the tight Teflon pestle (0.20 mm clearance). The differential centrifugations were the same as described previously for the preparation of rat liver mitochondria (see Methods, I, 2). The final suspension had a protein concentration of approximately 20 mg/ml.

5. Preparation of pigeon heart mitochondria:

The pigeon was decapitated, the heart removed and placed in ice-cold Stock Medium. Heart mince (3.5 grams) was incubated in 50 ml Homogenizing Medium for a total time of 16 minutes. This was followed by the same homogenizing procedure and differential centrifugations procedure as that described above for the preparation of hamster heart mitochondria (see Methods, I, 4). The final suspension had a mitochondrial protein concentration of approximately 20 mg/ml.

6. Preparation of pigeon heart homogenate:

(a) For experiments with the SZ-14 reorienting density gradient zonal rotor (the rotor is described in Methods, II, 3), the same procedure as for the preparation of pigeon heart mitochondria was followed except for these modifications.

The suspension after the second homogenization was filtered through three successive Nylon sieves (0.0064 mm^2 mesh) to remove large debris from the homogenate before it was layered on the gradient.

(b) For experiments on the isolation of mitochondria in a single step density gradient, centrifugation was done in an SW-41 and an HB-4 swinging-bucket rotor.

The amount of homogenate that could be layered on the density gradients was very limited (0.4 to 0.8 ml homogenate used with the SW-41 swinging-bucket rotor and 3 ml homogenate with the HB-4 swinging-bucket rotor). Therefore a concentrate homogenate had to be layered on the gradients in order to have enough material to do different tests on the fractions collected after the centrifugation.

Three grams of pigeon heart mince were incubated in 10 ml Homogenizing Medium, containing 2 mg Nagarse/ml, for a total incubation time of 16 minutes. The first homogenization was done, after 8 minutes, with the loose Teflon pestle (0.66 mm clearance) in an A.H. Thomas, size C, homogenizer. At the end of the incubation time, the second homogenization was done with the tight Teflon pestle (0.20 mm clearance) in the A.H. Thomas, size C, homogenizer, without prior dilution. The homogenate was then filtered through the Nylon sieves and the filtrate was ready for layering on the gradient.

II. Density gradient centrifugation.

1. Composition of the gradient solutions:

(a) Ficoll-sucrose density gradients:

Ficoll, a polymer of sucrose, was passed through a chelating resin, Chelex-100, 100-200 mesh, to

remove the calcium ions. Then the solution was concentrated using a flash evaporator (Buchler Instruments, Fort-Lee, N.J., U.S.A.). Because of its high molecular weight (400,000), the contribution of Ficoll to the osmotic pressure of the solution was very low ($<1\%$, even at the highest concentration used). The continuous density of the gradient was obtained by increasing linearly the Ficoll concentration. The osmolarity was made quasi-uniform by adding 0.25 M sucrose throughout the gradient. For this purpose, Aristar sucrose was used because of its low Ca^{++} content ($<$ 0.5 ppm). This iso-osmotic gradient solutions contained also 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2 and 0.1% or 0.5% bovine plasma albumin throughout.

(b) Sucrose density gradients:

Aristar sucrose was used. The solutions contained 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2, 0.1% or 0.5% bovine plasma albumin.

2. <u>Density gradient ranges</u>:

The density gradient ranges were chosen in order to have the mitochondria distributed throughout the gradient. The specific range for each experiment is given in the Results at the bottom of each respective table.

3. <u>Rotors used</u>:

(a) 2 types of swinging-bucket rotors were used:

(i) Beckman SW-41 is a swinging-bucket titanium

rotor which holds six buckets of 13.2 ml capacity each. This rotor was used on a Beckman Ultracentrifuge L 3-40 (Beckman Instruments Inc., Palo Alto, California, U.S.A.).

A Ficoll solution (0.4 ml), containing the same additions as the gradient solution was used as a cushion and, on it, was layered the gradient solution prepared with a Beckman Linear Gradient Former. The material (0.4 to 0.8 ml) was layered on the top of the gradient solution and the centrifugation performed. The specific conditions for each experiment are given in the Results, at the bottom of each table. After the centrifugation period, the gradient was fractionated using an Isco Density Gradient Fractionator, Model 183, at 0.6 ml per minute, monitored with a Model UA-2 Ultraviolet Analyzer (Isco, Lincoln, Nebraska, U.S.A.) at 280 nm. Fractions of 1 ml were collected using a LKB 7000 Ultro Rac Fraction Collector (U.S.A. LKB Instruments, Inc., Rockville, Md.). The linearity of the gradient was confirmed by reading an aliquot of each fraction, at 20°C, in an Abbe Refractometer, Model A (Carl Zeiss Canada Ltd.); the sucrose percentages were transformed from W/W to W/V, using a conversion graph supplied by the Beckman Co.

Cytochrome oxidase activity was determined in an aliquot of each fraction. Tubes containing peak cytochrome oxidase activity were pooled, diluted and centrifuged at 15,000 x G for 10 minutes. The pellet recovered was studied polarographically.

Another method was also used to recover the mitochondrial zone from the gradient. It was collected directly into a syringe through an L-shaped needle. The material thus obtained was diluted, centrifuged at 15,000 x G for 10 minutes using an SS-34 rotor in a Sorvall RC 2-B Refrigerated Centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.). The pellet was studied polarographically. The material collected from the density gradient was diluted in a ratio of 1 in 6. The dilution was done using one of two techniques (in Results, II): a fast dilution and a slow dilution (10). The fast dilution was done by mixing directly the material from the density gradient with Suspending Medium. The slow dilution (10), done by progressively increasing the rate of dilution, is illustrated in Figure 2. The material from the gradient was mixed using a Vortex mixer or a stirrer, during the process of slow dilution; the dilution medium was the Suspending Medium containing 0.1% bovine plasma albumin.

(ii) Sorvall HB-4 is a 4 x 50 ml swinging-bucket rotor which was used in a Sorvall RC 2-B Refrigerated Centrifuge. The advantage of this rotor is its greater capacity which allows more homogenate to be layered on the gradient.

A 4 ml Ficoll cushion, containing the same additions as the gradient, was first introduced in the tube. The gradient solution was prepared as described for the Beckman SW-41 swinging-bucket rotor. Then 3 ml homogenate was

Figure 2: Dilution volume vs dilution time for the slow dilution of the mitochondrial suspension recovered from hyperosmotic sucrose solutions. This type of slow dilution was done using the Beckman Density Gradient Former.

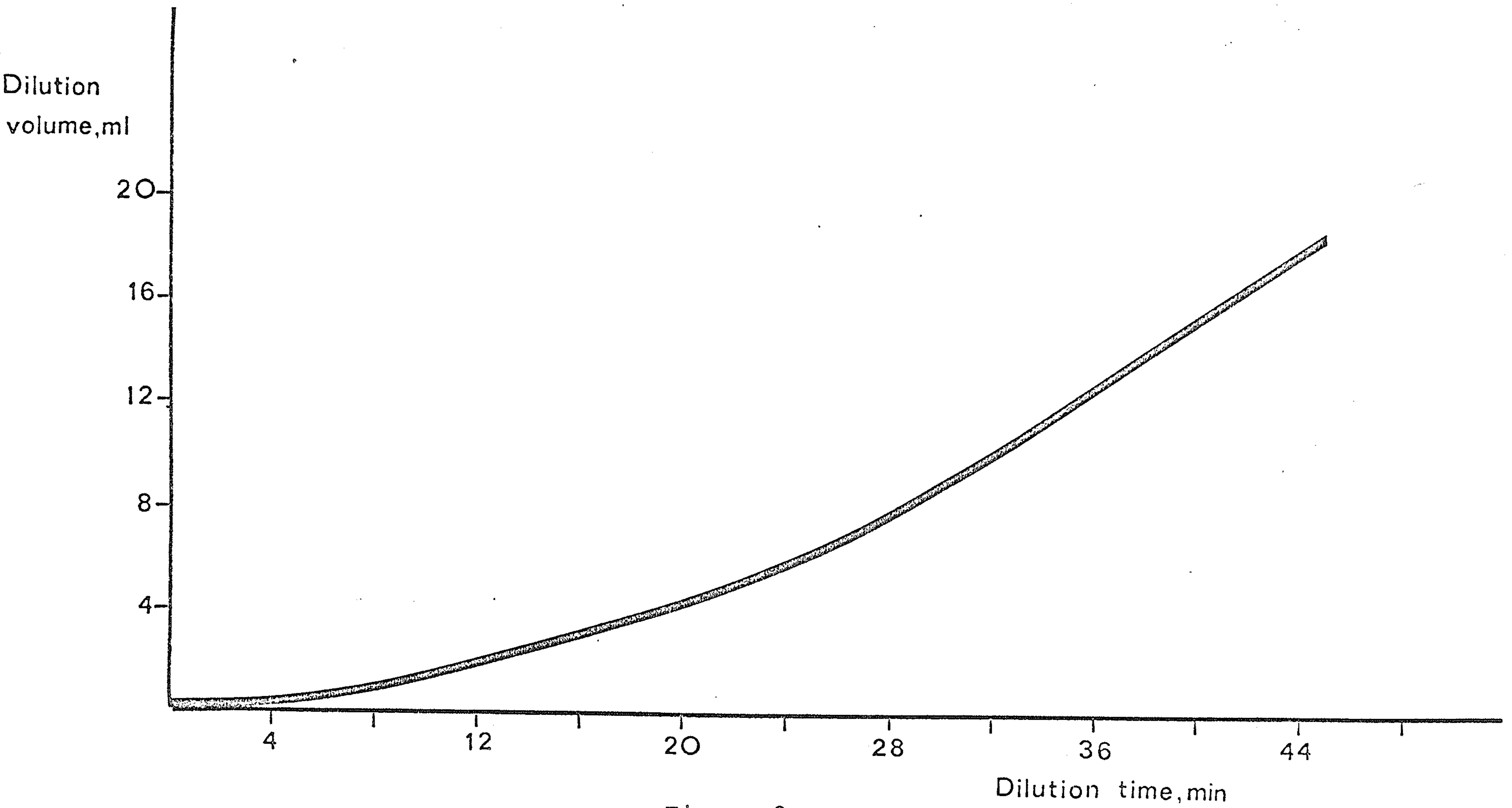


Figure 2

layered on the gradient and the centrifugation performed as described below in the Results of each experiment. Then the density gradient fractions were collected from the bottom of the tube through a needle fitted to the Sorvall Peristaltic Pump. Approximately 2 ml fractions were collected, dense end first. Refractometer readings, total protein readings at 280 nm and cytochrome oxidase activity were determined on each fraction. Again when oxidative phosphorylation was studied, the tubes containing the mitochondrial suspension were pooled, diluted and centrifuged at 15,000 x G for 10 minutes, and the pellet was studied polarographically. In some experiments the mitochondrial zone was collected with a L-shaped needle fitted to a syringe.

(b) Sorvall SZ-14 reorienting density gradient zonal rotor:

This rotor consists of a large cylindrical chamber, containing 1,400 ml, divided into 6 sector-shaped compartments by vertical septa radiating from the axial core to the rotor wall. In these experiments, the gradient and the sample were loaded dynamically, as follows.

The rotor was installed in a Sorvall RC 2-B Refrigerated Centrifuge and centrifuged at 2,500 r.p.m. A 50 ml portion of Ficoll solution (24%) and a 1,000 ml gradient solution in the range of 3.4% to 12% Ficoll containing throughout 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2 and 0.1% bovine plasma albumin were then introduced with a Sorvall

Peristaltic Pump, heavy solution first. The gradient was made using a modified density gradient Mixer, a mechanical stirrer assuring proper mixing of the 2 solutions (see Figure 3). Then 100 ml of pigeon heart homogenate was introduced with the peristaltic pump and the centrifugation was done for 10 minutes. The force developed on the sample ranged from 3,600 x G at the free fluid surface of the gradient to 6,100 x G at the interface of the gradient and cushion.

After an appropriate centrifugation time, the rotor was slowly decelerated with a rate controller to avoid gradient disruption during the gradient reorientation. At rest, the rotor was emptied from the bottom (i.e. the cushion and the dense end first) using a peristaltic pump, and 40 ml fractions were collected. An aliquot of each fraction was read on the refractometer to determine the Ficoll concentration (% W/V) of each fraction and the total protein determination done at 280 nm on each fraction also. The sedimentable proteins recovered, after dilution and centrifugation at 14,500 x G for 10 minutes, were determined by the Lowry method (182) on each fraction. Oxidative phosphorylation was measured on each pellet of sedimentable protein.

Figure 3: SZ-14 reorienting density gradient zonal rotor and accessories.

A: light solution

B: heavy solution

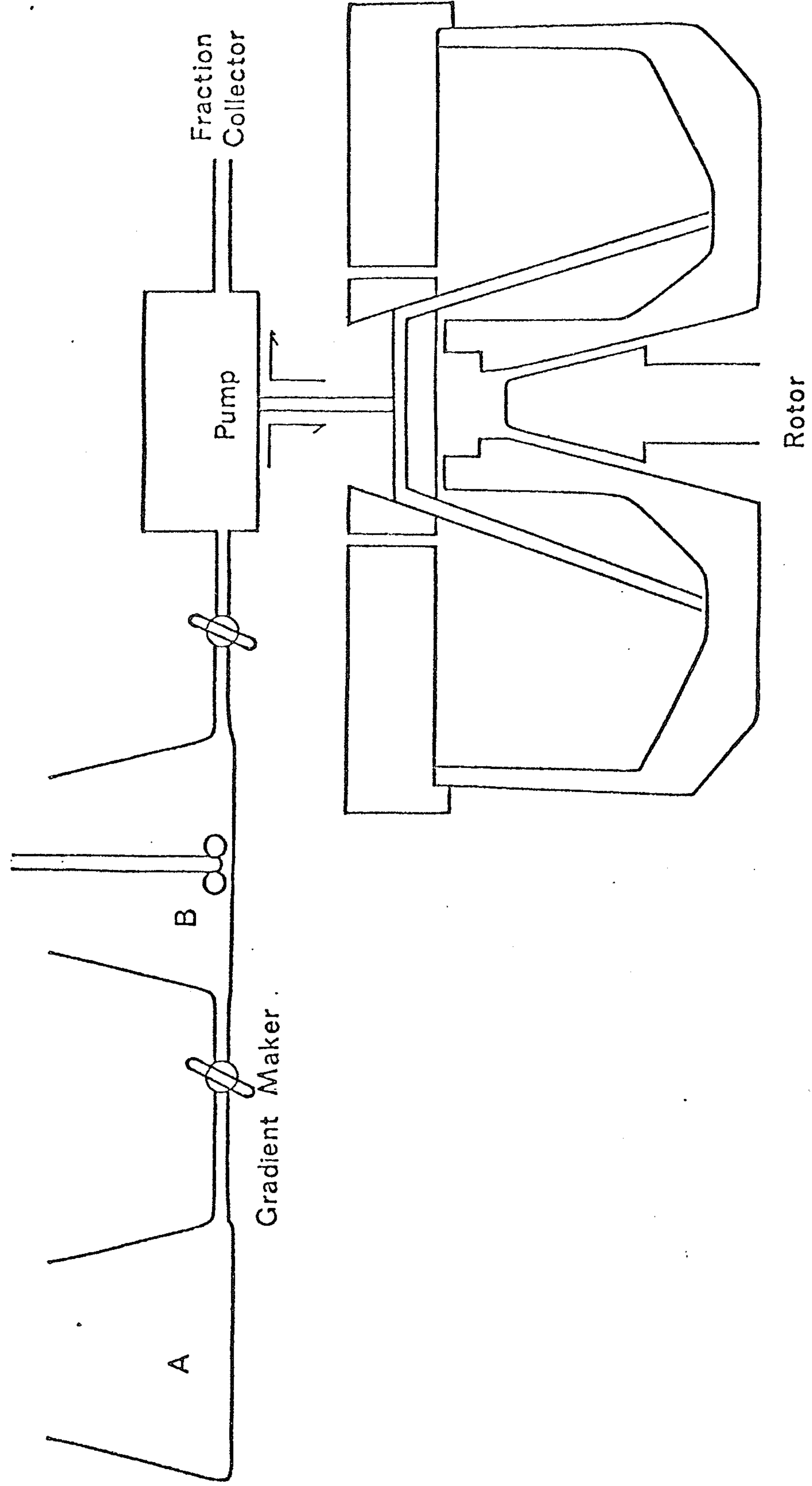


Figure 3

III. Polarographic measurements.

1. Instrument:

The polarographic determinations were done using a Gilson Oxygraph, Model KM (Gilson Medical Electronics, Middletown, Wisconsin, U.S.A.), fitted with an oxygen electrode (Clark-type electrode, Yellow Springs Instrument Co.) with a Teflon membrane (174, 175), connected to an amplifier and recording system. A polarizing voltage of -0.8 volts was applied. The volume of the reaction chamber was 1.5 - 1.6 ml. Figure 4 gives a schematical representation of the Oxygraph cuvette fitted with an oxygen electrode.

2. Reaction Medium:

The Reaction Medium had the following composition (176):

0.23 M mannitol

0.07 M sucrose

0.02 M Tris-Cl, pH 7.2

0.02 mM EDTA

5 mM Pi

The Reaction Medium was pre-equilibrated with the oxygen tension of air at 28°C at atmosphere pressure. The concentration of oxygen was calculated using the solubility of oxygen in Ringer's solution at 76 cm Hg (178), corrected for the barometric pressure at the time of the experiment and also corrected by a factor of 0.9214, obtained by

Figure 4: Schematical representation of the Oxygraph cuvette fitted with an oxygen electrode.

W: circulating water at 28°C

C: reaction chamber

S: magnetic stirrer

T: Teflon membrane of electrode

F: chimney

O: oxygen electrode

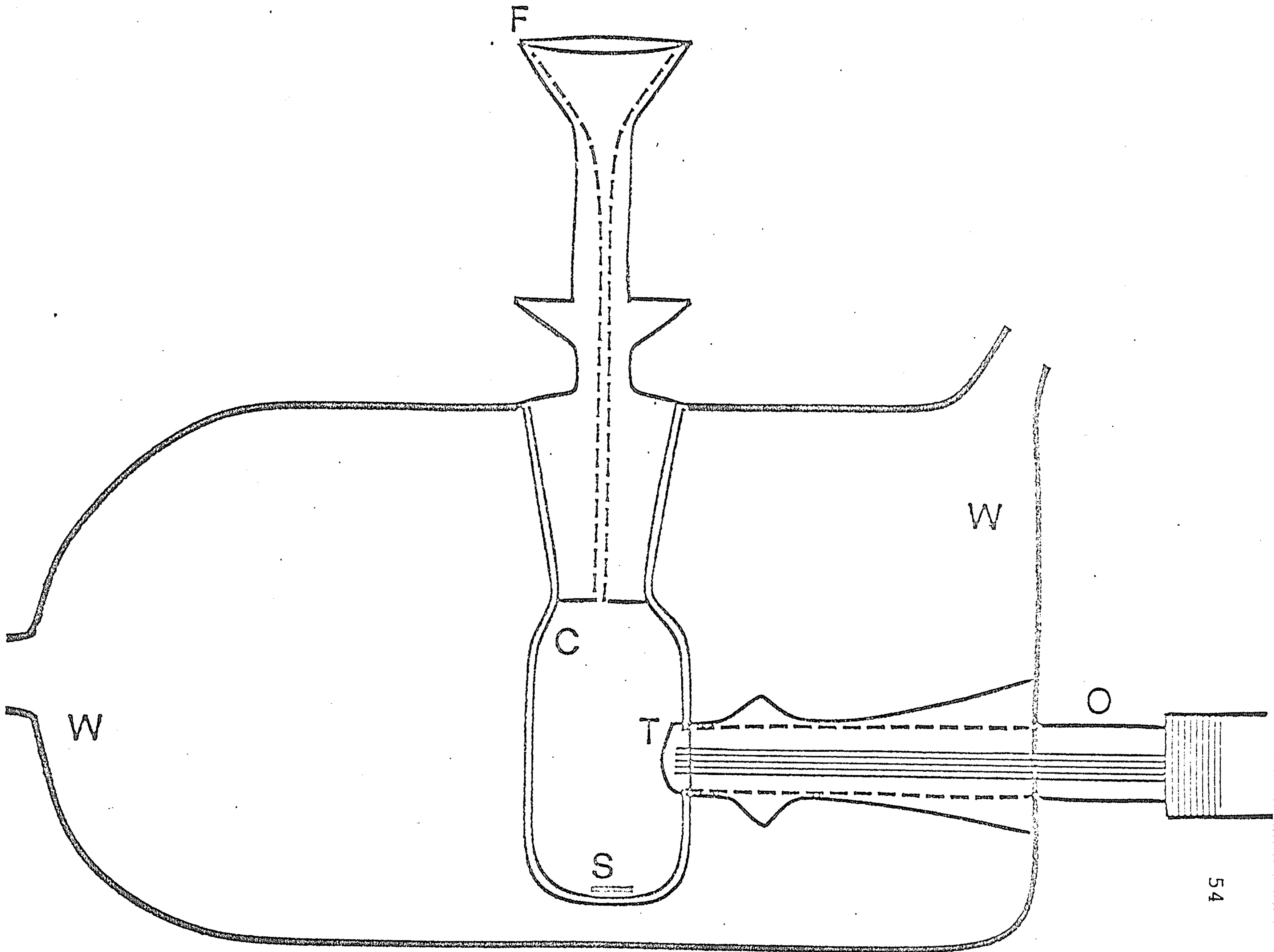


Figure 4

titrating the oxygen concentration of the medium with enzymatically determined NADH in the presence of sonicated mitochondria.

3. <u>Example of a typical polarographic test (see Figure 5):</u>

   1. addition of the Reaction Medium.
   2. addition of an aliquot of the mitochondrial suspension.
   3. addition of a known amount of ADP.

      A consumption of oxygen may occur if endogenous substrate(s) is (are) present in the mitochondria.
   4. addition of an excess substrate, e.g. pyruvate-malate. There is a fast utilization of oxygen, defined (177) as State 3. After depletion of the added ADP, there is a decrease in the rate of the oxygen consumption, defined as State 4, due to the absence of Pi acceptor.
   5. addition of ADP. The fast respiration rate (presence of Pi acceptor) and the slow respiration rate (absence of Pi acceptor) can be observed again.

After few similar cycles of State 3 and State 4, the oxygen in the Reaction Medium was exhausted. From such an experiment, the O_2 rates, the RCR's and the ADP/0 ratios can be calculated. Unless otherwise mentioned, the measurement

Figure 5: Example of a typical polarographic experiment.

1: addition of Reaction Medium

2: addition of mitochondria

3: addition of ADP (248 μM)

4: addition of substrate (5 mM pyruvate - 1 mM malate)

5: addition of ADP (248 μM)

O_2 rate is expressed in μmoles O_2 per minute per gram mitochondrial protein.

RCR = Respiratory control ratio

= State 3 respiration rate/State 4 respiration rate.

$$ADP/O = \frac{\text{Concentration of ADP } (\mu M)}{\mu MO_2 \text{ consumed} \times 2}$$

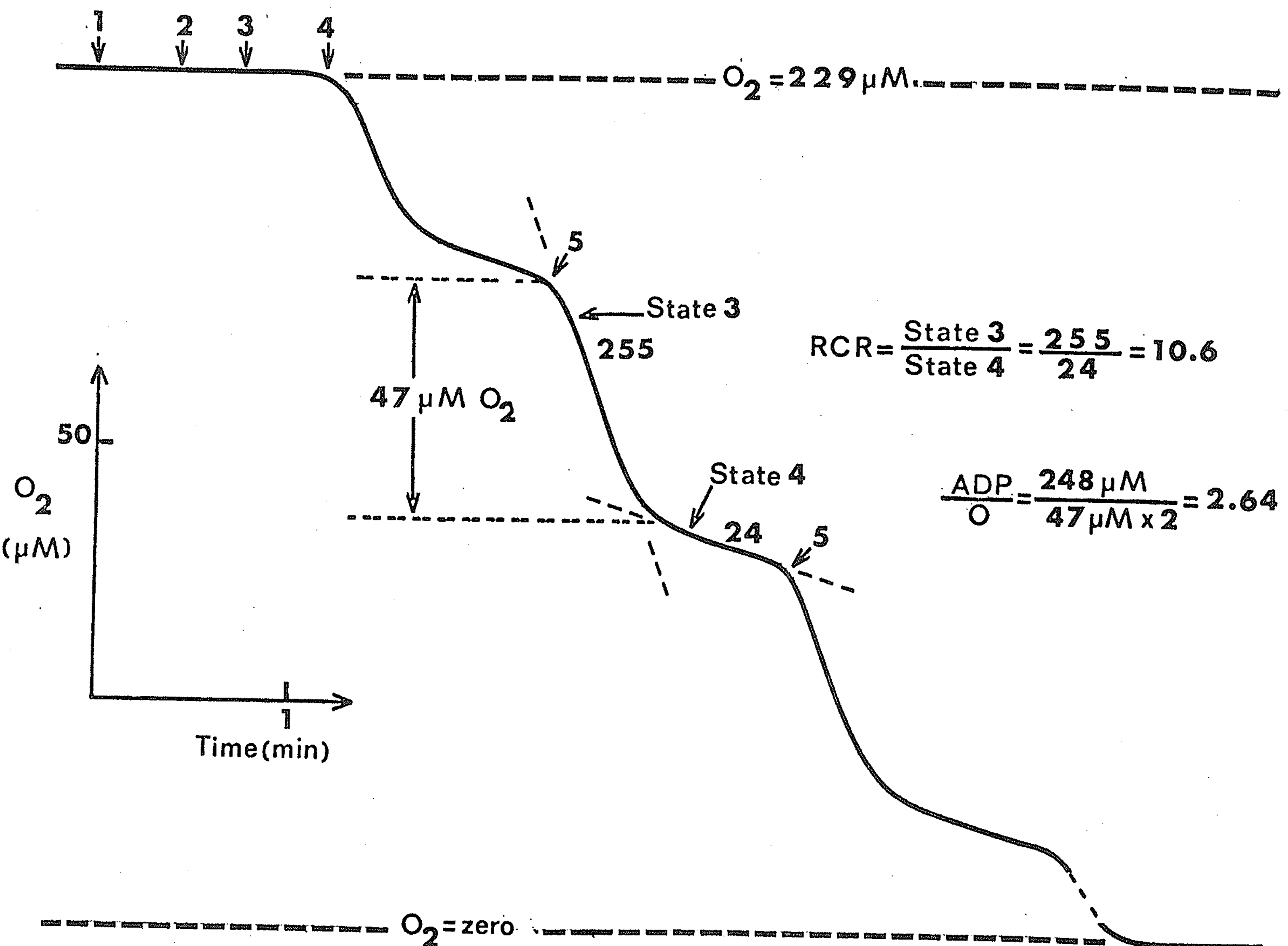


Figure 5

values presented in Results were taken from the second cycle of State 3 - State 4 respiration.

4. Calculations:

- Respiratory control ratio (177, 179)

$$RCR = \frac{\text{State 3 respiration rate}}{\text{State 4 respiration rate}}$$

This is a good indication of the intactness of the mitochondria (177, 180).

- O_2 rates

Oxygen consumption during State 3 or State 4 respiration and was expressed in µmoles O_2/min/g mitochondrial protein.

- ADP/0 ratio (177, 179)

This is the ratio of molecules of ADP esterified per atom of oxygen consumed.

$$\frac{ADP}{0} = \frac{\text{concentration of ADP } (\mu M)}{\mu M\ O_2 \text{ consumed} \times 2}$$

IV. Assays.

1. ADP assay:

It is important to know the exact concentration of ADP for the calculation of the ADP/O ratios.

The concentration of ADP was estimated by ultraviolet absorbance on a Beckman DU spectrophotometer at 259 nm. The stock ADP, approximately 50 µM, was diluted 1,250 times in 0.02 M phosphate buffer at pH 7.0. The zero was adjusted

with a buffer blank. At 259 nm and pH 7.0, the molar absorptivity of ADP is 15.4 x 10^3 mM (181).

2. Protein estimation:

(a) Direct measurement at 280 nm:

In some experiments, the total protein was determined at 280 nm on a Beckman DU spectrophotometer. However, the gradient solution interfered with this reading (see Figure 6), but by diluting the protein to bring the readings into an acceptable range (O.D. $<$ 1) the interference was minimized to a point where correction was unnecessary ($<$ 5%).

(b) Colorimetric method:

Mitochondrial protein was determined by the method of Lowry *et al* (182).

(i) Reagents:

1) 1N NaOH
2) 2% Na_2CO_3
3) 0.5% $CuSO_4 \cdot 5H_2O$ in 1% Na- or K-tartrate.
4) Alkaline copper solution, prepared by mixing 50 ml of 2) plus 1 ml of 3).
5) Dilute Folin Reagent, prepared by making a 1 in 3 dilution of the commercial reagent with distilled water.

Figure 6: Effects of different concentrations of Ficoll-sucrose gradient solutions on the protein determination at 280 nm and on the protein determination by the Lowry method.

Each concentration of Ficoll studied contained:

0.25 M sucrose
0.1 mM EDTA
10 mM Tris-Cl, pH 7.2
0.1% albumin

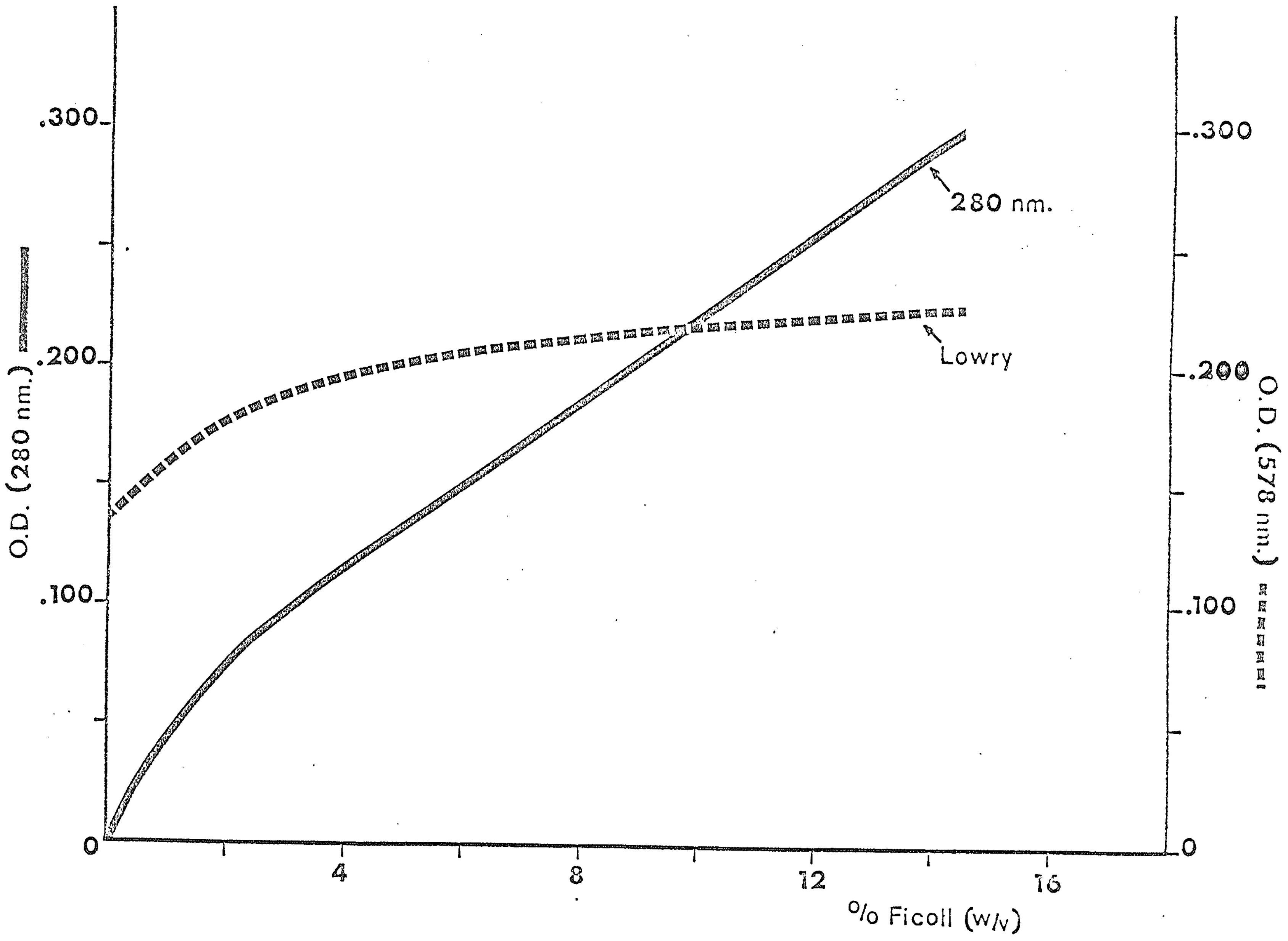


Figure 6

(ii) Procedure:

The mitochondrial suspension was diluted with 0.9% NaCl to obtain a suitable protein concentration range. The protein determination of each sample was done in triplicate. The diluted suspension, 10 µl, was digested in 0.1 ml NaOH for 0.5 hr. Then 1 ml of a freshly prepared alkaline copper solution was added and followed by a 10 minutes incubation. Finally, 0.1 ml of diluted Folin reagent was added. After 30 minutes of incubation, the optical density of the colour developed was read, against a blank, at 578 nm on an Eppendorf Photometer, model 1100 M. The protein standard curve was prepared as described above using 0-75 µg of crystallized bovine plasma albumin. The protein concentration of the unknown sample was determined on the standard curve from the optical density of the sample.

When the mitochondria were prepared in the presence of albumin, the aliquots for the protein determination were washed three times in 10 volumes of Suspending Medium in order to prevent the interference of albumin in the determination of mitochondrial protein. However, it is known (183) that around 8% of the albumin remains bound to the mitochondria even after extensive washing. The contribution of this albumin to the mitochondrial protein readings was not subtracted.

The effect of increasing concentrations of Ficoll gradient solutions on the protein determination was also checked by the Lowry method (see Figure 6). Because of the interference, the mitochondria recovered from the gradients were washed in Suspending Medium to prevent any interference of the gradient solutions.

3. Cytochrome oxidase activity:

Cytochrome oxidase activity was measured using the method of Schnaitman *et al* (184). The activity was assayed polarographically by measuring the oxygen consumption with a Clark electrode at 28°C. The results were corrected for the auto-oxidation of ascorbic acid.

(i) Reagents:

0.075 M Na-Phosphate buffer, pH 7.2

1 mM cytochrome C

5 mM 2,4-DNP

30 mM TMPD

0.375 M Na-ascorbate, pH 6.1-6.3.

(ii) Procedure:

The buffer was added first in the cuvette. Then the cytochrome C, the 2,4-DNP and the TMPD were added to their respective final concentrations: 33 µM, 50 µM and 0.30 mM. When a stable base line was obtained on the

Oxygraph chart, a final concentration of 3.75 mM ascorbate was added and the reaction was allowed to run for one minute. Then the mitochondria, either as isolated or after incubation with Triton X-100 or after successive freezing and thawing, were added and the oxygen consumption was allowed to proceed until a measurable rate was obtained. The cytochrome oxidase activity was expressed in μmoles O_2 per minute per gram mitochondrial protein.

4. Calcium determination:

Calcium was measured using a Perkin-Elmer Atomic Absorption Spectrophotometer, model 303, with the HGA 2000 graphite furnace attachment. Ten μl of each of 6 standard solutions (0.05 μg Ca^{++}/ml to 0.5 μg Ca^{++}/ml) were used to prepare a standard curve; 10 μl of each sample were also used for the Ca^{++} determination of the unknown. Keeping the volume constant reduced errors due to background readings from water and other constituents.

V. Electron micrographs.

Mitochondria were fixed in State 3 by adding 2% glutaraldehyde (final concentration) to the suspension of mitochondria in the Oxygraph cuvette (211). After one hour in 2% glutaraldehyde, the mitochondria were post-fixed

in 1% OsO_4 in acetate-Veronal buffer, pH 7.0 for an additional hour and then block-stained with 2% uranyl acetate in acetate-Veronal buffer, pH 7.0 for another hour. The blocks then were dehydrated through changes of alcohol/H_2O and embedded in Epon-Araldite resin. Sections were cut to a thickness of 80 nm using a Sorvall Porter-Blum Ultra-Microtome. These sections were poststained with Pb_2NO_3 (Reynold's stain). Electron microscopy was performed using a Zeiss 9-S instrument.

VI. Statistical treatment.

The t-test for paired and unpaired observations, the analysis of variance and the correlation value were calculated according to Dixon and Massey (212).

IV. RESULTS

I. Mitochondria isolated from pigeon heart homogenate in a single step density gradient centrifugation.

1. Introduction:

The first part of this project was to develop a method of isolating mitochondria with a higher yield than the conventional method which involved three successive differential centrifugation steps (136, 137). Only 18% to 25% of all the mitochondria present in the skeletal muscle and 50% to 70% of all the mitochondria presented in the heart are isolated by the conventional Nagarse procedure (137). The remaining organelles might either be broken during the homogenization procedure, trapped in the cell debris and sedimented during the first centrifugation or sedimented in the first centrifugation due to a higher density. By layering the whole homogenate on the gradient, a higher yield of mitochondria was expected due to the elimination of the differential centrifugation steps of the conventional procedure, as well as a shorter preparation time. Pigeon heart was used in these experiments.

Separation of mitochondria from a homogenate layered on a density gradient have been reported by several workers. Day *et al* (94) reported that it was possible to stabilize the

zonal centrifuge absorbancy profiles of adult rat brain homogenate by substituting an iso-osmotic Ficoll-sucrose density gradient for a hyperosmotic sucrose density gradient. Blokhuis et al (58) have demonstrated, after sucrose density gradient centrifugation, the heterogeneity of rat brain mitochondria by showing that the peaks of different mitochondrial enzyme activities were not superimposable.

Rat liver has generally been used to demonstrate the separation of mitochondria directly from a homogenate after centrifugation on Ficoll-sucrose (29) or sucrose density gradients (21, 35, 57, 59, 90, 155, 156). In these studies, the mitochondrial zone was determined, either by measuring the cytochrome oxidase activity (21, 29, 35, 57) or the succinate dehydrogenase activity (156), or by identifying the mitochondrial band by microscopy (59, 156).

However, the functional intactness of the mitochondria isolated in a density gradient centrifugation was never studied. In the following sections is described a method developed to isolate functionally intact pigeon heart mitochondria by a single step density gradient centrifugation in a Ficoll density gradient.

2. Comparison of the effectiveness of sucrose Stock Medium and sucrose-mannitol Stock Medium used for the conventional Nagarse procedure of isolating mitochondria.

The first study compared oxidative phosphorylation of mitochondria isolated in a sucrose Stock Medium with those isolated in a sucrose-mannitol Stock Medium. It was important to know which Stock Medium yielded the best quality of mitochondria before proceeding to further studies involving density gradient centrifugation.

The composition of the sucrose Stock Medium was as follows: 0.25 M sucrose, 0.1 mM EDTA for an osmolality of 250 mosmoles. The sucrose-mannitol Stock Medium had the composition given in Methods (I, 1) for an osmolality of 280 mosmoles. The mitochondria were isolated using the conventional procedure of differential centrifugation given in Methods (I, 5), the final suspension being made in their respective Suspending Mediums.

Table 1 shows the respiration and oxidative phosphorylation calculations for both preparations. The results show that both media give a similar quality of mitochondria, with high RCR's, ADP/O ratios, and similar respiration rates. Therefore, it was concluded that either Stock Medium could be used to isolate similar quality of mitochondria.

TABLE 1

Comparison of oxidative phosphorylation measurements by pigeon heart mitochondria isolated in sucrose Stock Medium and in sucrose - mannitol Stock Medium.

Medium used	RCR	0_2 rate	ADP/O
Sucrose	7.7	166	2.70
Sucrose-mannitol	7.1	171	2.73

N = 1

Substrate: 5 mM pyruvate - 1mM malate. Results taken from the second cycle of State 3 - State 4 respiration, with 217 μMADP.

O_2 rate is expressed in μmoles O_2 per minute per g mitochondrial protein.

Yield of mitochondria:

Sucrose medium : 7.9 mg mitochondrial protein/g heart.

Sucrose-mannitol medium: 6.2 mg mitochondrial protein/g heart.

One pigeon heart was used; half of the heart was used for the mitochondrial isolation in sucrose Stock Medium and the other half was used for the mitochondrial isolation in sucrose-mannitol Stock Medium.

3. Sedimentation pattern of previously isolated mitochondria subjected to rate zonal centrifugation.

In the previous section, it was shown that sucrose Stock Medium and sucrose-mannitol Stock Medium yielded qualitatively similar mitochondria. In the following section, Ficoll-sucrose density gradient and Ficoll-sucrose-mannitol density gradient will be compared to determine which of these gradient solutions yielded mitochondria with greater metabolic integrity.

The experiment consisted of preparing pigeon heart mitochondria, again using both Stock Media, and layering an aliquot of each mitochondrial suspension on density gradients to determine the sedimentation pattern of isolated mitochondria. Two different density gradients were used, for each mitochondrial suspension, both of them in the range of 15-30%(W/V) Ficoll (0.375-0.75 mM). The Ficoll-sucrose density gradient contained 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2 and 0.5% bovine plasma albumin throughout, for a total osmolality of 270 mosmoles. The Ficoll-sucrose-mannitol density gradient contained 0.21 M mannitol, 0.07 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2, and 0.5% bovine plasma albumin throughout, for a total osmolality of 300 mosmoles. The contribution of Ficoll to the total osmolality of the gradient

solution was less than 0.3%. An aliquot of the mitochondria isolated in the sucrose Stock Medium was layered on a Ficoll-sucrose density gradient and on a Ficoll-sucrose-mannitol density gradient. An aliquot of the mitochondria isolated in the sucrose-mannitol Stock Medium was layered on a Ficoll-sucrose density gradient and on a Ficoll-sucrose-mannitol density gradient. Rate zonal centrifugation was performed using a SW-41 swinging-bucket rotor. The gradients were fractionated as described in Methods (II, 3a), while monitoring the absorbancy profiles at 280 nm. Figure 7 shows a typical sedimentation pattern, with the mitochondria suspended in the 17%-24% Ficoll zone. The pattern of all four experiments were identical. In each case, the fractions corresponding to the absorbancy peak were pooled, diluted with Suspending Medium and recovered by centrifugation at 17,300 x G for 10 minutes. In each case, the mitochondria were resuspended in Suspending Medium of the same composition as the density gradient, e.g. mitochondria isolated in sucrose Stock Medium, and layered on a Ficoll-sucrose-mannitol density gradient were resuspended, after the rate zonal centrifugation, in sucrose-mannitol Suspending Medium. The four mitochondrial suspensions were assayed polarographically. Table 2 shows the results obtained.

Again, mitochondria isolated in sucrose Stock Medium and in sucrose-mannitol Stock Medium proved to be of

Figure 7: Sedimentation pattern of mitochondria isolated in sucrose-mannitol Stock Medium and subjected to rate zonal centrifugation in a Ficoll-sucrose-mannitol density gradient.

The O.D. base line was adjusted with the Suspending Medium containing 0.5% bovine plasma albumin. Centrifugation was performed at 18,400 x G for 20 minutes. A Ficoll solution (46%) was used as "cushion" at the bottom of the tube.

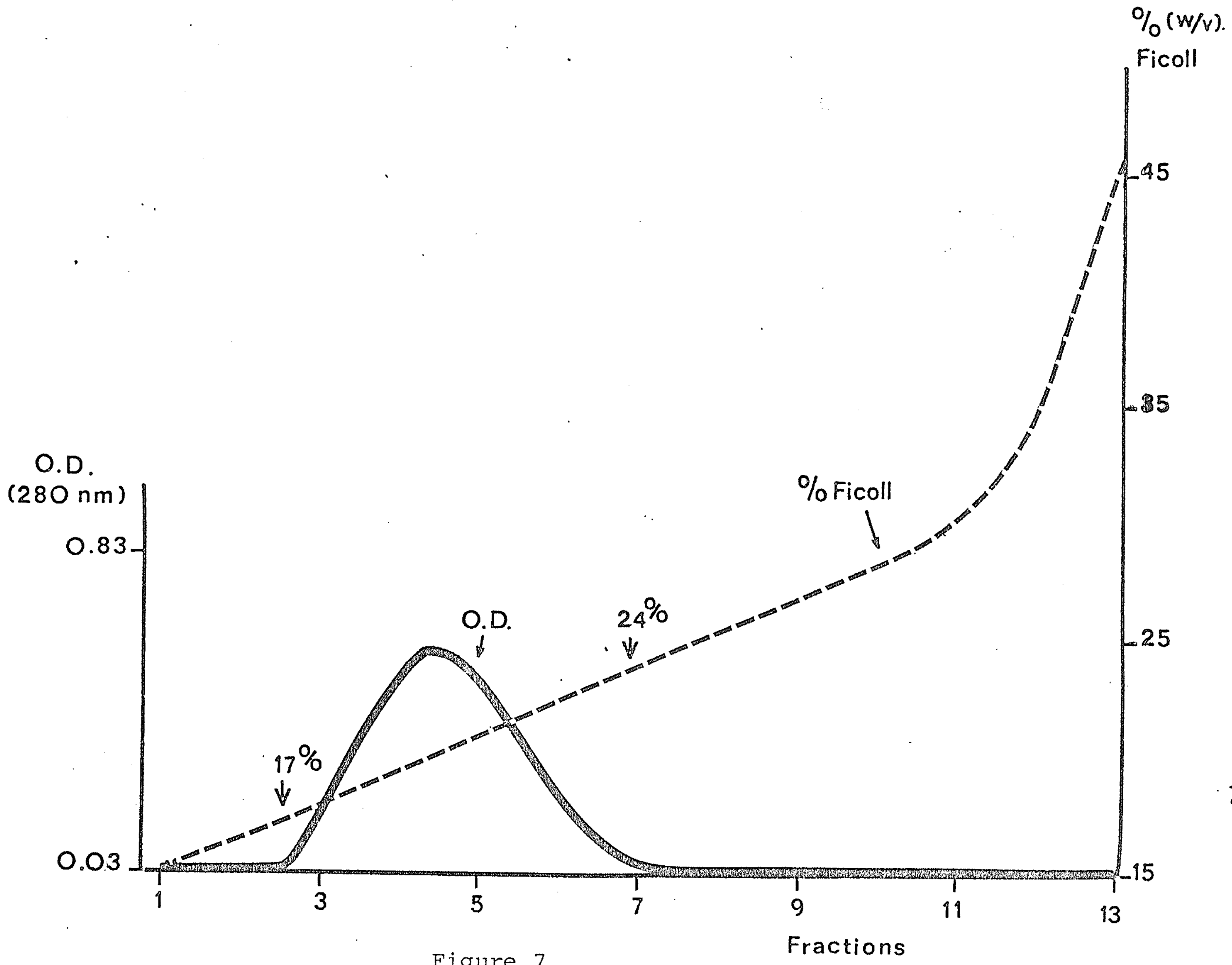


Figure 7

TABLE 2

Comparison of oxidative phosphorylation by mitochondria isolated in sucrose Stock Medium and in sucrose-mannitol Stock Medium and subjected to rate zonal centrifugation in different density gradient solutions.

	RCR		O_2 rate		ADP/O	
	S	S-M	S	S-M	S	S-M
Before gradient	9.3	9.1	161	162	2.60	2.69
After gradient: Ficoll-sucrose	5.0	4.3	89	86	2.40	2.45
Ficoll-sucrose-mannitol	4.9	5.4	98	114	2.71	2.38

S: Sucrose Stock Medium S-M: Sucrose-mannitol Stock Medium.

N = 1

Substrate: 5mM pyruvate-1mM malate. Results taken from second cycle of State3-State 4 respiration, with 216 µmADP.

O_2rate is expressed in µmoles O_2per min. per g mitochondrial protein.

Yield of mitochondria:
Sucrose medium: 7.8 mg mitochondrial protein/g heart.

Sucrose-mannitol medium: 9.2 mg mitochondrial protein/g heart.

Rate zonal centrifugation 18,400 x G for 20 minutes.

Suspending Medium contained 0.5% bovine plasma albumin.

The mitochondrial recovery after the gradient varied from 54% to 68%.

similar quality (referred as "Before gradient" in the Table). Ficoll-sucrose and Ficoll-sucrose-mannitol density gradients yielded mitochondria of a similar quality, the RCR's and ADP/O ratios being comparable; however, the O_2 rates in all cases were low, probably due to the ageing of the mitochondria, the fractionation procedure being lengthy. From this experiment, it was concluded that either gradient solution could be used for purification of mitochondria. In subsequent experiments sucrose-mannitol Stock Medium was chosen for the isolation of mitochondria, since it was the solution used previously in this laboratory. However, iso-osmotic Ficoll-sucrose density gradients were used for the experiments with isolated mitochondria subjected to rate zonal centrifugation.

The above experiment incidentally showed that it is possible to recover coupled heart mitochondria after density gradient centrifugation. This is the first time that heart mitochondria have been found to be coupled after centrifugation in a Ficoll density gradient.

4. Mitochondria obtained directly from a homogenate layered on a density gradient and subjected to rate zonal centrifugation.

Once it was determined that previously isolated mitochondria could be recovered functionally intact from a density gradient, work was begun on the experimental conditions

required to isolate mitochondria directly from a whole homogenate layered on a density gradient. By layering the homogenate on the density gradient, a higher yield of mitochondria was expected than that obtained by the conventional procedure; all the mitochondria present in the homogenate should be recovered in the density gradient. Pigeon heart was used. The procedure involved rate zonal centrifugation in order to shorten the total time for isolation of the organelles, thus minimizing the detrimental effects of ageing of the mitochondria.

This study was divided into three parts:

(a) to determine the best gradient solutions.

(b) to locate the mitochondria in the gradient using cytochrome oxidase as a marker enzyme (157, 159). In this case, it was necessary to determine if the enzyme activity was affected by different concentrations of Ficoll. It was also necessary to devise a preliminary treatment of the organelles to maximize the cytochrome oxidase activity, since the outer mitochondrial membrane when intact is poorly permeable to cytochrome c (160).

(c) to determine the optimal conditions with respect to speed of centrifugation, at a fixed centrifugation time.

(a) Density gradient solutions.

The composition of the gradient solutions used to separate the mitochondria from the whole homogenate was varied in order to identify the solution which would prevent the aggregation of myofibrils and of cell membranes that was observed in preliminary experiments. Such a gradient solution should also give a mitochondrial preparation of good quality.

Pigeon heart homogenate was prepared as previously described in Methods (I, 6). Rate zonal centrifugation was performed at 18,400 x G for 20 minutes using an SW-41 swinging-bucket rotor. A 50% Ficoll solution was used as "cushion", this dense solution should prevent the sedimentation of any material to the bottom of the tube. Six media were tested. In each the gradient density was obtained by increasing linearly the Ficoll concentration from 15% to 30% (W/V). The remaining constituents of the six media were constant throughout the gradient:

1) 0.21 M mannitol, 0.07 M sucrose, 10 mM Tris-Cl, pH 7.2, 0.1 mM EDTA, 0.5% albumin.
2) 0.25 M sucrose, 10 mM Tris-Cl, pH 7.2, 0.1 mM EDTA, 0.5% albumin, 0.5% heparin.
3) 0.25 M sucrose, 10 mM Tris-Cl, pH 7.2, 1.0 mM EDTA, 0.5% albumin.
4) 0.25 M sucrose, 10 mM Tris-Cl, pH 7.2, 1.0 mM EDTA, 0.5% albumin, 0.5% heparin.

5) 0.18 M KCl, 1 mM $MgSO_4$, 10 mM Tris-Cl, pH 7.2, 1.0 mM EDTA, 0.5% albumin.

6) 0.18 M KCl, 1 mM $MgSO_4$, 10 mM Tris-Cl, pH 7.2, 0.1 mM EDTA, 0.5% albumin.

Assuming a similar mitochondrial sedimentation pattern obtained after centrifugation on a density gradient of previously isolated mitochondria (shown in Figure 7), the first milliliter was discarded and 4.5 - 5.0 ml of the upper (less dense) portion of the gradient were collected with a syringe after the centrifugation. The Ficoll-sucrose-mannitol density gradient (i.e. no. 1) was discarded due to the large amount of aggregates; the Ficoll-KCl density gradients (i.e. no. 5 and 6) were also discarded because all the material was packed on the top of the cushion. (This problem will be discussed later). The Ficoll-sucrose density gradients, numbers 2,3 and 4, had some aggregates but the presence of EDTA and/or heparin gave an improvement. These three mitochondrial fractions, from gradients numbers 2,3 and 4, were diluted, centrifuged and suspended for polarographic studies, see Table 3.

The three gradient solutions gave a very good quality of mitochondria. The RCR's were very high, the ADP/O ratios were near 3 and the O_2 rates were only slightly lower than those obtained with the conventional procedure. The latter could mean that the preparations were contaminated by non-mitochondrial material. Also, the aggregation was still a problem.

TABLE 3

Oxidative phosphorylation by mitochondria recovered, from heart homogenate subjected to rate zonal centrifugation, in different density gradient solutions.

Solutions	RCR	O_2 rate	ADP/O
2	14.6	192	2.82
3	9.2	169	2.65
4	14.8	171	2.99

N = 1

Substrate: 5mM pyruvate-1mM malate. Results taken from second cycle of State3-State 4 respiration, with 214 μMADP.

O_2 rate is expressed in μmoles O_2/min/g protein.

Rate zonal centrifugation: 18,400 x G for 20 minutes.

Suspending Medium contained 0.5% albumin.

Yield in mg protein per g heart: solution no.2:7.27; solution no.3:6.71; solution no.4:7.49.

Composition of the density gradients:

2-0.25M sucrose, 10mM Tris-Cl pH7.2, 0.1mMEDTA,0.5% albumin, 0.5% heparin.

3-0.25M sucrose, 10mM Tris-Cl pH7.2, 1.0mMEDTA,0.5% albumin.

4-0.25M sucrose, 10mM Tris-Cl pH7.2, 1.0mMEDTA,0.5% albumin, 0.5% heparin.

As reported below, all the material was sedimented at the bottom of the Ficoll-KCl density gradients. The solutions numbers 5 and 6, were approximately 380 mosmolar each whereas all the other solutions (numbers 1 to 4) were between 270 and 300 mosmolar. The higher osmolality of the Ficoll-KCl gradient might be responsible for the different pattern of sedimentation. A 270 mosmolar solution containing 61.5 mM KCl, 125 mM sucrose, 1 mM $MgSO_4$, 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2 and 0.5% albumin was therefore tried. The gradient range again was 15% to 30% Ficoll (W/V). Some preliminary experiments showed that a shorter time and a slower speed of centrifugation was desirable (4,340 x G for 8 minutes) because KCl, in contrast to sucrose, permeates the inner mitochondrial membrane. Therefore, in hypotonic sucrose medium, mitochondria probably in a swollen configuration and, due to their larger size sediment at a faster rate. In the following experiment, on one gradient were layered previously isolated pigeon heart mitochondria; on the second gradient was layered a pigeon heart homogenate. After the centrifugation, the isolated mitochondria suspended in the 23% - 30% Ficoll zone were collected with a syringe; the same Ficoll zone was collected from the tube in which the homogenate had been layered. The latter gradient contained some aggregates. Both fractions were diluted, centrifuged and suspended for polarographic studies (Table 4).

The previously isolated mitochondria (referred as "Before gradient") recovered after rate zonal centrifu-

TABLE 4

Oxidative phosphorylation by previously isolated pigeon heart mitochondria and by mitochondria isolated from pigeon heart homogenate on a Ficoll-sucrose-KCl density gradient.

	RCR	O_2 rate	ADP/O
Before gradient	9.1	164	2.67
After gradient:			
mitochondria	4.9	145	2.50
homogenate	7.1	157	2.74

N = 1

Substrate: 5 mM pyruvate-1mM malate. Results taken from second cycle of State 3 - State 4 respiration (219 μM ADP).

O_2 rate is expressed in μmoles O_2/min/g protein.

4,340 x G for 8 minutes

Suspending Medium contained 0.5% albumin.

Yield of isolated mitochondria: 9.67 mg mitochondrial protein/g heart.

Mitochondrial recovery after the gradient: 29%.

Yield of protein from the homogenate: 10.4 mg protein/g heart.

gation on a Ficoll-sucrose-KCl density gradient were not as active as the mitochondria isolated directly from the homogenate on the density gradient; but the aggregation problem still was not resolved.

All the data presented so far for the homogenate layered on density gradients and subjected to rate zonal centrifugation indicated satisfactory oxidative phosphorylation. The yield of mitochondria isolated directly from the density gradient was similar to that obtained by the conventional procedure for mitochondrial isolation. Therefore, it was concluded that the density gradient technique did not improve the mitochondrial yield. It appears that the homogenization procedure, including the incubation with Nagarse, may be the factor responsible for a yield which is considerably below the tissue mitochondrial content.

Because high concentrations of EDTA and/or heparin seem to decrease the amount of aggregates, the Ficoll solution was passed through an ion exchange resin, Chelex-100, 100-200 mesh, to give a Ficoll solution essentially free of calcium (2.8 ppm). This Ca^{++}-free Ficoll was used to make a gradient (14-28% W/V) containing 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl pH 7.2 and 0.5% bovine plasma albumin. A pigeon heart homogenate was layered on the gradient and rate zonal centrifugation was carried out at

18,400 x G for 15 minutes. In this case, very few small aggregates were observed in the gradient. Thus, this proved to be the method of choice as far as the aggregation was concerned. The gradient was fractionated as follows, The upper fraction (22% to 25% Ficoll zone) and the lower fraction (25% to 28% Ficoll zone) were collected, diluted, centrifuged at 12,000 x G for 10 minutes and suspended in Suspending Medium containing 0.5% albumin for polarographic studies (Table 5).

The mitochondria recovered appeared functionally intact (177, 180), having high RCR's and ADP/O ratios. However, the O_2 rates were low when compared with those of conventionally isolated heart mitochondria. Since the O_2 rates can be regarded as specific activity of the mitochondria, the lower value may reflect some contamination by non-mitochondrial material, especially in the lower fraction . Therefore, the mitochondria pellet should be carefully washed before the final suspension. The amount of aggregation could not be decreased by using 4 mM EGTA instead of 0.1 mM EDTA in the gradient solutions and there was no improvement in the quality of the mitochondria by EGTA (data not shown).

From the foregoing preliminary results studying the isolation of mitochondria directly from a homogenate by rate zonal centrifugation, it was decided to use Ca^{++}-

TABLE 5

Oxidative phosphorylation by mitochondria isolated from heart homogenate by rate zonal centrifugation in a Ca^{++}-free Ficoll-sucrose density gradient.

Fractions	RCR	O_2 rate	ADP/O
Upper	14	160	2.73
Lower	16	120	2.55

N = 1

Substrate: 5mM pyruvate-1mM malate. Results taken from second cycle of State 3-State 4 respiration with 208 μMADP.

O_2 rate is expressed in μmoles O_2/min/g protein

18,400 x G for 15 minutes. SW-41 swinging-bucket rotor.

Yield of mitochondria : 4.37 mg protein/g heart.

Upper fraction : 22%-25% Ficoll. Lower fraction: 25%-28% Ficoll.

free Ficoll and to have in the gradient solutions: 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2, and 0.1% or 0.5% bovine plasma albumin. This gradient solution gave a good quality of mitochondria with a minimum amount of aggregation. Table 6 shows that this method gave reproducible results.

Up to this time, those fractions of the gradient which contained the mitochondria isolated from the homogenate were identified by comparison with the sedimentation pattern of previously isolated mitochondria subjected to the same rate zonal centrifugation.

For a more reliable method, cytochrome oxidase, an enzyme bound to the inner mitochondrial membrane (157, 159) was used to locate the distribution of the mitochondrial population throughout the gradient in all further studies.

(b) Cytochrome oxidase activity studies.

It was necessary to determine if cytochrome oxidase was affected by different concentrations of Ficoll. The experiments were done with previously isolated pigeon heart mitochondria (Methods I, 5); the mitochondrial protein (2.22 mg) was incubated, at $0^{\circ}C$, in one ml of Ficoll solution having different concentration: 0%, 15%, 20%, 25% and 30%, each of them having the same additions: 0.25 M sucrose, 10 mM Tris-Cl, pH 7.2, 0.1 mM EDTA and 0.5% bovine

TABLE 6

Oxidative phosphorylation by mitochondria isolated from pigeon heart homogenate in a single step density gradient centrifugation.

Fractions	RCR	O_2 rate	ADP/O
Upper	9.4 ± 1.5	231 ± 75	2.36 ± 0.11
Lower	9.0 ± 1.9	207 ± 52	2.41 ± 0.04

Mean ± S.E. ; N = 5

Substrate: 5 mM pyruvate-1mM malate. Results taken from second cycle of State 3-State 4 respiration.

O_2 rate is expressed in μmoles O_2/min/g protein

18,400 xG for 20 minutes. SW-41 swinging-bucket rotor.

Gradient range: 14% - 28% Ficoll (W/V).

plasma albumin, to simulate the gradient conditions. Because the outer mitochondrial membrane is poorly permeable to cytochrome c (160), an aliquot of the suspension was subjected to different treatments to obtain the maximum activity of the cytochrome oxidase:

- no treatment
- sonication (4 x 15 seconds) in 0.075 M Na-PO_4 buffer pH 7.2
- incubation at 0^oC, for 30 minutes, in 0.8% Triton X-100, a non-ionic surfactant, in 10 mM K-PO_4 buffer pH 7.2.

The cytochrome oxidase activity was measured polarographically using the technique explained in Methods (IV, 3). In each of the tests, 54 to 65 µg of mitochondrial protein were used. The Table 7 gives the enzyme activity, expressed in µmoles O_2 per minute per g mitochondrial protein for each concentration of Ficoll, with the three treatments for each concentration.

Table 7 shows that there was no inhibition of cytochrome oxidase at any concentration of Ficoll with non treated mitochondria. With sonicated mitochondria, the presence of a high concentration of Ficoll decreased by 40% the efficiency of the ultra-sound disruption of the organelles, probably due to the viscosity of the solutions. However, in all cases, the activity was higher than with untreated mitochondria. With Triton X-100, the activity was

TABLE 7

Cytochrome oxidase activity measured in the presence of different concentrations of Ficoll.

% Ficoll solutions	Cytochrome oxidase activity after treatment		
	None	Sonication	Triton X-100
0	662	1339 (0)	318 (0)
15	662	982 (5)	280 (3)
20	612	879 (7)	259 (4)
25	574	817 (8)	233 (5)
30	656	827 (10)	222 (6)

Cytochrome oxidase activity is expressed in µmoles O_2/min/g mitochondrial protein.

Sonication: 200 µl of mitochondria (434 µg) incubated in Ficoll solutions added to 400 µl 0.075M Na-PO_4 buffer pH7.2. Sonicated for 4 x 15 seconds.
Numbers in ( ); concentration of Ficoll during the sonication.

Triton X-100: 200 µl of mitochondria (434 µg) incubated in Ficoll solutions added to 800 µl of 1% Triton X-100 in 10mM K-PO_4 buffer pH7.2.
Incubation at 0°C for 30 minutes.
Numbers in ( ): concentration of Ficoll during the Triton X-100 treatment.

much lower than with untreated mitochondria, irrespective of the Ficoll concentration. This implied that the inhibition was due to the Triton X-100 itself. It was therefore decided to make a study to determine the concentration of Triton X-100 which would give optimal enzymatic activity.

The above procedure was modified to allow a better temperature control and a shorter time of incubation. To determine the optimal concentration of Triton X-100 for full enzyme activity, the following experimental conditions were used: an incubation time of 15 minutes, at 25°C, in an Eppendorf Heater (thermostat) Block 3401 (Brinkmann Instruments Canada Ltd., Toronto). This experiment was done again with previously isolated pigeon heart mitochondria incubated in 0% and 20% Ficoll solutions containing 0.25 M sucrose, 10 mM Tris-Cl, pH 7.2, 0.1 mM EDTA and 0.5% bovine plasma albumin. The mitochondrial protein (2.18 mg) were incubated at 0°C in one ml of each of these solutions. Again the mitochondrial suspensions were subjected to three treatments: none, sonication and Triton X-100. Table 8 gives the cytochrome oxidase activity for the two different concentrations of Ficoll. Fifty four to 65 µg of mitochondrial protein were used per assay.

For the conditions selected the optimal activity of the cytochrome oxidase of pigeon heart mitochondria, in the presence of 0% and 4% Ficoll, was obtained with a final

TABLE 8

Cytochrome oxidase activity of pigeon heart mitochondria measured in the presence of two different concentrations of Ficoll and varying concentrations of Triton X-100.

Treatment		μmoles O_2/min/g mitochondrial protein	
		0% Ficoll	20% Ficoll
No		680	643
Sonication		1407	824
% Triton X-100, final			
concentration:	0.04	2999	2614
	0.05	3589	2915
	0.06	3235	2548
	0.07	3337	2630
	0.10	2611	1647

Sonication: 437 μg mitochondrial protein in 0% or 20% Ficoll added to 400 μl of 0.075M Na-PO_4 buffer PH7.2. Sonicated 4 x 15 seconds in a final concentration of 0% and 6% Ficoll, respectively.

Triton X-100: 218 μg mitochondrial protein in 0% or 20% Ficoll incubated in 400 μl of Triton X-100, in 10 mM K-PO_4 buffer PH7.2, for 15 minutes at 25°C. Final concentration of Ficoll during the incubation with Triton X-100: 0% and 4% respectively.

concentration of 0.05% Triton X-100. As the concentration of Triton X-100 increased, there was a decrease in the enzyme activity, which would explained the low activity observed in the presence of 0.8% Triton X-100 in the previous table.

A similar study was done with hamster heart mitochondria and hamster skeletal muscle mitochondria. The experiments were done exactly as the one described for pigeon heart mitochondria. The results are shown in Tables 9 and 10.

For the conditions selected, a concentration of 0.04% Triton X-100 was sufficient to obtain maximal activation of the cytochrome oxidase of hamster heart mitochondria whereas 0.05% Triton X-100 was necessary to obtain the optimal activity of cytochrome oxidase from hamster skeletal muscle mitochondria. For practical purpose, it was assumed that the maximal cytochrome oxidase activity would be obtained in all these different types of mitochondria under the same Triton X-100 incubation conditions.

Once these conditions were settled, this method was used to determine the distribution, throughout the gradient, of the mitochondria from the homogenate subjected to rate zonal centrifugation. A pigeon heart homogenate was layered on a 15% - 28% Ficoll-sucrose density gradient, containing 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl,

TABLE 9

Cytochrome oxidase activity of hamster heart mitochondria measured in the presence of two different concentrations of Ficoll and varying concentrations of Triton X-100.

Treatment		µmoles O_2/min/g mitochondrial protein	
		0% Ficoll	20% Ficoll
No		840	686
Sonication		1466	872
% Triton X-100, final			
concentration:	0.03	2420	1558
	0.04	2875	2559
	0.05	2197	2006
	0.06	2202	1769
	0.07	1844	1470

46 to 56 µg mitochondrial protein were used per assay.

Same conditions as described for Table 8.

TABLE 10

Cytochrome oxidase activity of hamster skeletal muscle mitochondria measured in the presence of two different concentrations of Ficoll and varying concentrations of Triton X-100.

Treatment		μmoles O_2/min/g mitochondrial protein	
		0% Ficoll	20% Ficoll
No		1016	954
Sonication		1301	1097
% Triton X-100, final			
concentration:	0.03	1512	1397
	0.04	1731	1321
	0.05	2165	1768
	0.06	1991	1759
	0.07	1939	1528
	0.10		757

56 to 71 μg mitochondrial protein were used per assay.

Same conditions as described for Table 8.

pH 7.2, and 0.1% albumin throughout. An HB-4 swinging-bucket rotor was used, which can accomodate large centrifuge tubes and therefore a larger sample volume than the SW-41 swinging-bucket rotor. After a rate zonal centrifugation at 18,300 x G for 20 minutes, the gradient was fractionated from the bottom of the tube using a peristaltic pump, as described in Methods II, 3. Two ml fractions were collected. The material which adhered to the wall of the tubes (due to the wall effect) interfered with the fractionation (3, 4, 6). Therefore, the sedimentation pattern of pigeon heart homogenate was not reproducible.

The cytochrome oxidase activity was measured on each fraction, after incubation in 0.05% Triton X-100 for 15 minutes at 25°C. Because the amount of mitochondrial protein present in each fraction was unknown, the enzyme activity was expressed in μmoles O_2 per minute per 150 μl of mitochondrial suspension (Table 11).

Sixty per cent of the total cytochrome oxidase activity was still present on the top of the gradient. Thus, only 40% of the mitochondrial population sedimented into the gradient because of the packing of material on the top of the gradient, which prevents a total sedimentation of the material. Pretlow and Boone (158) reported that if the beginning of the gradient was too dense, cell aggregation occurred at the sample-gradient interface;

TABLE 11

Cytochrome oxidase activity of pigeon heart mitochondria isolated from a homogenate using rate zonal centrifugation.

Fractions	cyt.oxi.activity	Fractions	cyt.oxi.activity
1 (bottom)	0.1	12	16.4
2	4.3	13	9.8
3	7.2	14	9.0
4	6.2	15	8.5
5	11.6	16	6.8
6	9.2	17	7.3
7	7.5	18	11.4
8	19.4	19 (top)	21.1
9	9.9	20 (layered sample)	32.4
10	15.1	21 (layered sample)	34.0
11	8.1	22 (layered sample)	161.8

Enzyme activity is expressed in µmoles O_2 /minute/150 µl of mitochondrial suspension used in each assay.

Gradient: 15% - 28% Ficoll

18,300 x G for 20 minutes. HB-4 swinging-bucket rotor.

2 ml per fraction, except #22, 0.4 ml.

100 µl of each fraction were added to a final concentration of 0.05% Triton X-100. Incubation of 15 minutes at 25°C. Used 150 µl per assay.

these workers used no more than 4-5% Ficoll at the beginning of the gradient to prevent that blockage. This idea was therefore tested, using a flatter gradient and varying centrifugation speed. The details follow.

(c) Centrifugation speed study.

The density gradients used were in the range of 4% to 12% Ficoll, having 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl pH 7.2 and 0.1% bovine plasma albumin throughout. The HB-4 swinging-bucket rotor was used again and a pigeon heart homogenate was layered. The same time of centrifugation was used, i.e. 10 minutes, and the centrifugal force was the only variable. After the centrifugation, 2 ml fractions were collected with a needle fixed to the peristaltic pump. The cytochrome oxidase activity and the determination of total protein at 280 nm were done on each fraction.

The centrifugal forces used were: 221 x G
434 x G
762 x G
1334 x G
2705 x G
4572 x G

Table 12 and Figures 8 give examples of the type of results obtained for two different centrifugal forces, i.e. 762 x G and 2,705 x G for 10 minutes in both cases.

TABLE 12

Cytochrome oxidase activity and total protein determination after a rate zonal centrifugation of pigeon heart homogenate at two centrifugal forces.

Fractions	Cytochrome oxidase activity, μmoles O_2/min/150 μl mitochondrial suspension		O.D. at 280 nm	
	762 x G	2,705 x G	762 x G	2,705 x G
1 (bottom)	2.4		1.38	2.98
2	5.3	35.6	3.32	9.72
3	3.9	31.1	1.89	9.45
4	3.9		2.75	5.99
5	5.1		3.67	4.49
6	8.7	6.7	4.69	3.78
7	9.3		4.34	3.68
8	7.4	5.1	3.77	3.47
9	8.0		3.83	3.47
10	5.5		3.37	3.61
11	5.4	4.9	3.47	3.44
12	12.8	11.1	4.59	5.46
13	10.8	19.3	4.44	7.10
14	14.9	11.4	5.61	5.63
15	35.2	9.1	7.96	4.54
16	61.6		10.35	4.62
17	52.9	17.4	9.69	6.72
18	64.9	37.2	10.61	10.08
19	20.0	24.3	5.87	9.24
20 (top)	43.5	21.0	9.44	10.71
21 } sample layered	28.4	40.0	11.83	16.32
22 } sample layered	27.6	42.9	21.68	21.42
23 } sample layered	22.2		18.97	

Gradient :5% - 12% Ficoll (W/V)

762 x G for 10 minutes and 2,705 x G for 10 minutes. HB-4 swinging-bucket rotor.

2 ml per fraction, except #23, 0.7 ml.

100 μl of each fraction were added to a final concentration of 0.05% Triton X-100. Incubation of 15 minutes at 25°C. Used 150 μl per assay.

As the centrifugal force was increased, the distribution of the cytochrome oxidase shifted towards the denser part of the gradient. At 762 x G (Figure 8A), there was a large peak of cytochrome oxidase activity in the 4.0% to 6.6% Ficoll zone; there was also a large peak of total protein as determined at 280 nm. By increasing the centrifugal force to 2,705 x G (Figure 8B), the peak of cytochrome oxidase was more spread out with a small peak in the 4.8% to 5.8% Ficoll zone, and in the 6.7% to 7.5% Ficoll zone; also an increase in the amount of activity in the denser part of the gradient (9.8%-12% Ficoll) with a packing on the cushion was observed. The same general pattern was observed with the total protein determined at 280 nm.

From all the experiments, 2,705 x G for 10 minutes was accepted as the best compromise to obtain optimal mitochondrial distribution in the gradient. It was then necessary to examine oxidative phosphorylation and the purity of the mitochondrial preparations obtained from the density gradients. The same type of experiment was repeated; the 5.4% - 7.4% Ficoll zone (upper fraction) and the zone around the cushion (8.7% - 12% Ficoll: lower fraction) were collected with a syringe. The two fractions were diluted, centrifuged and suspended in Suspending Medium containing 0.1% bovine plasma albumin. Table 13 gives the oxidative phosphorylation results of the two fractions.

Figure 8: Cytochrome oxidase activity and total protein determination after rate zonal centrifugation of pigeon heart homogenate at two centrifugal forces.

8A: 762 x G

8B: 2,705 x G

The total protein was determined at 280 nm.

The cytochrome oxidase activity is expressed in µmoles O_2 per minute per 150 µl of mitochondrial suspension. The fractions were incubated in 0.05% Triton X-100 for 15 minutes, at 25°C, prior the assay.

The centrifugation was done on an HB-4 swinging-bucket rotor. 2 ml per fraction, except #23, 0.7 ml.

▪▪▪▪▪▪▪ total protein

------ density gradient

▬▬▬ cytochrome oxidase activity

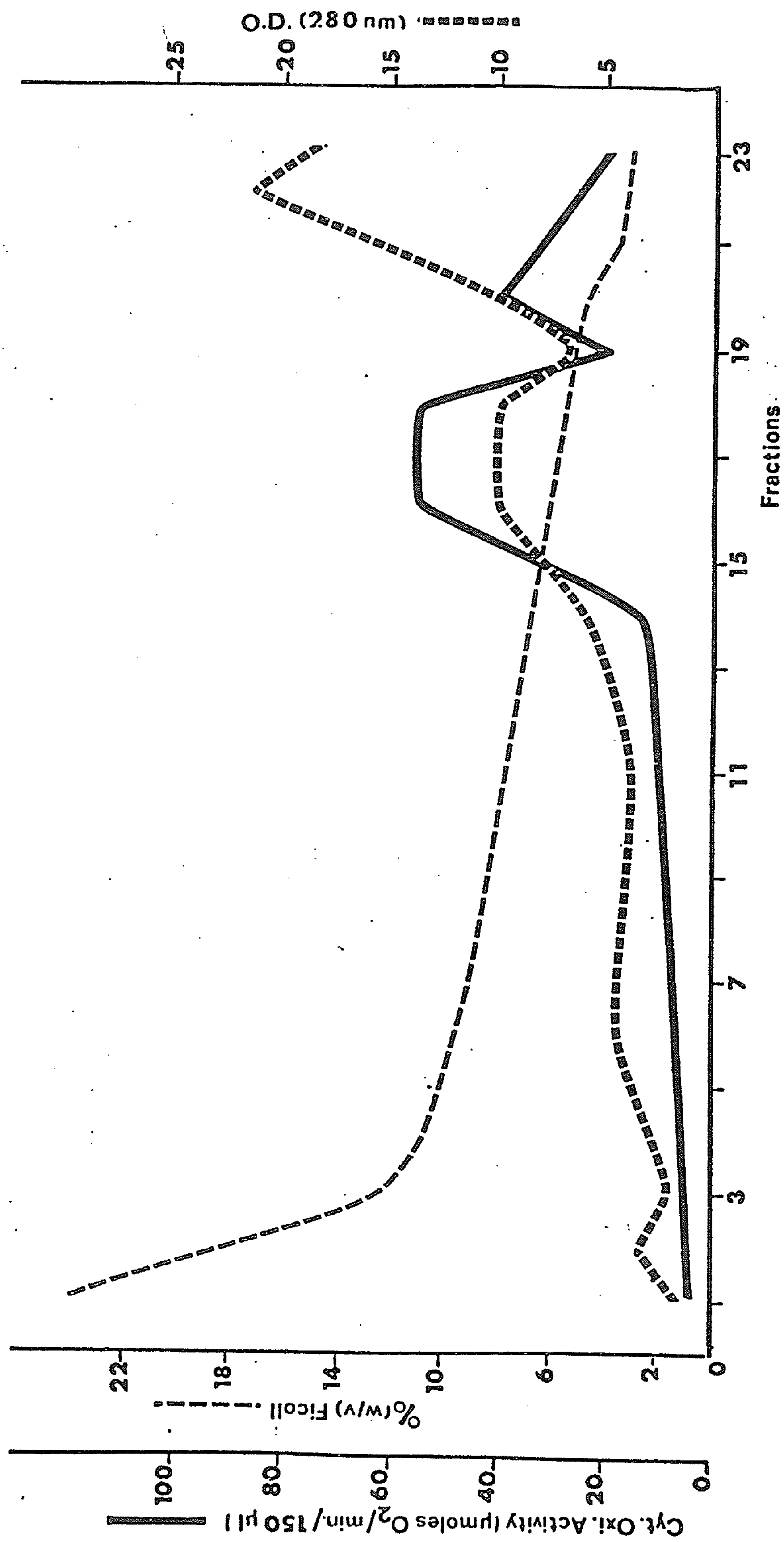


Figure 8A

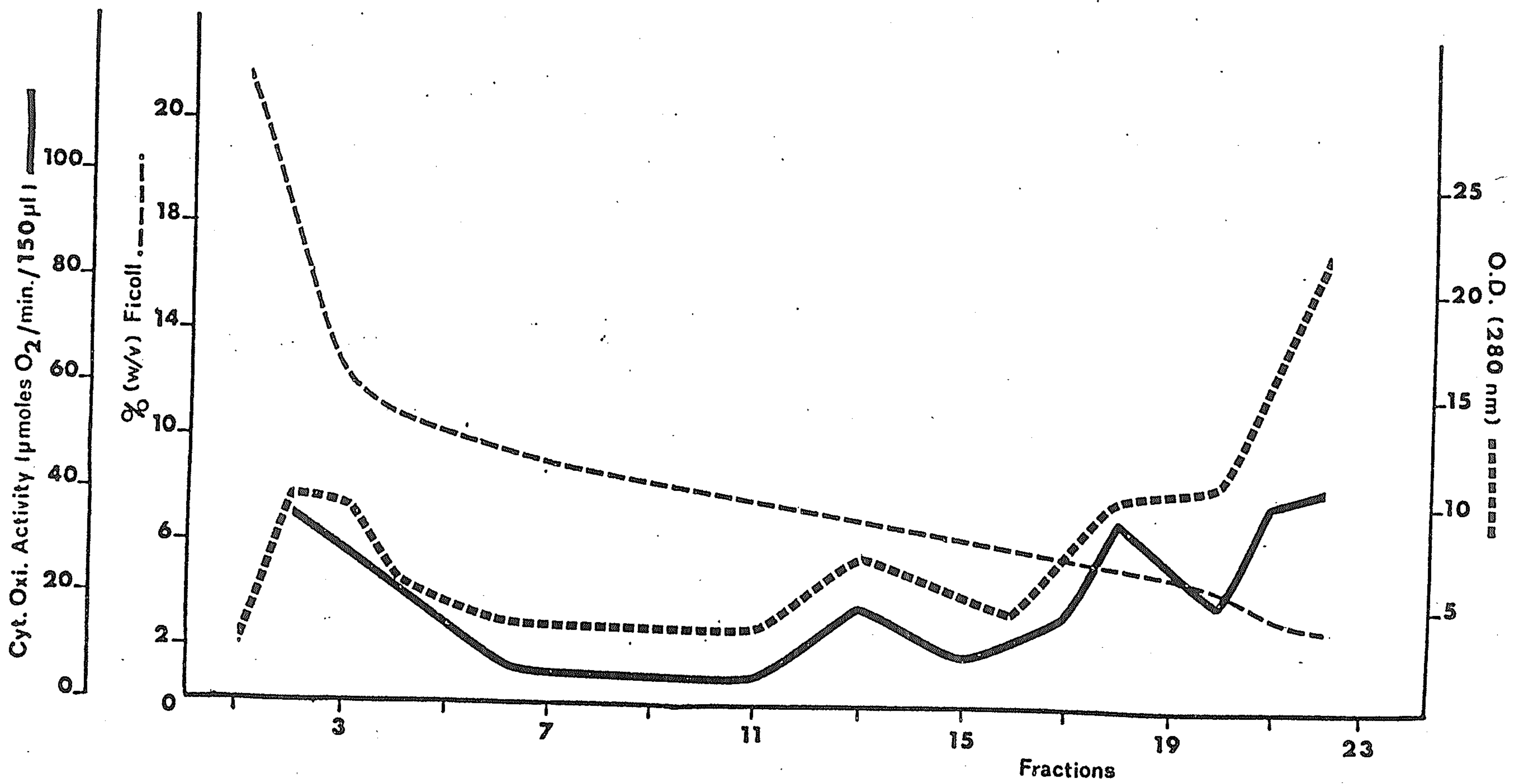


Figure 8B

TABLE 13

Oxidative phosphorylation in the two fractions, from pigeon heart homogenate, recovered after rate zonal centrifugation on a Ficoll-sucrose density gradient.

Fractions	RCR	O_2 rate	ADP/O
Upper	12.4	157	2.50
Lower	8.9	134	2.52

Substrate: 5mM pyruvate - 1mM malate. Results taken from the second cycle of State 3-State 4 respiration with 231 μMADP.

O_2 rate is expressed in μmoles O_2/minute/g protein.

2.705 x G for 10 minutes. HB-4 swinging-bucket rotor.

Yield of protein : 2.0 mg protein/g heart. (5.4-7.4% fraction)
1.9 mg protein/g heart. (8.7-12% fraction).

Upper fraction = 5.4% - 7.4% Ficoll zone.

Lower fraction = 8.7% - 12% Ficoll zone.

The RCR's and the ADP/O ratios of such preparations indicated functionally intact mitochondria (177, 180). The O_2 rates, however, were lower than those obtained with the conventional preparation of pigeon heart mitochondria. This might partly be explained by non-mitochondrial contamination present, particularly in the fraction above the cushion (see Figure 9).

Figure 9A and Figure 9B are electron micrographs of the mitochondria recovered in the 5.4% - 7.4% Ficoll zone; this mitochondrial suspension is largely free of contamination. Figure 9C is a picture of the mitochondria recovered in the 8.7% - 12% Ficoll zone; the area chosen to be photographed was selected to show the type of contamination (mainly by myofibrils) present in the suspension. This area grossly overemphasizes the extent of the contamination. This experiment too was not an improvement over the conventional procedure with respect to the mitochondrial yield, the yield being low in both fractions.

From these experiments, it was concluded that it is possible to prepare pigeon heart mitochondria by layering on a density gradient heart homogenate and by doing a single step density gradient centrifugation. This method yielded a good quality of mitochondria with a reasonable degree of purity in the upper fraction, as shown by the electron micrographs.

Figure 9: Electron micrographs of mitochondria isolated from a pigeon heart homogenate using a single step density gradient centrifugation in a Ficoll-sucrose density gradient.

9A: Upper fraction (5.4-7.4% Ficoll zone)
Magnification 6,700 x.

9B: Upper fraction (5.4-7.4% Ficoll zone)
Magnification 22,600 x.

9C: Lower fraction (8.7-12% Ficoll zone)
Magnification 20,800 x.

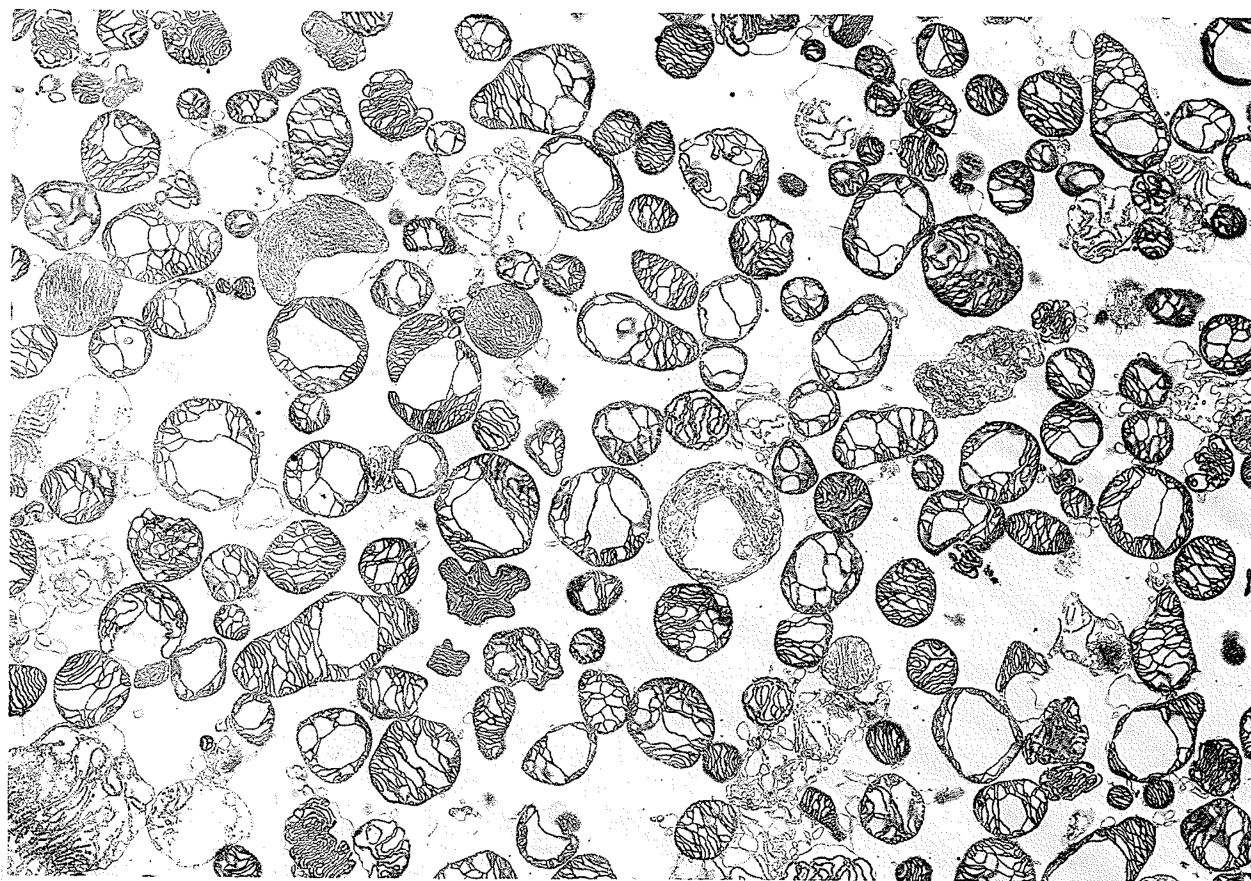


Figure 9A

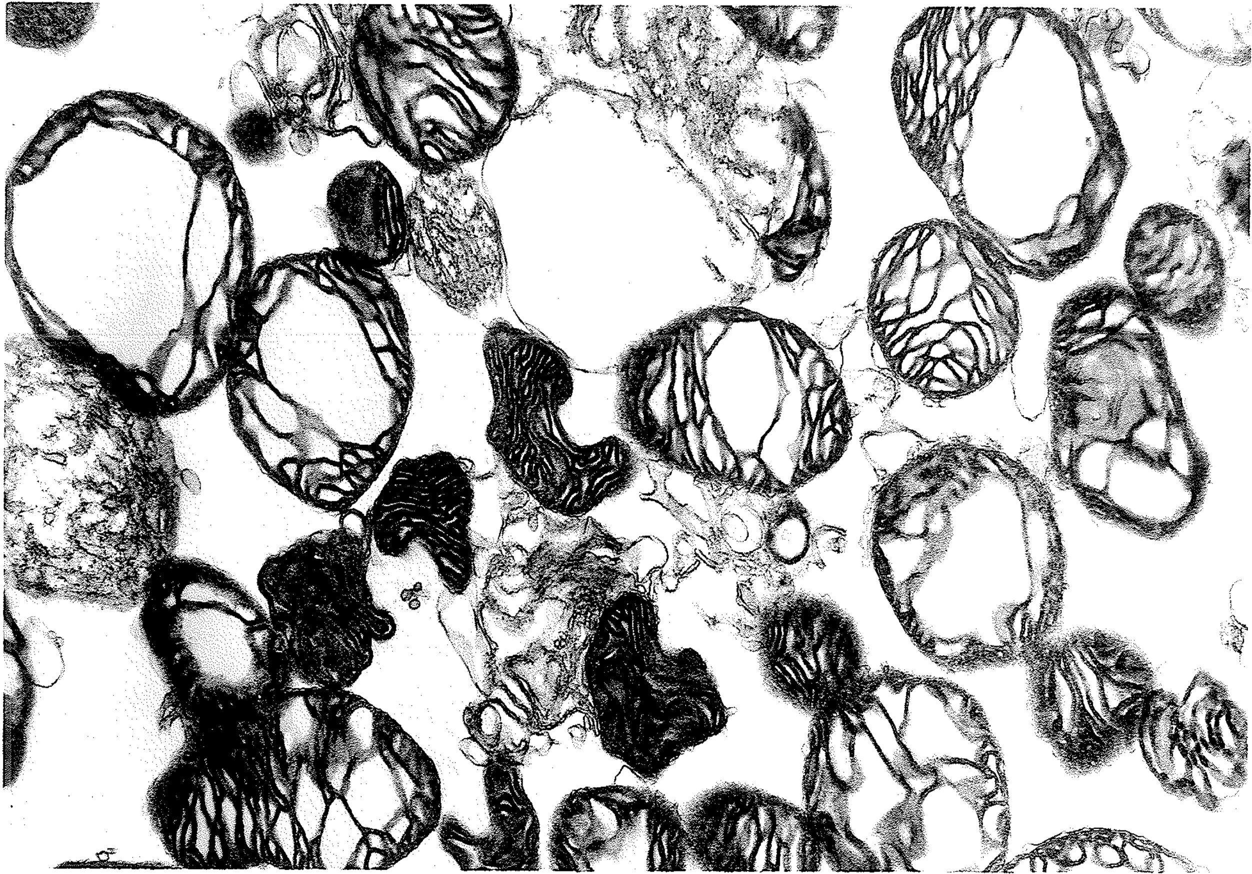


Figure 9B

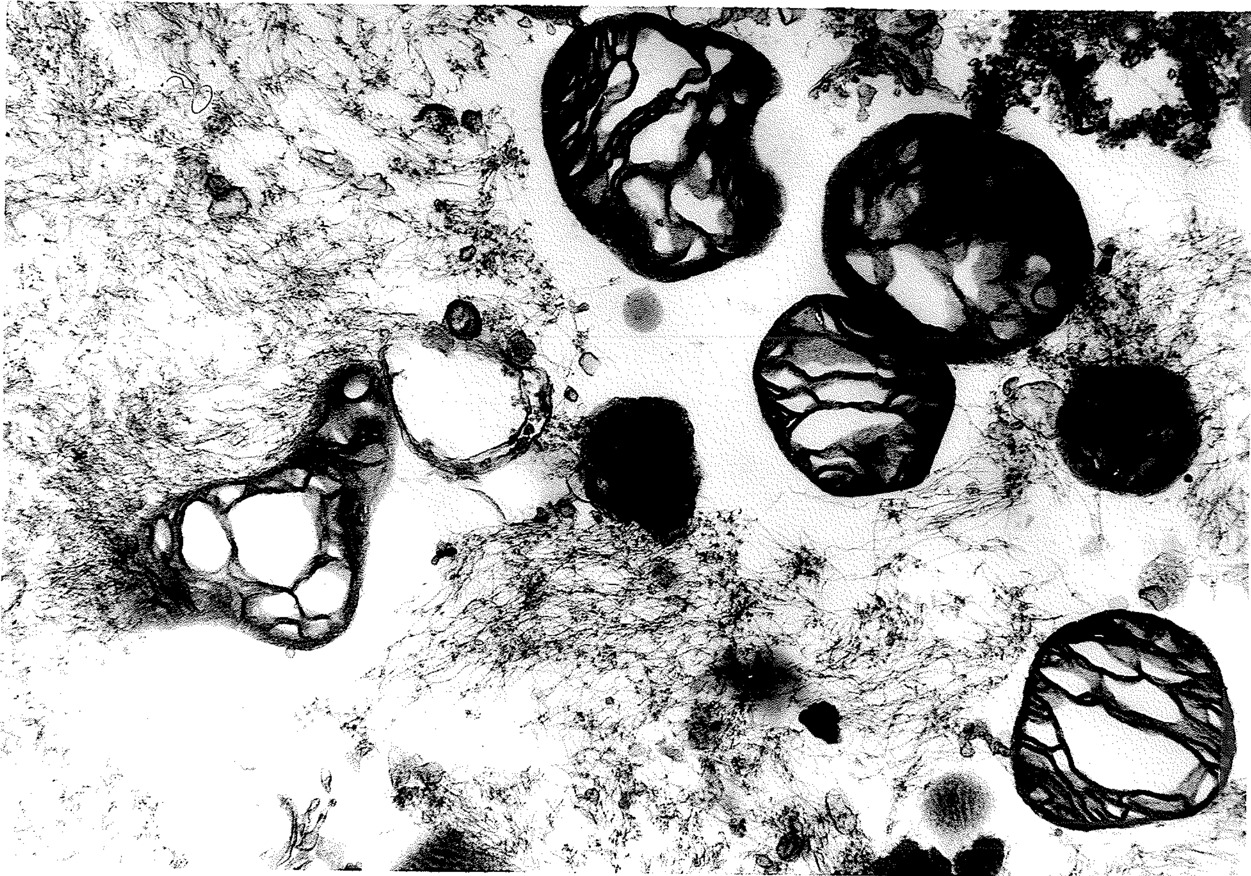


Figure 9C

However, the material which sediment on the wall of the tubes interfered with the fractionation procedure and the sedimentation pattern presented in Figure 8 is not very reproducible. Therefore, the SZ-14 reorienting density gradient zonal rotor was used for the further experiments. This rotor prevents the wall effect (15) because the centrifugation is performed in sector-shaped compartments.

(d) SZ-14 reorienting density gradient zonal rotor experiments.

This rotor holds 1.4 liter of gradient solution and therefore a larger volume of heart homogenate can be layered on it. For that reason, this rotor is useful for large scale isolation of mitochondria. As explained in Methods (II, 3), 100 ml of 25% Ficoll was used as a cushion, and 1000 ml of gradient solution (3.2% - 12.5% Ficoll) was introduced into the rotor, the cushion and the gradient solution having 0.25 M sucrose, 10 mM Tris-Cl, pH 7.2, and 0.1 mM EDTA throughout. Then 100 ml of pigeon heart homogenate was layered on the top of the gradient. The centrifugation was performed as described in Methods II, 3.

The rotor was statically unloaded by collecting 40 ml fractions. Figure 10 shows a series of measurements done on each fraction. The determination of the total protein was done at 280 nm. The sedimentable protein

Figure 10: Sedimentation pattern of a pigeon heart homogenate after a rate zonal centrifugation in a Ficoll-sucrose density gradient, using a SZ-14 reorienting density gradient zonal rotor.

- - - - - - : gradient shape

▪▪▪▪▪▪▪▪ : total protein determined at 280 nm

———— : sedimentable protein determined by the Lowry method.

Experiment done in the absence of albumin.

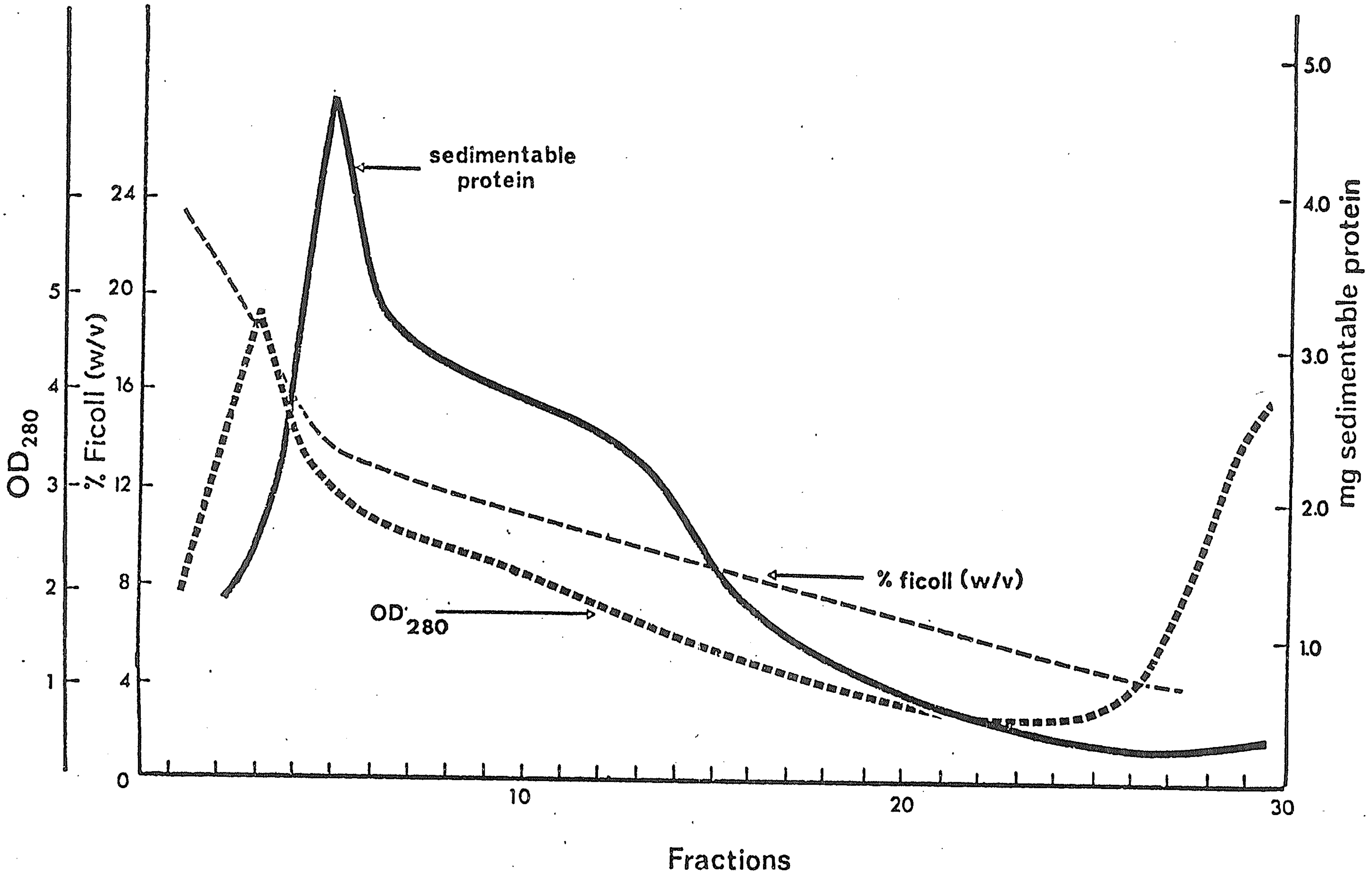


Figure 10

(Methods II, 3) were determined by the Lowry method (182). Oxidative phosphorylation was examined on each fraction. This type of experiments was repeated twice: the first was done in the absence of albumin, and the second in the presence of 0.1% albumin in the Stock Medium and in the gradient solution, including the cushion.

In the experiment without albumin, the fractions 2 to 16 contained 86% of the total amount of sedimentable protein; oxidative phosphorylation in these fractions was similar and therefore the data of these 15 fractions were combined (Table 14). In the experiment done in the presence of 0.1% albumin, the fractions 2 to 11 contained 76% of the total amount of sedimentable protein; oxidative phosphorylation in these 10 fractions was similar and the data were also combined (Table 14).

In the experiment done in the presence of albumin, the RCR's, the ADP/O ratios and chiefly the O_2 rates were higher than those obtained in the absence of albumin ($p < 0.005$). This is a good example of the beneficial effect of albumin on oxidative phosphorylation by binding the free fatty acids (161-164). The yield of mitochondrial protein was similar to that obtained by the conventional procedure (7.69 ± 0.56 mg mitochondrial protein/g heart): 8.04 mg protein/g heart in the experiment done in the presence of albumin and 11.04 mg protein/g heart in the experiment done in the absence of albumin.

TABLE 14

Respiration and oxidative phosphorylation by mitochondria isolated from pigeon heart homogenate in a SZ-14 reorienting density gradient zonal rotor.

Medium	RCR	O_2 rate	ADP/O
0.1% albumin (10 fractions)	6.8 $\pm$ 0.2	218 $\pm$ 9	2.39 $\pm$ 0.02
No albumin (15 fractions)	4.0 $\pm$ 0.1	91 $\pm$ 4	2.16 $\pm$ 0.03
	$p < .005$	$p < .005$	$p < .005$

Mean $\pm$ S.E.

Substrate: 5mM pyruvate - 1mM malate. Results taken from second cycle of State 3 - State 4 respiration with 230 μMADP.

O_2 rate is expressed in μmoles O_2/min/g protein

Gradient :3.2% - 12.5% Ficoll (W/V).

3,600 x G - 6,100 x G , 10 minutes.

$p < .005$, unpaired t-test.

5. Conclusion.

We have demonstrated that it is possible to isolate mitochondria directly from a pigeon heart homogenate using a single step density gradient centrifugation. In such experiments, a 15% heart homogenate is layered on a 5% - 12% Ficoll (Ca^{++}-free) density gradient, with 0.25 M sucrose, 10 mM Tris-Cl pH 7.2, 0.1 mM EDTA and 0.1 or 0.5% bovine plasma albumin throughout; the centrifugal force was 2,705 x G for 10 minutes. Mitochondria with good oxidative phosphorylation activity were suspended in the 5.4% - 7.4% Ficoll zone and in the 8.7% - 12% Ficol zone (Table 13). The degree of purity of the mitochondrial preparations was fairly good as shown by the O_2 rates, especially in the 5.4% - 7.4% Ficoll zone. The electron micrographs showed also the degree of purity of this mitochondrial fraction. The upper fraction (5.4% - 7.4% zone) was largely free of any contamination (Figure 9A, 9B), the mitochondria having the same configuration as ones isolated by the conventional procedure. The lower fraction (8.7% - 12% zone) showed much more contamination. However, this picture (Figure 9C) probably overemphasized the amount of contamination as the oxidative phosphorylation results indicated that there was a fairly good quality of mitochondria in that fraction.

These experiments were repeated several times. The sedimentation pattern was reproducible "visually"; however

the fractionation procedure was no longer reproducible due to the interference of the material which sedimented on the wall. The results shown in Figure 8B are pattern of a single experiment and not necessarily characteristic.

By removing the mitochondrial fraction from the gradient with a syringe, interference by the material sedimented on the wall could be largely avoided and thus the suspensions collected were representative of the mitochondria band in the gradient.

At this point, the principal aim of the study had been accomplished except that the yield of mitochondria was not higher than that obtained by the conventional procedure of mitochondria isolation.

By using a SZ-14 reorienting density gradient zonal rotor, the wall effect could be avoided and a large amount of homogenate could be studied. The mitochondrial yield obtained with this technique was at least as good as the yield obtained with the conventional procedure which involved differential centrifugation. This technique necessitated a large volume of gradient and the fractionation procedure was relatively long, the recovery of the sedimentable protein being also time consuming due to the large number of fractions to be handled.

This procedure of isolation of mitochondria by a single step density gradient centrifugation was not used in further experiments presented in this thesis. Because mitochondria isolated from different tissues and subjected to rate zonal centrifugation on Ficoll-sucrose and sucrose density gradients were to be studied, the standardization of the technique for all the different tissues would be too time consuming. Nevertheless, it was clearly established that it is possible to isolate directly from the homogenate mitochondria of good quality and purity, in spite of the omission of the three steps of differential centrifugation of the conventional procedure.

II. Comparison of oxidative phosphorylation by previously isolated mitochondria after having been subjected to rate zonal centrifugation either in a sucrose density gradient or in a Ficoll-sucrose density gradient.

1. Introduction.

Sucrose density gradients have been used successfully by several workers to separate different mitochondrial populations (9, 45, 63-73, 143) and for a further purification of previously isolated mitochondria (10, 21, 74, 75). However, oxidative phosphorylation by such mitochondria were not studied by these workers, probably because they assumed that the hypertonic sucrose concentration used to make the density gradient damaged the mitochondria due to the osmotic stress (53), as shown by electron micrographs and by solubilization of mitochondrial enzymes(55, 56).

In spite of the osmotic effect described, oxidative phosphorylation by mitochondria, after sucrose density gradient centrifugation, was studied. Rat liver mitochondria (10, 83, 84) and pigeon heart mitochondria (10) lose respiratory control after centrifugation through a sucrose density gradient. Mitochondria isolated from Euglena Gracilis (89), after purification on a sucrose density gradient, exhibited phosphorylation and respiration. Mitochondria, isolated from a large variety of plants (10), were reported to be coupled after purification on a sucrose

density gradient. The latter workers (10) suggested also that mitochondria, in hypertonic solutions, should be diluted slowly to normotonic conditions to avoid an osmotic shock that could occur by a rapid dilution; they also reported that rat liver and pigeon heart mitochondria were uncoupled after a sucrose density gradient even if the slow dilution technique was used. However, rat skeletal muscle mitochondria have been reported (68, 165) to have high RCR after purification on a sucrose density gradient. These reports contain the only published data on coupled mammalian mitochondria after a sucrose density gradient.

To prevent the osmotic effect of hypertonic sucrose solutions, Ficoll-sucrose density gradients have been used. At least on theoretical grounds, this type of iso-osmotic density gradient should offer some advantages over a simple sucrose density gradient. However, Ficoll-sucrose density gradients have been reported to inhibit the mitochondrial phosphorylation ability (88, 89, 92). But, such density gradients have also been reported to allow the recovery of coupled mitochondria which were previously isolated from rat brain (92, 93) and rat liver (83, 84).

As shown above, there are some discrepancies in the literature on the effect of sucrose density gradient and Ficoll-sucrose density gradient on the mitochondrial function of oxidative phosphorylation. There are no

studies which compare objectively the effect of both types of density gradients on the mitochondria previously isolated from different tissues using the same isolation procedure. Therefore, this second section compares oxidative phosphorylation by mitochondria isolated by differential centrifugation from a variety of tissues and then subjected to rate zonal centrifugation, either on sucrose density gradients or on Ficoll-sucrose density gradients. Ca^{++}-free Ficoll was used. The tissues studied were: rat liver, hamster heart and hamster skeletal muscle.

The following method was used: the mitochondria were prepared as described in Methods(I, 2-4). After conventional isolation of the organelles, an aliquot of the suspension was kept for oxidative phosphorylation studies. Another aliquot of the suspension was layered on the gradient. The sucrose density gradients contained 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2 and 0.1% bovine plasma albumin throughout; the Ficoll-sucrose density gradients contained 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-Cl, pH 7.2 and 0.1% bovine plasma albumin throughout. After rate zonal centrifugation in a SW-41 swinging-bucket rotor, the mitochondrial suspension was recovered, diluted and centrifuged at 17,300 x G for 10 minutes. The pellet was resuspended and studied polarographically. As it appeared that the slow dilution technique (10) might be beneficial to the mitochondria, this technique was also applied.

2. <u>Isolated rat liver mitochondria subjected to rate zonal centrifugation</u>.

Previously isolated rat liver mitochondria were layered on the density gradients and rate zonal centrifugation was performed as described in Table 15. From the sucrose density gradients, the 47% - 50% (W/V) sucrose zone was collected, and from the Ficoll-sucrose density gradients, the 17% - 22% (W/V) Ficoll zone was collected. These two preparations were studied polarographically (see Table 15).

There was no significant differences between the quality of the RCR's and the ADP/O ratios of the mitochondria freshly isolated, referred as "Before" in the table, and the mitochondria recovered from the Ficoll-sucrose density gradients. However, the mitochondria recovered from the sucrose density gradient, after the fast dilution, were almost uncoupled. The slow dilution procedure did not improve the mitochondrial measurements which is in agreement with the data published by Douce <u>et</u> <u>al</u> (10).

The percentage of recovery (mg mitochondrial protein recovered from the gradient over mg mitochondrial protein layered on the gradient x 100) from the Ficoll-sucrose density gradient was 38% ± 5; from the sucrose density gradient, 50% ± 5, after the fast dilution and 52% ± 5 after the slow dilution.

TABLE 15

Previously isolated rat liver mitochondria subjected to rate zonal centrifugation on sucrose and Ficoll-sucrose density gradients.

	RCR	O_2 rate	ADP/O
Before	4.8 ± 0.4	56 ± 6	2.12 ± 0.07
After Ficoll	3.6 ± 0.7	44 ± 6	1.77 ± 0.44
	N.S.	$p < .05$	N.S.
After sucrose:			
-fast dilution:	1.2 ± 0.1	31 ± 4	0.24 ± 0.24
	$p < .005$	$p < .01$	$p < .005$
* -slow dilution	1.0	29 ± 4	0

Mean ± S.E.: N = 5, except * N=4

Substrate: 4 mM glutamate - 0.2mM malate; results taken from second cycle of State 3-State 4 respiration (210 μMADP).

O_2 rate is expressed in μmoles O_2/min/g mitochondrial protein.

Yield of mitochondria: 9.3 ± 0.8 mg mitochondrial protein/g liver

Sucrose : 35% - 52% (W/V); Ficoll :13% - 22% (W/V).

23,000 x G ; 15 minutes

The p values represent the comparison between the isolated mitochondria("Before") and the mitochondria recovered from the gradient (paired t-test).

From these experiments, it can be concluded (i) that rat liver mitochondria, after Ficoll-sucrose density gradient, have oxidative phosphorylation activity similar to that of freshly isolated mitochondria, and (ii) that rat liver mitochondria, after a sucrose density gradient, were essentially uncoupled. Clearly, Ficoll-sucrose density gradient is far superior to sucrose density gradient in the case of rat liver mitochondria.

3. Isolated hamster heart mitochondria subjected to rate zonal centrifugation.

A similar study was done with hamster heart mitochondria again to compare the quality of oxidative phosphorylation by freshly isolated mitochondria, referred as "Before" in Tables 16 and 17, with those of mitochondria recovered from a sucrose density gradient and from a Ficoll-sucrose density gradient (composition given in the Introduction of this section).

In this series, the effect of bovine plasma albumin on the mitochondrial activities (161-164) was tested by using 0.1% bovine plasma albumin in the gradient solutions and in the Suspending Medium. These results were compared with another set of experiments in which albumin was omitted. Again the effect of the slow dilution after recovery from the sucrose density gradients was compared with the effect of the fast dilution. The results are shown in Tables 16 and 17.

TABLE 16

Previously isolated hamster heart mitochondria subjected to rate zonal centrifugation on Ficoll-sucrose density gradients, in the presence and absence of 0.1% bovine plasma albumin.

	RCR		O_2 rate		ADP/O	
	Alb	*no Alb	Alb	*no Alb	Alb	*no Alb
Before	7.2±0.2	7.4±0.2	229±16	243±22	2.56±0.05	2.79±0.07
After	6.3±0.3	6.2±0.2	253±27	173±12	2.55±0.05	2.63±0.01
	$p < .025$	$p < .05$	NS	$p < .05$	NS	NS

Mean ± S.E.; N = 5, except * N=3

Substrate: 5mM pyruvate - 1mM malate; results taken from second cycle of State 3-State 4 respiration (229 μMADP).

O_2 rate is expressed in μmoles O_2/min/g mitochondrial protein.

Yield of mitochondria : 20.8±2.0 mg mitochondrial protein/g heart.

Ficoll gradient: 13% - 30% (W/V).

23,000 x G ; 15 minutes

The p values represent the comparison between the isolated mitochondria ("Before") and the mitochondria recovered from the gradient (paired t-test).

TABLE 17

Previously isolated hamster heart mitochondria subjected to rate zonal centrifugation on sucrose density gradient, in the presence and the absence of 0.1% bovine plasma albumin.

	RCR		O_2 rate		ADP/O	
	Alb	*no Alb	Alb	*no Alb	Alb	*no Alb
Before	7.8±0.4	7.9	223±6	195	2.56±0.05	2.75
After fast dilution	5.6±0.5	5.7	214±7	187	2.38±0.07	2.59
	$p < .005$		NS		$p < 0.01$	
**slow dilution	3.3±0.5		146±19		2.05±0.06	

Mean ± S.E.; N = 5, except * N = 2, **N = 4.

Substrate: 5mM pyruvate - 1mM malate; results taken from second cycle of State 3-State 4 respiration (229 μMADP).

O_2 rate is expressed in μmoles O_2/min/g mitochondrial protein

Yield of mitochondria : 23.3±0.7 mg mitochondrial protein/g heart.

Sucrose gradient : 22% - 39% (W/V)

23,000 x G ; 15 minutes.

The p values represent the comparison between the isolated mitochondria ("Before") and the mitochondria recovered from the gradient (paired t-test).

The mitochondria recovered after a Ficoll-sucrose density gradient, in the 14.4% - 23% Ficoll zone in the presence of albumin, were of a very good quality as shown by high values for RCR's, O_2 rates and ADP/O ratios. However, a similar high mitochondrial quality was observed also in the absence of albumin for the mitochondria recovered in the 15%-20% Ficoll zone. The RCR being a criterion of the functional intactness of the mitochondria (177, 180), the Ficoll-sucrose density gradient allowed the recovery of mitochondria functionally similar to the freshly isolated organelles.

The mitochondria recovered from the sucrose density gradient, in the 31%-39% sucrose zone (in the presence of albumin) had oxidative phosphorylation activities comparable to those of freshly isolated mitochondria. Similar observations were made in the experiments done in the absence of albumin, where the mitochondria were recovered in the 32% - 39% sucrose zone. In this case, mitochondrial function after the slow dilution was poorer than that after the fast dilution.

The percentage of recovery after the rate zonal centrifugation was 48% ± 6 in the Ficoll-sucrose density gradient , 76% ± 3 in the sucrose gradient followed by a fast dilution and 73% ± 3 in the sucrose density gradient followed by a slow dilution.

To conclude, Ficoll-sucrose density gradients and sucrose density gradients both allow isolation of mitochondria with oxidative phosphorylation activity similar to that obtained with freshly isolated organelles. The heart mitochondria before and after being subjected to density gradient centrifugation had high oxidative phosphorylation activity. Therefore, the known beneficial effect of albumin (161-164) to improve the coupling of the mitochondria was not observed in these experiments. The slow dilution technique did not improve the quality of the mitochondria after they had been in hyperosmotic conditions; this could be either due to the long dilution time, i.e. an ageing effect, or due to the dilution technique itself. Finally, these results show that heart mitochondria can be subjected to rate zonal centrifugation in sucrose density gradient and in Ficoll-sucrose density gradient without losing their phosphorylation ability.

4. Isolated hamster skeletal muscle mitochondria subjected to two different centrifugal forces.

Mitochondria isolated from hamster skeletal muscle were also subjected to Ficoll-sucrose density gradients and to sucrose density gradients in order to compare oxidative phosphorylation by freshly isolated mitochondria with that of the mitochondria recovered from these two density gradients.

The experiments were done with 0.1% bovine plasma albumin in the gradient solutions and in the Suspending Medium. Also two different centrifugal forces were used: 23,000 x G for 15 minutes and 108,500 x G for 60 minutes, the first one being a rate zonal centrifugation and the second one being an isopycnic centrifugation.

The experimental protocol was the following: the mitochondria were isolated from the skeletal muscle as described in Methods(I, 3). The mitochondrial suspension was assayed polarographically, indicated as "Before" in the Tables; an aliquot of the suspension was layered, for example, on the sucrose density gradient, and the rate zonal centrifugation was performed. After its completion, the mitochondria were recovered and assayed polarographically. Another aliquot of the original suspension was layered on a second sucrose density gradient and the 108,500 x G centrifugation was performed. After this centrifugation, the original mitochondrial suspension was again studied polarographically in order to account for any ageing effect in the "Before" preparation presented in Table 18, and the results obtained were referred as the values of the mitochondrial measurements "before" the isopycnic centrifugation (Table 19). After completion of the centrifugation, the mitochondria were recovered and assayed polarographically. Therefore, the sucrose density gradient

experiments and the Ficoll-sucrose density gradient experiments were two different sets of experiments. Fast and slow dilutions were also used to return the mitochondria from hyperosmotic to normotonic conditions. Tables 18 and 19 contain the results.

After the rate zonal centrifugation (Table 18), the mitochondria were recovered from the Ficoll-sucrose density gradients in the 13% - 19% Ficoll zone. The mean RCR value was decreased from the "Before" value by one third, while the O_2 rates and the ADP/O ratios shown little change. The RCR of the mitochondria recovered from the sucrose density gradients, in the 25% - 38% sucrose zone, was less than half of the "Before" value, the O_2 rate and the ADP/O ratio being much lower than the O_2 rate and the ADP/O ratio of the freshly isolated mitochondria.

After the isopycnic centrifugation, the RCR of the mitochondria recovered from the Ficoll-sucrose density gradient, in the 19.8% - 25% zone, was more than half of the RCR value of the "Before" mitochondria; the O_2 rate and the ADP/O ratio being also lower than the measurements of the "Before" organelles. The RCR of the mitochondria recovered from the sucrose density gradient was less than one third of the original value; the O_2 rate and the ADP/O ratio were also very low for a skeletal muscle mitochondria preparation.

TABLE 18

Previously isolated hamster skeletal muscle mitochondria subjected to rate zonal centrifugation on sucrose and Ficoll-sucrose density gradients, in the presence of 0.1% bovine plasma albumin.

	Sucrose			Ficoll		
	RCR	O_2 rate	ADP/O	RCR	O_2 rate	ADP/O
Before	7.6±2.0	183±18	2.39±0.13	9.0±0.3	218±10	2.48±0.06
After						
-fast dilution	3.1±0.6	110±13	2.05±0.13	6.0±0.6	195±3	2.40±0.06
	NS	$p < .005$	$p < .01$	$p < .05$	$p < .05$	NS
-slow dilution	1.3±0.3	42±18	0.62±0.62			

Mean ± S.E.; N = 3.

Substrate: 5mM pyruvate - 1mM malate; results taken from second cycle of State 3-State 4 respiration (130-229 μMADP).

O_2 rate is expressed in μmoles O_2/min/g mitochondrial protein.

Yield of mitochondria: 1.10±0.04mg mitochondrial protein/g muscle.

Sucrose gradient: 22%-39% (W/V); Ficoll gradient: 13%-25% (W/V)

23,000 x G ; 15 minutes.

The p values represent the comparison between the isolated mitochondria ("Before") and the mitochondria recovered from the gradient (paired t-test).

TABLE 19

Previously isolated hamster skeletal muscle mitochondria subjected to isopycnic centrifugation on sucrose and Ficoll-sucrose density gradients, in the presence of 0.1% bovine plasma albumin.

	Sucrose			Ficoll		
	RCR	O_2 rate	ADP/O	RCR	O_2rate	ADP/O
Before	5.5±1.4	151±28	2.26±0.15	6.5±0.6	181±3	2.56±0.08
After -fast dilution	1.7±0.2 NS	88±5 NS	1.41±0.23 $p < .005$	3.6±0.3 $p < .025$	130±4 $p < .005$	2.25±0.12 NS
-slow dilution	1.4±0.2	65±11	0.35±0.35			

Mean ± S.E. ; N = 3

Substrate: 5mM pyruvate - 1mM malate; results taken from second cycle of State 3-State 4 respiration (130 - 229 µMADP).

O_2rate is expressed in µmoles O_2/min/g mitochondrial protein

Yield of mitochondria: 1.10±0.04mg mitochondrial protein/g muscle.

Sucrose gradient: 39%-51% (W/V); Ficoll gradient: 19.5%-25% (W/V).

108,500 x G ; 60 minutes.

The p values represent the comparison between the isolated mitochondria ("Before") and the mitochondria recovered from the gradient (paired t-test).

The percentages of recovery were 28% ± 3 after Ficoll-sucrose density gradients, 30% ± 3 after sucrose density gradients (fast dilution) and 34% ± 2 after sucrose density gradients (slow dilution).

Thus, Ficoll-sucrose density gradients allow the recovery of good quality of skeletal muscle mitochondria, at both centrifugal forces. The sucrose density gradients allow the recovery of an inferior quality of mitochondria after rate zonal centrifugation if compared with freshly isolated mitochondria. However, after isopycnic centrifugation on sucrose density gradient, the mitochondria showed "poor" coupling of oxidative phosphorylation, the RCR indicating that these mitochondria were not functionally intact. The slow dilution did not improve the quality of the mitochondria recovered from the sucrose density gradients.

5. Conclusion.

Rat liver mitochondria, subjected to rate zonal centrifugation on a Ficoll-sucrose density gradient, were functionally similar to freshly isolated mitochondria, as also shown by Dimini and Hoch (83), and by Grimwood and Wagner (84). However, such mitochondria, when subjected to rate zonal centrifugation on a sucrose density gradient, were poorly coupled and lost their phosphorylation ability (88, 89).

Heart mitochondria could be subjected to rate zonal centrifugation in a sucrose or in a Ficoll-sucrose density gradient without losing their phosphorylation ability. No difference was observed between the mitochondrial oxidative phosphorylation measurements when the experiments were done in the presence or in the absence of 0.1% albumin.

Hamster heart mitochondria were more resistant than rat liver mitochondria to the osmotic effect of high sucrose concentrations. Oxidative phosphorylation activity of heart mitochondria recovered from sucrose density gradient was comparable to that of freshly isolated mitochondria, but this was not the case for rat liver mitochondria. This observation might be explained by a greater elasticity of heart mitochondrial membranes than the liver mitochondrial membranes, the former then being less susceptible to osmotic damage in hypertonic sucrose solution.

Skeletal muscle mitochondria subjected to rate zonal centrifugation were more active in oxidative phosphorylation after recovery from a Ficoll-sucrose density gradient than after recovery from a sucrose density gradient. After an isopycnic centrifugation, similar results were found, i.e. Ficoll-sucrose density gradient allowed the recovery of skeletal muscle mitochondria of a better quality than sucrose density gradient.

The slow dilution procedure to return the mammalian mitochondria from hypertonic to normotonic solution did not improve the quality of the mitochondria recovered after centrifugation on sucrose density gradients. A similar observation has been reported by Douce *et al* (10). Control experiments shown that the results obtained after the slow dilution were not due to ageing effects. The technique itself was responsible for a decrease of the RCR by 40%. However, the combined effect of the ageing and the slow dilution technique has not been tested.

The percentage of mitochondrial recovery from sucrose density gradients was always higher than from Ficoll-sucrose density gradients. This might be explained by the higher viscosity of the Ficoll solution (the viscosity of Ficoll solution will be discussed in Results III). To improve the mitochondrial recovery, the dilution of the gradient zone collected should be larger and the centrifugation performed at a higher centrifugal force.

III. Differently sedimenting populations of mitochondria, previously isolated from skeletal muscle of normal and dystrophic hamsters, in sucrose and Ficoll-sucrose density gradients.

1. Introduction.

A Ca^{++}-associated defect of oxidative phosphorylation has been observed with mitochondria isolated from skeletal muscle of BIO-14.6 dystrophic hamsters, as young as 30 days of age (139, 140). This phosphorylation defect could be ameliorated by adding, to the polarographic test system, either Mg^{++} (140-142), a chelator which binds Ca^{++} (140) or ruthenium red, an inhibitor of mitochondrial Ca^{++} transport (144). However, the Mg^{++} level in the dystrophic skeletal muscle mitochondria was normal (145). Thakar et al (144), using ruthenium red as an inhibitor of mitochondrial Ca^{++} transport, showed that the elevated Ca^{++} level in the dystrophic mitochondria was not an artifact of isolation but represented the in vivo situation. Wrogemann et al (145) suggested the possibility of different mitochondrial populations from dystrophic mitochondria, one normal population and an abnormal one(s) with a high Ca^{++} content. This concept would be more consistent with histological pictures of dystrophic muscle, which shows normal-appearing muscle areas surrounded by necrotic areas. Mezon et al (143, 146), using continuous sucrose density gradients, showed the existence of a distinct population of mitochondria, in the

dystrophic muscle, with high Ca^{++} content, that population being absent in the normal muscle. Greenawalt _et al_ (147) had already shown the high specific gravity of Ca^{++}-loaded mitochondria.

As shown in the previous section of the Results (II, 4), continuous Ficoll-sucrose density gradients yielded better quality of skeletal muscle mitochondria than continuous sucrose density gradients. Therefore, this density gradient centrifugation technique was applied to study the skeletal muscle mitochondrial populations in the disease model of dystrophic hamsters.

2. _Determination of the Ficoll-sucrose density gradient shape and its application_.

Initial experiments with continuous Ficoll-sucrose density gradients (19.5% - 30% W/V) showed subtle differences between the sedimentation pattern of normal mitochondria and Ca^{++}-loaded mitochondria (data not shown). However, the resolution obtained with such gradients was not satisfactory. Therefore, discontinuous Ficoll-sucrose density gradients were used to separate differently sedimenting populations of skeletal muscle mitochondria in order to compare the sedimentation pattern of normal and dystrophic organelles.

The method used was as follows: the skeletal muscle mitochondria were isolated as described in Methods (I,3) except that the Stock Medium contained 5 mM pyruvate-1mM

malate (146). As the mitochondrial Ca^{++} content was to be measured, substrates were added in all the media used (Stock Medium and gradient solutions) in order that the mitochondria would retain their endogenous Ca^{++} (144), the retention of Ca^{++} being an energy-dependent process (147, 185, 187). During the oxidation of the substrates, a high energy intermediate is formed and utilized for Ca^{++} uptake (187). Essentially Ca^{++}-free Ficoll ($<$ 2.8 ppm) and sucrose ($<$ 0.5 ppm) were used to prepare the gradient solutions. The discontinuous Ficoll-sucrose density gradient was prepared by layering successively on top of each other with a syringe, the following concentrations (W/V) of Ficoll solutions: 50%, 35%, 28% and 15%, containing throughout 0.25 M sucrose, 0.01 M Tris-Cl, pH 7.4, and 5 mM pyruvate - 1 mM malate, thus making four bands. After the sample had been layered on the density gradient, the centrifugation was performed using a Beckman SW-41 swinging-bucket rotor at 108,500 x G for 60 minutes. The different bands were collected, diluted with Suspending Medium and centrifuged at 16,000 x G for 10 minutes. The final pellets were resuspended and studied polarographically. The Ca^{++} content was measured in the freshly isolated mitochondria and in each mitochondrial fraction recovered from the density gradient.

A continuous sucrose density gradient (39% - 51% W/V) was also used to separate mitochondrial populations from dystrophic skeletal muscle. The reason why this type of density gradient was also used will be explained later. The method used was essentially the same as that for the discontinuous Ficoll-sucrose density gradient. The gradient was fractionated with a syringe: the mitochondrial band (40% - 45% sucrose, referred as "upper" fraction) was taken as one fraction, the rest of the gradient solution (45% - 51% sucrose, referred as "lower" fraction) as a second fraction, the pellet, if present, being considered as a third fraction.

A preliminary experiment was done to test if the discontinuous Ficoll-sucrose density gradient could be used to show additional population(s) in the dystrophic mitochondria. Tables 20 and 21 contain the results obtained.

Table 20 shows the mitochondrial distribution as well as the Ca^{++} distribution in the different bands after the centrifugation. The normal mitochondria exhibited two mitochondrial populations, one sedimenting in the "15%" Ficoll band and one in the "28%" Ficoll band. One interesting finding is the fact that the mitochondrial Ca^{++} content, expressed in nmoles Ca^{++}/mg mitochondrial protein, was the same in each of these two bands. A small Ca^{++} leakage is evident if one compares the Ca^{++} content of the freshly isolated mitochondria (referred as "Before") with that of the mitochondria recovered after the density gradient centrifugation.

TABLE 20

Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band.

Fractions	Normal			Dystrophic		
	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}
"Before"	100	25.6	100	100	178.3	100
"15%"	8	19.5	6	6	103.1	3
"28%"	45	21.6	38	49	118.9	33
"35%"	-	-	-	7	191.8	7
"50%"	-	-	-	-	-	-

Normal : 114 days old. Dystrophic : 64 days old

%T.L.M. = percentage of total layered mitochondria, measured as protein, recovered in each band.

Mitochondrial Ca^{++} : expressed in nmoles Ca^{++}/mg mitochondrial protein.

%T.M.Ca^{++}=percentage of total mitochondrial calcium recovered in each band.

"Before": these values were measured on the mitochondria prior to centrifugation at 108,500 x G for 60 minutes on discontinuous Ficoll-sucrose density gradient.

The dystrophic mitochondria were distributed into three bands, the 15%, the 28% and also the 35% Ficoll, thus showing an additional mitochondrial population which was not present in the normal preparation. This finding seemed to justify the use of the technique to study dystrophic mitochondrial populations.

Table 21 shows oxidative phosphorylation results from mitochondria recovered from the discontinuous Ficoll-sucrose density gradient.

The isolated normal mitochondria (referred as "Before", also see Figure 11A) had oxidative phosphorylation activity similar to that of the organelles recovered in the "15%" band. The same was observed with the dystrophic mitochondria (Figure 11B). In the normal case, oxidative phosphorylation was drastically decreased in the "28%" band when compared with the freshly isolated mitochondria. In the dystrophic preparation, the mitochondria recovered in the "28%" band (Figure 11C) and in the "35%" band were uncoupled. However, by adding 4.5 mM $MgCl_2$ in the polarographic Reaction Medium, the dystrophic mitochondria from the "28%" band (Figure 11D) were coupled (data in brackets), at least to the same extent as the normal mitochondria recovered in the "28%" band. This behaviour of the mitochondria is similar to that of the Ca^{++}-associated Mg^{++}-responsive defect of

TABLE 21

Oxidative phosphorylation and cytochrome oxidase activity of normal and dystrophic mitochondria before and after centrifugation on a discontinuous Ficoll-sucrose density gradient.

Fractions	Normal				Dystrophic			
	RCR	O_2 rate*	ADP/O	cyt.oxi*	RCR	O_2 rate*	ADP/O	cyt.oxi*
"Before"	5.4	217	2.49	1000	3.3(3.5)	186(198)	1.90(2.02)	996
"15%"	6.0	316	2.22	1629	3.6	157	2.00	1220
"28%"	1.9	64	1.49	1193	1.00(1.62)	57(94)	0(1.62)	991
"35%"	-	-	-	-	1.00(1.00)	33(57)	0(0)	786
"50%"	-	-	-	-	-	-	-	-

* Expressed as µmoles O_2/min/g mitochondrial protein.

Results taken from the second cycle of State 3 - State 4 respiration, with 150 - 200 µMADP.

Prior testing for cytochrome oxidase activity, the mitochondrial suspension was frozen and thawed four times.

The numbers in brackets are the values when Mg^{++} was added to the Reaction Medium prior to mitochondria.

"Before": these values were measured on the mitochondria prior to centrifugation at 108,500 x G for 60 minutes on discontinuous Ficoll-sucrose density gradient.

Figure 11: Polarographic tracings of respiration by freshly isolated normal and dystrophic mitochondria and by dystrophic mitochondria recovered from the "28%" Ficoll band.

11A: Isolated normal mitochondria.

11B: Isolated dystrophic mitochondria.

11C: Dystrophic mitochondria recovered from the 28% Ficoll band.

11D: Dystrophic mitochondria recovered from the 28% Ficoll band, with 4.5 mM $MgCl_2$ added to the Reaction Medium.

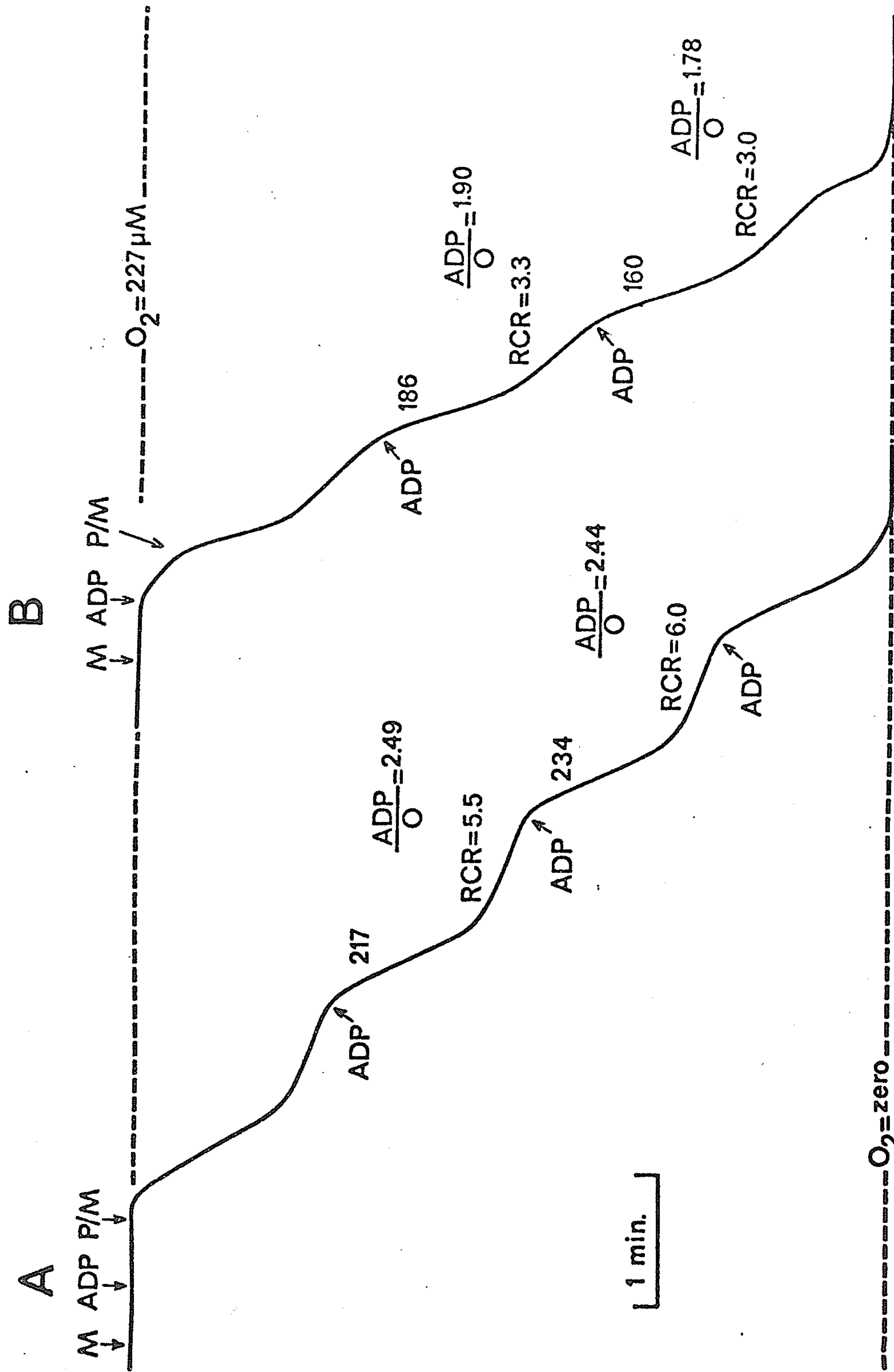


Figure 11

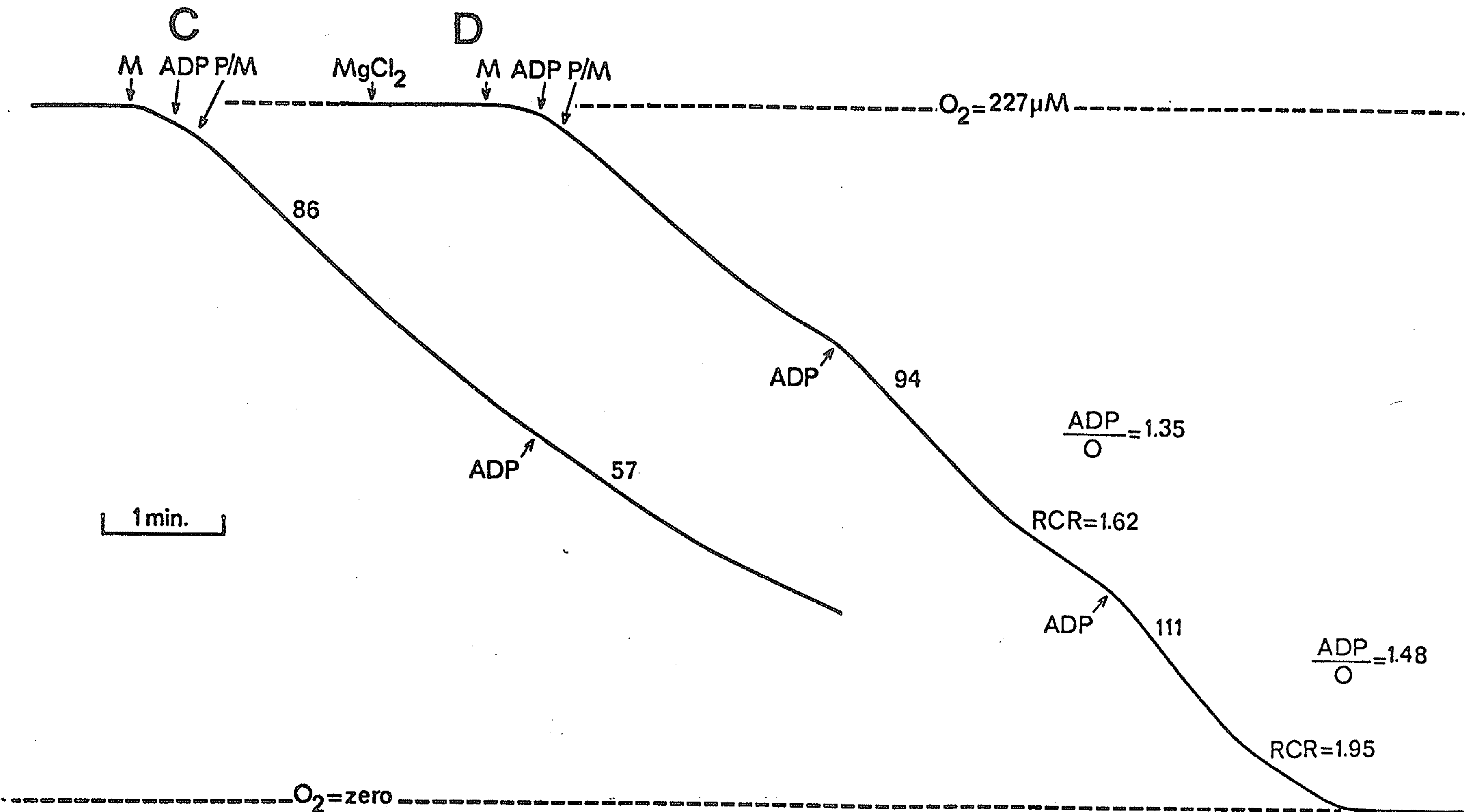


Figure 11

respiration and oxidative phosphorylation described previously in this laboratory (140, 141, 145). But Mg^{++} was not tested with the normal mitochondria recovered from the "28%" band to see if it would have improved oxidative phosphorylation. However, from previous experiments with normal mitochondria, it is known that Mg^{++} will lower rather than raise the RCR (141). Mg^{++} in fact did fail to recouple the dystrophic mitochondria recovered from the "35%" band. This failure of Mg^{++} to restore the oxidative phosphorylation was also observed when the mitochondria were isolated from very necrotic dystrophic muscle (K. Wrogemann, personal communication).

These preliminary experiments showed that this method seems to be applicable to isolate a mitochondrial fraction present in the dystrophic mitochondria but absent in the normal mitochondria.

3. Comparison of the sedimentation pattern of previously isolated normal and dystrophic skeletal muscle mitochondria subjected to discontinuous Ficoll-sucrose density gradient centrifugation.

In the previous section it was established that the discontinuous Ficoll-sucrose density gradient technique was suitable to study the difference in the sedimentation pattern of normal and dystrophic skeletal muscle mitochondria. This technique was used to study dystrophic hamsters of different ages (a) to determine at which age a different sedimentation

pattern of the dystrophic mitochondria could first be observed and (b) to correlate the Ca^{++} content of the previously isolated mitochondria with the appearance of abnormal mitochondrial populations after the density gradient centrifugation. The macroscopically observable muscle necrosis, identifiable mainly by the occurrence of white streaks, might be associated with the high Ca^{++} content of freshly isolated dystrophic skeletal muscle mitochondria.

(a) Comparative study of the sedimentation pattern of isolated normal and dystrophic mitochondria, without considering the age of the animals.

The mitochondria were isolated from the skeletal muscle of dystrophic hamsters from 32 to 133 days old.

Table 22 contains the results obtained from all the experiments done to compare the sedimentation pattern of normal mitochondria and dystrophic mitochondria without considering the age of the animals. Results for each group of age will follow thereafter.

The normal mitochondria were equally divided between the "15%" band and the "28%" band as seen before. The total mitochondrial recovery was 62%. The mitochondria in these two bands had a similar Ca^{++} content, expressed in nmoles Ca^{++}/mg mitochondrial protein; these values of Ca^{++} content are comparable to those of freshly isolated mitochondria, thus, showing that the organelles do not lose their endogenous Ca^{++}. The percentage of total mitochondrial Ca^{++} recovered in the two bands are also similar, for a total Ca^{++} recovery of 57%. Analysis of variance (ANOVA) shows no difference between the two bands for any of the parameters studied.

TABLE 22

Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band, 33 to 133 day old dystrophic hamsters.

Fractions	Normal (45 to 136 days old)			Dystrophic (32 to 133 days old)		
	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}
"Before"	100	17.3 ± 1.6^{c}	100	100	298.9 ± 78.7^{l}	100
"15%"	30 ± 4^{a}	13.8 ± 1.6^{d}	25 ± 4^{f}	10 ± 3^{h}	131.1 ± 23.1^{m}	7 ± 3^{q}
"28%"	32 ± 4^{b}	17.3 ± 2.9^{e}	32 ± 6^{g}	39 ± 4^{i}	204.5 ± 30.5^{n}	39 ± 9^{r}
"35%"	-	-	-	$*22 \pm 5^{j}$	$*298.6 \pm 64.5^{o}$	$*17 \pm 4^{s}$
"50%"	-	-	-	**4	**615	**3

Mean $\pm$ S.E.; N = 9, except *N = 5, ** N = 1

%T.L.M. = percentage of total layered mitochondria, measured as protein, recovered in each band.

Mitochondrial Ca++: expressed in nmoles Ca++/mg mitochondrial protein.

%T.M.Ca^{++} = percentage of total mitochondrial calcium recovered in each band.

"Before" : these values were measured on the mitochondria prior to centrifugation at 108, 500 x G for 60 minutes on discontinuous Ficoll-sucrose density gradient.

The superscript letters (a,b,c, etc.) identify the data compared statistically in various ways, as summarized on p. 139.

Paired t-test to compare the mean value of each measurement of normal and dystrophic preparations:

$p < 0.005$: a vs h, c vs l, d vs m, e vs n, f vs q.

N.S. : b vs i, g vs r.

Paired t-test to compare the mean value of each measurement of the dystrophic preparation. Also, the correlation value (r) shows the interdependence of these measurements:

$p < 0.005$: h vs q, with an r value of 0.975 ($\alpha < 0.01$)

N.S. : i vs r, with an r value of 0.873 ($\alpha < 0.01$)

: j vs s, with an r value of 0.372 ($\alpha > 0.1$)

Analysis of variance (ANOVA) was done to determine whether the mean value of the measurements varied from one band to the other(s), within the centrifuge tube:

a vs b, d vs e, f vs g: at $\alpha = 0.05$, the mean values of the measurements from band to band are not significantly different.

h vs i vs j, m vs n vs o, q vs r vs s: at $\alpha = 0.05$, the mean values of the measurements from band to band are different.

The sedimentation pattern of the dystrophic mitochondria differed greatly from the pattern of the normal mitochondria. There was a shift of the layered mitochondria towards the bands of high Ficoll concentrations. The dystrophic mitochondria were distributed on the gradient between the "15%", the "28%", the "35%" and the "50%" bands. In all nine experiments, there were mitochondria present in the "15%" and in the "28%" bands; however, in five cases, there was an extra mitochondrial population in the "35%" band and in one case, there was yet another extra mitochondrial population in the "50%" band. The total mitochondrial recovery was 62%, the total mitochondrial Ca^{++} recovery was 56% (weighted sums: sum of the measurements from the 4 bands taking into consideration the importance attached to each measurement).

The percentage of dystrophic mitochondria recovered in the "15%" band is significantly lower ($p < 0.005$) than the percentage recovered from the same band with the normal mitochondria (10% vs 30%). In the experiments with dystrophic animals, the percentage of mitochondrial protein recovered from the "15%" band was significantly higher than the percentage of the total mitochondrial Ca^{++} recovered; however, these two values were interdependent as shown by an r value of 0.975 ($\alpha < 0.01$).

There was no difference between the percentage of mitochondrial protein recovered from the "28%" band in the dystrophic experiment and in the normal experiment (39% vs 32%). Also there was no difference between the percentage of mitochondrial protein recovered and the percentage of total mitochondrial Ca^{++} recovered from the "28%" band; again these data were interdependent as shown by an r value of 0.873 .

The percentage of mitochondrial protein recovered in the "35%" band was not significantly different from the percentage of total mitochondrial Ca^{++} recovered; however these two values did not covary ($r = 0.372$, $\alpha > 0.1$). This might be due to too large a variation between the individual values. However, the percentages of total mitochondrial Ca^{++} recovered in the "35%" and the "50%" band seem too low. If a mitochondrial fraction sedimented into such concentrated Ficoll solutions, the amount of mitochondrial Ca^{++} present in those bands should represent a higher percentage of the total mitochondrial Ca^{++} layered. Such low Ca^{++} level might have been due to leakage of this ion from damaged mitochondria which had partly lost the ability to retain their endogenous Ca^{++}. Also as shown later, oxidative phosphorylation by the mitochondria recovered in these bands was uncoupled. There was a significant difference between the mean value, from band to band, for each of the measurements studied so far (ANOVA) in the dystrophic mitochondria.

One more aspect which has to be considered is the influence of the mitochondrial Ca^{++} content on the sedimentation pattern. By ANOVA, there was a significant difference in the mitochondrial Ca^{++} content from band to band in the dystrophic preparations. However, the amount of Ca^{++} in the dystrophic mitochondria of the "15%" band was 10 times higher than that present in the same band with normal mitochondria. The same observation was made in the "28%" band. It therefore seems that the mitochondria were not separated only according to their Ca^{++} content. This might be explained either (i) by the fact that the centrifugation was not truly isopycnic, due to a short time and/or slow speed of centrifugation with respect to the viscosities of the Ficoll solutions used or (ii) that the Ca^{++} content is not the only factor influencing the density of these mitochondria.

However, there was an overall shift towards the high Ficoll concentrations in the case of the dystrophic mitochondria. The two extra populations of mitochondria had a higher Ca^{++} content than the two normal-sedimenting populations. The percentage of dystrophic mitochondrial protein recovered in the "28%" fraction remained constant when compared with the normal preparation, while the percentage of dystrophic mitochondrial protein recovered is decreased in the "15%" fraction but is increased in the "35%" and the "50%" fractions. No mitochondrial population with normal Ca^{++} levels was observed in the dystrophic muscle.

(b) Comparative study of the sedimentation pattern of normal and dystrophic mitochondria, considering the age of the animals.

The following Tables 23, 24, 25 contain the data of each age group studied in order to correlate the occurrence of the extra band(s) with the age of the hamsters.

Table 23 contains the results obtained from three different experiments using 32, 35 and 36 day old dystrophic hamsters. The distribution of the normal mitochondria between the "15%" and the "28%" bands corresponded to the results presented in Table 22, for a total protein recovery of 65%, whereas the percentage of total mitochondrial Ca^{++} recovered from the gradient was only 44%.

Several white streaks were observable in the muscle mass of these dystrophic hamsters. The sedimentation pattern of the dystrophic mitochondria contained two extra mitochondrial populations. The percentage of total mitochondria recovered was 66%, the percentage of total mitochondrial Ca^{++} recovered was 43% (weighted sums). The observations given for the "15%" and the "28%" bands in Table 22 also applied in this case. Of the three experiments, two presented a mitochondrial population in the "35%" band. In one experiment, a small mitochondrial population was present in the "50%" band. Ca^{++} leakage is suggested by lower values for the percentages of total mitochondrial Ca^{++} recovered from

TABLE 23

Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band, 32 - 36 day old dystrophic hamsters.

Fractions	Normal (45 - 49 days old)			Dystrophic (32 - 36 days old)		
	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}
"Before"	100	22.1 ± 4.8^{c}	100	100	473.8 ± 188.9^{l}	100
"15%"	32 ± 10^{a}	15.0 ± 3.5^{d}	24 ± 10^{f}	7 ± 3^{h}	140.4 ± 42.6^{m}	4 ± 3^{q}
"28%"	33 ± 11^{b}	14.3 ± 1.7^{e}	20 ± 5^{g}	39 ± 8^{i}	202.2 ± 55.6^{n}	31 ± 22^{r}
"35%"	-	-	-	$^{*}28^{j}$	$^{*}209.6^{o}$	$^{*}10^{s}$
"50%"	-	-	-	** 4	**615.3	** 3

Mean $\pm$ S.E.; N = 3 except * N = 2, ** N = 1

% T.L.M. = percentage of total layered mitochondria, measured as protein, recovered in each band.

Mitochondrial Ca^{++}: expressed in nmoles Ca^{++}/mg mitochondrial protein.

%T.M.Ca^{++} = percentage of total mitochondrial calcium recovered in each band.

"Before" : these values were measured on the mitochondria prior to centrifugation at 108, 500 x G for 60 minutes on discontinuous Ficoll-sucrose density gradient.

Paired t-test : $p < 0.05$: a vs h; $p < 0.005$: c vs l; $p < 0.025$: d vs m, e vs n.
N.S. : b vs i, f vs q, g vs r, h vs q, i vs r.

ANOVA : a vs b, d vs e, f vs g, m vs n vs O, q vs r vs s: no significant difference
h vs i vs j : at $\alpha = 0.05$, the mean values of these measurements are different.

these two bands, when compared to the percentages of total mitochondrial protein recovered. Ca^{++} retention by mitochondria is an energy-dependent process (147, 185, 187); these mitochondria, at least in the "35%" band were uncoupled probably due to the centrifugation procedure, as shown later in Table 26. If the energy production was impaired, the Ca^{++} could have leaked out. The mitochondrial Ca^{++} content did not increase significantly from band to band.

Table 24 contains the results of four dystrophic hamsters between 71-74 days old. Some white streaks were observable in the muscle mass of these dystrophic hamsters.

Nothing was unusual in the sedimentation pattern of the normal mitochondria. The percentage of total layered mitochondria recovered was 62%, the percentage of total mitochondrial Ca^{++} recovered was 58%.

In the dystrophic mitochondria, one additional mitochondrial population was observed in three of the four cases. The percentage of total layered mitochondria recovered was similar to the percentage of total mitochondrial Ca^{++} recovered, i.e. 58% and 62% (weighted sums) respectively. In each of these three bands, the r values showed that both percentages were closely interdependent ($\alpha < 0.05$), these r values being 0.862, 0.910, 0.995 respectively for the "15%", the "28%" and the "35%" bands. By ANOVA, it was shown that the mitochondrial Ca^{++} content increased significantly from band to band ($\alpha < 0.05$).

TABLE 24

Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band, 71 - 74 day old dystrophic hamsters.

Fractions	Normal (69 - 72 days old)			Dystrophic (71 - 74 days old)		
	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}
"Before"	100	16.1 ± 1.2^{c}	100	100	272.9 ± 64.2^{k}	100
"15%"	27 ± 4^{a}	15.0 ± 1.9^{d}	25 ± 4^{f}	7 ± 2^{h}	155.7 ± 36.7^{l}	4 ± 1^{o}
"28%"	35 ± 3^{b}	16.0 ± 3.0^{e}	33 ± 6^{g}	37 ± 4^{i}	263.9 ± 24.4^{m}	42 ± 11^{p}
"35%"	-	-	-	$*19 \pm 6^{j}$	$*358.0 \pm 61.6^{n}$	$*21 \pm 4^{q}$
"50%"	-	-	-	-	-	-

Mean $\pm$ S.E.; N = 4, except * N = 3.

%T.L.M. = percentage of total layered mitochondria, measured as protein, recovered in each band.

Mitochondrial Ca^{++} : expressed in nmoles Ca^{++}/mg mitochondrial protein.

%T.M.Ca^{++}= percentage of total mitochondrial calcium recovered in each band.

"Before" : these values were measured on the mitochondria prior to centrifugation at 108,500 x G 60 minutes on discontinuous Ficoll-sucrose density gradient.

Paired t-test: $p < 0.005$: a vs h, c vs k, d vs l, e vs m, f vs O, g vs p.
$p < 0.05$: b vs i, g vs p, i vs p, j vs q.
N.S. : h vs O.

ANOVA : a vs b, d vs e, f vs g : no significant difference
h vs i vs j, l vs m vs n, o vs p vs q : at $\alpha = 0.05$, the mean values of these measurements are different.

Table 25 contains the results of two experiments with dystrophic hamsters, 130 and 133 days old. With these hamsters, few or no white streaks were observed indicating very little muscle necrosis.

The sedimentation pattern of the normal mitochondria was consistent with the one presented in Table 22. The percentage of mitochondrial protein recovered was 60%, the percentage of total mitochondrial Ca^{++} recovered was 76%. The mitochondrial Ca^{++} content, in nmoles Ca^{++} per mg mitochondrial protein, was two times higher in the 28% band than in the freshly isolated organelles. This might reflect a higher Ca^{++} content in these mitochondria or else that they picked up some surrounding Ca^{++} during the centrifugation; no conclusion can be given on that point.

The dystrophic mitochondria sedimented also in only two bands. The percentage of the total mitochondrial protein recovered was 67% and the percentage of total mitochondrial Ca^{++} recovered was 65%. Both the percentages of total mitochondrial Ca^{++} recovered and the mitochondrial Ca^{++} content were higher in the "28%" band than in the "15%" band. However, the mitochondrial Ca^{++} content in the "28%" was similar to that of freshly isolated dystrophic mitochondria. Thus, at this disease stage, there was no difference between the sedimentation pattern of the normal and the dystrophic mitochondria. Even if the Ca^{++} content in the dystrophic mitochondria

TABLE 25

Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered from each band; 130 - 133 day old dystrophic hamsters.

Fractions	Normal (133,136 days old)			Dystrophic (130,133 days old)		
	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}
"Before"	100	12.3	100	100	88.6	100
"15%"	34	9.3	27	24	68.1	19
"28%"	26	24.6	49	43	89.1	46
"35%"	-	-	-	-	-	-
"50%"	-	-	-	-	-	-

Means ; N = 2

%T.L.M. = percentage of total layered mitochondria, measured as protein, recovered in each band.

Mitochondrial Ca^{++} : expressed in nmoles Ca^{++}/mg mitochondrial protein.

%T.M.Ca^{++}= percentage of total mitochondrial calcium recovered in each band.

"Before" " these values were measured on the mitochondria prior to centrifugation at 108, 500 x G for 60 minutes on discontinuous Ficoll-sucrose density gradient.

was higher than that in the normal mitochondria, this was not sufficient for the separation of the abnormal mitochondrial population.

The next Table (Table 26) gives the oxidative phosphorylation values. The RCR's, O_2 rates and ADP/O ratios of the freshly isolated normal mitochondria agreed with those from normal skeletal muscle mitochondria obtained previously in this laboratory. Oxidative phosphorylation activity by the dystrophic mitochondria was low, probably due to the fact that several of the individual dystrophic mitochondrial preparations were uncoupled or "poorly" coupled. In the "15%" band, oxidative phosphorylation by the normal mitochondria was less active than that by freshly isolated organelles, while dystrophic mitochondria in this band, on the other hand, had an activity comparable to that of the freshly isolated organelles. There were no differences in the various parameters measured, between the normal and the dystrophic mitochondria recovered from the "15%" band. The mitochondria in the "28%" band, both normal and dystrophic, and in the "35%" band were almost or completely uncoupled, with very low O_2 rates; this might have been due to the centrifugation procedure itself since the viscosities between different bands varied abruptly (Table 27). The effect of Mg^{++} could not be tested, due to the small amount of mitochondrial material recovered.

TABLE 26

Oxidative phosphorylation by normal and dystrophic mitochondria before and after recovery from discontinuous Ficoll-sucrose density gradients.

	Normal			Dystrophic		
	RCR	O_2 rate	ADP/O	RCR	O_2 rate	ADP/O
"Before"	5.3±0.6[a]	256±13[d]	2.12±0.10[g]	3.0±0.8[j]	144±40[m]	1.30±0.34[p]
"15%"	*3.5±0.2[b]	*142±7[e]	*2.04±0.10[h]	**2.7±0.7[k]	**116±35[n]	**1.32±0.54[q]
"28%"	*1.4±0.2[c]	* 49±7[f]	*0.59±0.29[i]	+1.1±0.1[l]	+21±9[o]	+0.21±0.21[r]
"35%"	-	-	-	‡ 1±0	‡ 8±1	‡ 0
"50%"	-	-	-	-	-	-

Mean ± S.E.; N = 9, except for * N=8, **N=5, +N=7, ‡ N=3

O_2 rate expressed as µmoles O_2/min/g mitochondrial protein; results taken from the second cycle of State 3 - State 4 respiration.

Centrifugation at 108,500 x G for 60 minutes.

Unpaired t-test: $p < 0.025$: a vs j, g vs p, f vs 0.
$p < 0.01$: d vs m
N.S. : b vs k, c vs l, e vs n, h vs q, i vs r.

TABLE 27

Densities and viscosities of aqueous Ficoll and sucrose solutions, at different concentrations.

% (W/W)	*Ficoll		**Sucrose	
	Density, g/ml	Viscosity, centipoises	Density, g/ml	Viscosity, centipoises
15	1.053	20.97	1.062	2.51
28	1.098	117.08	1.122	4.80
35	1.123	271.21	1.161	8.24
50	1.175	1488.84	1.236	33.16

* Data at 4°C , from (148)

** Data at 5°C , from (149)

The sedimentation pattern of the normal skeletal muscle always showed two different populations, divided between the upper two arbitrarily chosen Ficoll concentrations. The percentages of the mitochondrial Ca^{++} recovered from each band were consistent with the percentages of mitochondrial protein recovered from the same band. This implies that the Ca^{++} leakage from the normal mitochondria during the centrifugation procedure on the discontinuous Ficoll-sucrose density gradient was relatively small, approximately 20% (cf. Table 22). Also the Ca^{++} content of the mitochondria recovered was the same in both the "15%" and "28%" bands.

The sedimentation pattern of the dystrophic mitochondria differed from the pattern of the normal mitochondria. With older hamsters with little muscle necrosis, the sedimentation pattern was similar to that obtained with normal hamsters, except that the dystrophic mitochondrial Ca^{++} content was higher than normal, even when the two populations were sedimented in bands of the same density. With younger hamsters, 32 to 74 days old, in which muscle necrosis was evident by the presence of white streaks (140, 141, 145) and by the generally pale appearance of the muscles, the sedimentation pattern did vary according to the degree of the muscle disease. One or two additional dystrophic mitochondrial populations were observed. The amount of dystrophic mitochondria recovered in the "15%" band was lower than in normals. In the "28%" band, there was no significant difference between the percentage

of total mitochondrial protein recovered and the percentage of total mitochondrial Ca^{++} recovered, when compared with the normal mitochondria. Abnormal mitochondrial fractions appeared in the "35%" and in the "50%" bands. The mitochondrial Ca^{++} content was higher in these two bands than that observed in the "15%" and in the "28%" bands. In none of these fractions, was a normal Ca^{++} content observed.

(c) Relation between the Ca^{++} content of the freshly isolated mitochondria and the number of populations obtained after discontinuous Ficoll-sucrose density gradient centrifugation.

In the previous section, a difference in the sedimentation pattern between normal and dystrophic mitochondria was observed. An attempt to correlate the Ca^{++} content of the freshly isolated dystrophic mitochondria and the presence of these extra mitochondrial populations is shown in Table 28.

The data indicate a close association ($r = 0.908$) between the mitochondrial Ca^{++} content and the number of mitochondrial populations obtained. The mitochondrial Ca^{++} content also paralleled the stage of the muscle necrosis, estimated from the extent of the white streaking. It has been shown (145) that the Ca^{++} content in the white streaks is 13 times higher than in the normal-appearing dystrophic muscle. By using a mitochondrial Ca^{++} transport inhibitor in the isolation medium (144) it has been shown that the high Ca^{++} levels in dystrophic mitochondria represented the *in vivo* situation. Therefore, the mitochondrial distribution shift towards high Ficoll concen-

TABLE 28

Calcium content of freshly isolated dystrophic mitochondria and number of dystrophic mitochondrial populations separated on discontinuous Ficoll-sucrose density gradients.

Age, days	nmoles Ca^{++}/mg mitochondrial protein	number of mitochondrial populations
32	130.7	2
35	508.5	3
36	782.3	4
71	165.7	3
72	384.7	3
73	383.8	3
74	157.7	2
130	88.9	2
133	88.2	2

Correlation factor (r) of 0.9079, ($p < 0.001$) between the Ca^{++} content, in nmoles Ca^{++} /mg mitochondrial protein, of freshly isolated dystrophic mitochondria and the number of dystrophic mitochondrial populations obtained on discontinuous Ficoll-sucrose density gradients.

trations appears to be related to the disease stage and the endogenous mitochondrial Ca^{++} *in situ*.

(d) Discussion.

The centrifugation procedure used was probably not isopycnic. Mezon *et al* (146) used the same centrifugation procedure with sucrose density gradients and reported that they achieved isopycnic conditions. However, the viscosities of the Ficoll solutions were much higher than the sucrose solutions (Table 27), and the separation obtained might therefore not be isopycnic. No attempt was made to determine the centrifugation conditions for a true isopycnic centrifugation with discontinuous Ficoll-sucrose density gradients.

From the separation of the mitochondria obtained, it appears that the Ca^{++} content is not the only factor affecting the density of the mitochondria. Lynn *et al* (152) have shown that Ca^{++} changes reversibly the semi-permeable properties of mitochondrial membranes so as to permit rapid equilibrium of sucrose and salts. Thus, in the present work, it seems probable that Ca^{++} might have affected mitochondrial density through influencing mitochondrial membrane permeability.

4. Comparison of the sedimentation pattern of previously isolated normal and dystrophic skeletal muscle mitochondria subjected to continuous sucrose density gradient centrifugation.

A continuous sucrose density gradient (39% - 51% W/V) also was used to compare the sedimentation pattern of skeletal muscle mitochondria from normal and dystrophic hamsters. Mezon *et al* (146) have reported an abnormal mitochondrial fraction with a high Ca^{++} content in each dystrophic animal studied, regardless of the extent of Ca^{++} elevation of the freshly isolated dystrophic mitochondria. Thus, this technique seemed to be a more sensitive means to separate the abnormal mitochondrial populations than the discontinuous Ficoll-sucrose density gradient which did not detect in the present work, an abnormal mitochondrial population in every dystrophic animal studied. Therefore, the sucrose density gradient technique was applied to skeletal muscle mitochondria from dystrophic hamsters of two age groups, 18-20 and 67-82 days old. Table 29 shows the results obtained with 67-82 day old dystrophic hamsters.

The normal mitochondria were mainly restricted to an upper fraction of the density gradient in the range of 40% - 45% sucrose where 63% of the total layered mitochondria protein was found. The percentage of total layered

TABLE 29

Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered in a sucrose density gradient; 67-82 day old dystrophic hamsters.

Fractions	*Normal (65-71 days old)			**Dystrophic (67-82 days old)		
	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}
"Before"	100	22.7 ± 7.0^{c}	100	100	225.0 ± 73.1^{j}	100
"Upper"	63 ± 3^{a}	8.3 ± 1.6^{d}	25 ± 3^{f}	36 ± 2^{h}	22.8 ± 4.0^{k}	6 ± 2^{m}
"Lower"	15 ± 3^{b}	14.1 ± 7.6^{e}	9 ± 5^{g}	17 ± 5^{i}	37.4 ± 11.4^{l}	2 ± 0.4^{n}
"Pellet"	-	-	-	10 ± 2	567.8 ± 109.6	28 ± 4

Mean $\pm$ S.E. ; * N = 3, ** N = 5

%T.L.M. = percentage of total layered mitochondria, measured as protein, recovered in each fraction

Mitochondrial Ca^{++}= expressed in nmoles Ca^{++}/mg mitochondrial protein

%T.M.Ca^{++}= percentage of total mitochondrial calcium recovered in each fraction

"Before" = these values were measured on the mitochondria prior to centrifugation at 108,500 x G for 60 minutes on a linear sucrose density gradient(39% - 51% W/V) containing 10mM Tris-Cl, pH7.2, and 5mM pyruvate - 1mM malate.

"Upper" = 40% - 45% (W/V) sucrose zone;

"Lower" = 45% - 51% (W/V) sucrose zone.

Unpaired t-test : $p < 0.05$: c vs j; $p < 0.005$: a vs h, f vs m; $p < 0.025$: d vs k.
N.S. : b vs i, e vs l, g vs n.

This table was provided to the author by B.J. Mezon.

mitochondria recovered was 78%; the percentage of total mitochondrial Ca^{++} recovered was 34%. The lack of relation between these two percentages was also observable in the mitochondrial Ca^{++} content. The isolated mitochondria contained 22.7 ± 7 nmoles Ca^{++} per mg mitochondrial protein whereas the mitochondria recovered in the 40% - 45% sucrose contained only 8.3 ± 1.6 nmoles Ca^{++} per mg mitochondrial protein, thus showing a Ca^{++} leakage of 63%. In contrast, in the discontinuous Ficoll-sucrose density gradients experiments, the Ca^{++} leakage was approximately 20% (Table 22). Obviously the mitochondria in the Ficoll-sucrose density gradient retained more of their endogenous Ca^{++} than in the sucrose density gradient. Even when the oxidative phosphorylation parameters were low, especially in the 28% Ficoll band, the Ficoll-sucrose density gradient did not subject the mitochondria to the osmotic shock reported in the sucrose density gradient (53).

The dystrophic mitochondria show a different sedimentation pattern from the normal organelles (Table 29). The Ca^{++} content of the freshly isolated dystrophic mitochondria was significantly higher than in the normal mitochondria. The percentage of total layered mitochondria recovered in the 40% - 45% sucrose band was significantly lower than the percentage recovered from the same band ($p < 0.005$) of the normal mitochondria. However, the

percentage of mitochondrial protein recovered in the lower fraction (45% - 51% sucrose) was the same in both cases. There was always a fraction of the dystrophic mitochondria which sedimented through the whole gradient and was found as a "pellet" at the bottom of the tube, representing about 10% of the layered mitochondrial protein. The percentage of the total layered mitochondria recovered was 63%, whereas the percentage of total mitochondrial Ca^{++} recovered was 36%. The mitochondrial Ca^{++} content was significantly higher in the dystrophic mitochondria of the "upper" fraction when compared with normal mitochondria, thus showing a higher Ca^{++} level in the normally-sedimenting dystrophic mitochondria. The Ca^{++} content in the pellet was very high and represented a clearly abnormal fraction not present in the normal muscle. The dystrophic mitochondria sedimenting in the 40% - 45% sucrose band contained three times more Ca^{++} than the normal mitochondria sedimenting in the same band, thus showing that the Ca^{++} content was not the only factor determining the increased density of the mitochondria, as also shown in the discontinuous Ficoll-sucrose density gradient studies described above.

The same sucrose density gradient technique was also used to study the mitochondrial populations of very young dystrophic hamsters (18-20 days old), where no signs

of muscle necrosis were observable. The purpose of the experiment was to determine how early the abnormal mitochondrial population(s) observed in the dystrophic muscle could be detected in the disease.

As shown in Table 30, three normal hamster (18-23 days old) were studied. The mitochondria recovered in the "upper" fraction (40% - 45% sucrose) had values similar to those presented in Table 29. However, in normal, as well as in the dystrophic animals, there was a small mitochondrial pellet with high Ca^{++} contents. Such a pellet had never been obtained before from isolated normal mitochondria. The percentage of total layered mitochondria recovered was 59%; the percentage of total mitochondrial Ca^{++} recovered was 22% (weighted sums). The dystrophic mitochondria had a slightly different sedimentation pattern than the ones presented in Table 29. The mitochondrial pellets (which represented only 3% of the total mitochondrial protein layered), with a high Ca^{++} content were present in all the three animals studied. The percentage of total layered mitochondria recovered was 48%, the percentage of total mitochondrial Ca^{++} recovered was 33%.

Using very young hamsters, the difference between the sedimentation pattern of normal and dystrophic mitochondria seen earlier was not observable. The mitochondrial Ca^{++} content was similar in the freshly isolated normal and

TABLE 30

Percentages of total layered mitochondria, mitochondrial calcium content and percentages of total mitochondrial calcium recovered in a sucrose density gradient; 18-20 day old dystrophic hamsters.

Fractions	Normal (18-23 days old)			Dystrophic (18-20 days old)		
	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}	%T.L.M.	Mitochondrial Ca^{++}	%T.M.Ca^{++}
"Before"	100	70.7±8.3	100	100	83.0±10.1	100
"Upper"	58±8	19.7±3.3	16±3	45±10	45.3±14.3	21±1
"Lower"	-	-	-	-	-	-
"Pellet"	*2	*598	*9	3±1	398.3±36.9	12±2

Mean ± S.E. ; N = 3, except *N = 2

%T.L.M. : percentage of total layered mitochondria, measured as protein, recovered in each fraction

Mitochondrial Ca^{++}: expressed in nmoles Ca^{++}/mg mitochondrial protein.

%T.M.Ca^{++} : percentage of total mitochondrial calcium recovered in each band.

"Before" "Upper", "Lower" : see explanations in Table 29.

dystrophic mitochondria. From these results it seems that either (a) at that age (18-20 days old), there is no significant difference in the sedimentation pattern between the normal and the dystrophic mitochondria, or (b) the pellet observed in the normal animals was an artifact of density gradient centrifugation. The Ca^{++} contents of the isolated normal mitochondria and the isolated dystrophic mitochondria being similar, it is possible that they have a similar sedimentation pattern, the pellet observed not being specific to the dystrophic mitochondria. It is known, in growing children, that there is a net retention of Ca^{++} necessary for skeletal growth (153), the Ca^{++} level being elevated in the plasma and in the interstitial fluid. A similar situation might be found in the growing hamsters. The high mitochondrial Ca^{++} content found in the normal skeletal muscle might be explained either by an artifact of the isolation procedure, in which the mitochondria accumulated the Ca^{++} present in the interstitial fluid of the muscle tissue or it may represent a true _in vivo_ situation, i.e. high endogenous Ca^{++} levels in some of the organelles. Therefore, a series of experiments was done using very young normal hamsters (11 to 31 days old) to examine the relation between the age of normal hamsters and the mitochondrial Ca^{++} content (Table 31). The results, compared to those in other Tables presented, show that the mitochondrial Ca^{++} content is higher in very young normal

TABLE 31

Mitochondrial calcium content and skeletal muscle calcium content at different ages of normal hamsters.

Age (days old)	Skeletal muscle Ca^{++} content (μmoles Ca^{++}/g wet weight muscle)	Mitochondrial Ca^{++} content (nmoles Ca^{++}/mg mitochondrial protein)
11	2.62	148.9
19	2.34	54.1
22	1.85	64.9
31	1.60	21.5

hamsters than in animals of 30 days old or older. The mitochondrial Ca^{++} content reported for normal "adult" hamsters is 25.8 ± 2.3 nmoles Ca^{++}/mg protein (145) and the skeletal muscle Ca^{++} content, 1.49 ± 0.03 μmoles Ca^{++}/g wet weight muscle (145).

Since such normal mitochondria had a sedimentation pattern similar to the one observed with artificially Ca^{++}-loaded mitochondria (146), it cannot be excluded that the high Ca^{++} content in the mitochondria isolated from young dystrophic hamsters may be due to an isolation artifact. On the other hand, it might represent the *in vivo* situation.

Therefore, at that stage, the sucrose density gradient technique was unable to detect, at least as applied in this study, a difference in the sedimentation pattern between the normal and the dystrophic skeletal muscle mitochondria, unless some modifications in the mitochondria isolation procedure and in the density gradient centrifugation technique are made. For example, one might use ruthenium red (a mitochondrial Ca^{++} transport inhibitor) together with a substrate (to allow the mitochondria to retain their endogenous Ca^{++}) in the isolation media. It might then be possible to determine if the Ca^{++} content in the mitochondria isolated from normal and dystrophic skeletal muscle of very young hamsters is an artifact of isolation or represents the *in vivo* situation. Also ruthenium red and substrate both should be

added in the gradient solutions for the same reason. However, it is known that ruthenium red binds to the mitochondria (144) and therefore the mitochondrial density is increased (Mezon *et al*, unpublished data). Thus, the sucrose density gradient centrifugation technique would have to be redesigned to account for the increase of the mitochondrial density. For that reason, this problem was not pursued further in this study.

5. Discussion.

The mitochondria separated on a Ficoll-sucrose density gradient had a better retention of endogenous Ca^{++} than those separated on a sucrose density gradient, as shown by similar Ca^{++} levels in the freshly isolated mitochondria and those recovered from the gradient solutions. Using both types of density gradients, it was shown that the Ca^{++} content cannot be the only factor causing the increased density of the mitochondria. Dystrophic mitochondria, with up to 10 times more Ca^{++} than normal mitochondria, sedimented in the same range of density (Table 22). The abnormal populations of mitochondria, having even higher Ca^{++} content than the normal sedimenting dystrophic mitochondria, probably were separated according to Ca^{++} content as well as other Ca^{++}-associated factors, i.e. altered mitochondrial membrane permeability. The Ca^{++} might effect the permeability of the

mitochondrial membranes by rendering the membrane more permeable to some solutes (152). These experiments do not demonstrate if such changes in the membrane permeability paralleled the increase in the Ca^{++} concentration, or if this change happens beyond a certain threshold of Ca^{++} concentration. Both types of gradients were useful to separate abnormal mitochondrial fractions from dystrophic skeletal muscle. The Ficoll-sucrose density gradient separated one or two abnormal mitochondrial fractions with high Ca^{++} content. This type of density gradient did not show an abnormality in the sedimentation pattern each time, the occurrence of the abnormal mitochondrial population being related to the Ca^{++} content of the freshly isolated mitochondria. The mitochondrial Ca^{++} content seemed to be related to the stage to which the muscle disease had progressed as shown macroscopically by the presence and extent of white streaking. The sucrose density gradients separated abnormal mitochondrial fractions, with high Ca^{++} content according to the Ca^{++} level of the freshly isolated dystrophic organelles ($r = 0.936, \alpha < 0.05$). This method therefore appears to be more sensitive than the Ficoll-sucrose density gradient to separate abnormal mitochondria from the dystrophic skeletal muscle with less muscle necrosis.

The presence of abnormal mitochondria in dystrophic skeletal muscle is consistent with the histological picture of this muscle disease. The presence of mitochondria with

high Ca^{++} content could be due to some changes in the Ca^{++} level of the intracellular milieu. The excess of Ca^{++} might be accumulate by the mitochondria in order to keep the intracellular Ca^{++} level within a physiological range, which is around 10^{-6} M (145). That possibility will be explored later in the General Discussion.

V. GENERAL DISCUSSION

I. Density gradient centrifugation.

I have demonstrated that it is possible to isolate mitochondria directly from a pigeon heart homogenate by a single step density gradient centrifugation in a Ficoll-sucrose density gradient. Oxidative phosphorylation measurements on these mitochondria indicated that they were functionally intact, as shown by high RCR's (177, 180), and largely free of any contamination, as shown by high O_2 rates and by electron micrographs. Using an SZ-14 reorienting density gradient zonal rotor, large scale isolation of mitochondria was achieved.

A Ficoll-sucrose density gradient was far superior to a sucrose density gradient to purify previously isolated rat liver mitochondria. The mitochondria recovered from the Ficoll-sucrose density gradient, after rate zonal centrifugation, were functionally similar to the freshly isolated organelles, as also shown by Dimino and Hoch (83) and by Grimwood and Wagner (84).

Hamster heart mitochondria could be subjected to rate zonal centrifugation either in a sucrose density gradient or in a Ficoll-sucrose density gradient, without losing their phosphorylation ability. The mitochondria recovered from both density gradients had similar oxidative

phosphorylation activity to that of freshly isolated organelles, either in the presence or in the absence of 0.1% bovine plasma albumin. Pigeon heart mitochondria, exhibiting good oxidative phosphorylation activity, were also recovered from sucrose-mannitol density gradients and from sucrose density gradients.

Previously isolated hamster skeletal muscle mitochondria subjected to rate zonal centrifugation and to isopycnic centrifugation had a higher oxidative phosphorylation activity after recovery from a Ficoll-sucrose density gradient than from a sucrose density gradient.

To the best of my knowledge, this is the first report of coupled heart mitochondria obtained after rate zonal centrifugation in a sucrose density gradient and in a Ficoll-sucrose density gradient, and of coupled skeletal muscle mitochondria after zonal centrifugation (rate zonal and isopycnic) in a Ficoll-sucrose density gradient.

II. Muscular dystrophy.

1. Discussion of and conclusion on the results presented above in Results III.

This is the first approach to separate mitochondrial populations, previously isolated from normal and dystrophic skeletal muscle, by centrifugation on a discontinuous Ficoll-sucrose density gradient. However,

Mezon *et al* (146) have shown the presence of one "extra" mitochondrial population from dystrophic skeletal muscle by centrifugation on a sucrose density gradient. By using a discontinuous Ficoll-sucrose density gradient, it was shown in my work that one or two "extra" mitochondrial population(s) could be isolated from dystrophic skeletal muscle, the appearance of this(these) mitochondrial population(s) being related to the stage of the disease as observed macroscopically by the presence of white streaks in the muscle. The presence of additional mitochondrial populations in the dystrophic skeletal muscle was shown *in vitro* and one must be careful in applying these results to the *in vivo* situation. The mitochondria isolated from the dystrophic skeletal muscle and the fractions recovered from the density gradient contained high Ca^{++} levels. However, Thakar *et al* (144) by using a mitochondrial Ca^{++} transport inhibitor showed that the Ca^{++} content of dystrophic mitochondria was indeed higher *in vivo* than that of the normal organelles. Therefore, the higher Ca^{++} content, reported in the results, in dystrophic mitochondria as compared with normal mitochondria in all likelihood reflects the *in vivo* situation.

With both types of density gradients, the sedimentation pattern of the dystrophic mitochondria was different from the sedimentation pattern of the normal mitochondria, the "extra" mitochondrial population(s)

having a high Ca^{++} content. This separation of dystrophic mitochondria agreed well with the histological picture of dystrophic muscle which shows, side by side, normal-appearing muscle areas and necrotic areas. However, the experiments presented here did not directly prove that the "extra" mitochondrial populations were derived from the white streaks.

Wrogemann et al (145) showed an inverse relation between the coupling of oxidative phosphorylation and the mitochondrial Ca^{++} content. From the data presented in this study, it appears that the dystrophic mitochondria recovered from the "15%", the "28%" and the "35%" bands should be coupled, on a Ca^{++} level basis, in the polarographic test system by addition of Mg^{++} to the Reaction Medium. Therefore, in vivo, with the ionic environment in the cell, these mitochondria might also be coupled. With older hamsters, the sedimentation pattern showed only two populations in a Ficoll-sucrose density gradient and the mitochondrial Ca^{++} content was lower than that in younger dystrophic hamsters (32-74 days old). Further work with these mitochondria might result in an improvement in their oxidative phosphorylation activity. At this age (130 days old), the mitochondria either did not retain their Ca^{++}, lost their ability to accumulate Ca^{++} or the intracellular Ca^{++} level was declining. Very likely, the cells with high Ca^{++} content at young ages were already dead.

2. Abnormalities of energy levels in the cell in relation to Ca^{++} accumulation by mitochondria.

In the absence of exogenous ATP, the Ca^{++} uptake by the mitochondria requires electron transport (186) and the accumulation of Ca^{++} to a variable extent replaces and is alternative to ATP formation by oxidative phosphorylation. During substrate oxidation, a high-energy intermediate has been postulated that would be utilized for Ca^{++} uptake. This process apparently competes with ADP as an energy acceptor and thus prevents the phosphorylation of ADP (187). Such uncoupling by Ca^{++} is, however, completely reversible if Ca^{++} is not present at too high a concentration (185). This partial uncoupling of oxidative phosphorylation might result in subnormal levels of ATP and creatine phosphate depending on their rates of utilization. Dhalla et al (188) have shown, in 215 day old dystrophic hamsters that the creatine phosphate and ATP levels, and the ATP/ADP and ATP/AMP ratios were decreased in the dystrophic skeletal muscle when compared to normal skeletal muscle. Changes in the high energy phosphate stores may therefore indicate a defect in both the processes of energy production and of energy utilization (188).

Caulfield (189) has shown that during the process of lesion formation in the striated muscle of dystrophic hamsters, mineral deposits containing Ca^{++} and phosphate appear in the mitochondria after breakdown of the plasma

membrane, indicating an influx of high Ca^{++} concentrations from the interstitial fluid into the cell. The mineral deposits observable in electron micrographs appear as precipitate of calcium phosphate salts, without any evidence of crystal formation (147, 187). The mitochondria accumulate the Ca^{++} by forming an "inert pool" of calcium phosphate. Lehninger (187) described at least three stages in the energy-linked deposition of Ca^{++}. In dystrophy, the Ca^{++} accumulation by mitochondria might not be a primary defect of this genetic disease but a response to an increase of Ca^{++} levels within the cell. Because the interstitial fluid contains 1000 times more free Ca^{++} than the intracellular milieu (185), cell membrane damage might be responsible for the increased Ca^{++} level within the cell.

3. Membrane defect in muscular dystrophy.

Membrane defects have been studied and postulated to be an early defect in muscular dystrophy.

Alteration of mitochondrial membranes have been shown (190); the accelerated loss of soluble protein from mitochondria in dystrophic muscle seems to be a response to the abnormal permeability of the mitochondrial membrane and is consistent with an altered microscopic appearance (131).

Martonosi (191) has shown that the uptake of Ca^{++} by the sarcoplasmic reticulum of dystrophic mouse muscle was significantly lower than normal. Peter and Worsfold

(123) have shown that the Ca^{++} accumulation capacity was generally lower in the sarcoplasmic reticulum of severely affected human muscle than in normal or in sarcoplasmic reticulum from muscle less affected by Duchenne dystrophy; the rate of Ca^{++} uptake by the sarcoplasmic reticulum also tended to be lower than normal (123). Caulfield (189, 192) has shown a certain degree of distension of the sarcotubular system in dystrophic hamster muscle. Low initial and total Ca^{++} uptake, low ATPase activity and normal efficiency have been reported by Samaha and Gergely (193) in fragmented sarcoplasmic reticulum from the muscle of patients with Duchenne dystrophy. Sylvester (194) reported that Ca^{++} was accumulated in dystrophic sarcoplasmic reticulum at a faster initial rate than in normal sarcoplasmic reticulum, that the net Ca^{++} uptake was normal or slightly higher than normal; no decrease in the ability of dystrophic sarcoplasmic reticulum to accumulate Ca^{++} was found.

Erythrocyte ghost preparations, showing a characteristic transport adenosine triphosphatase, have been studied in the hope of finding a disease-related change in membrane integrity (197). Peter et al (195) reported that ATPase from red blood cell ghosts of patients with Duchenne muscular dystrophy was stimulated by ouabain but inhibited in normal subjects; these workers suggested that the change in the membrane properties of erythrocytes from dystrophic patients

was due to a serum factor. Araki and Mawatari (196), Chattapadhyay and Brown (197) showed similar results of stimulation by ouabain of ATPase of erythrocyte ghosts in human dystrophies and inhibition by ouabain in normal subjects. However, Jacobson et al (198) reported that ouabain consistently inhibited the ATPase activity of dystrophic as well as normal hamster red blood cell membrane.

Alterations of muscle plasma membranes have also been studied. Changes in membrane permeability, shown by leakage of intracellular aldolase and by a greater K^+ efflux and influx than normal, were reported by Zierler (199). Hazlewood and Ginski (200, 201) showed similar results of ionic imbalance, based on altered permeability of muscle plasma membrane. An increased leakage of sarcoplasmic enzymes into the plasma suggested also a permeability defect of the sarcolemma. Dubois (119) reported enzymes mainly found inside the muscle cells such as creatine kinase, lactate dehydrogenase, aldolase, transaminases etc. seem to be released from the dystrophic muscle into the plasma where their level was higher than normal. The serum enzyme abnormality is an often documented indirect evidence of membrane abnormality in Duchenne dystrophy but has some limitations. For example, this permeability seemed to be independent of the size of the molecules and some enzymes such as AMP deaminase were not elevated in the serum and the phosphofructokinase was not found in the serum at all (119, 202).

Sulakhe *et al* (203) using isolated sarcolemmal membranes from old hamsters showed increased Mg^{++}- and Na^{+}-K^{+}-ATPase activities. Wrogemann *et al* (204) confirmed their finding, but in young hamsters, they did not find any difference between normal and dystrophic Na^{+}-K^{+}-ATPase activities in sarcolemmal membrane of the same strain of hamsters. Bray (205) reported that ouabain-sensitive Na^{+}-K^{+}-ATPase activity of dystrophic mice muscle preparations was significantly lower than that of control preparations.

None of the membrane defects or abnormalities could be considered with certainty as the primary defect in muscular dystrophy. The high Ca^{++} content in one mitochondrial population from skeletal muscle of young dystrophic hamsters might be a response to an increased intracellular Ca^{++} level to maintain it within the physiological range (206). The relation of this high mitochondrial Ca^{++} content to the primary defect of the disease is still unknown.

4. Hypothetical sequence of biochemical disorders occurring in the progress of muscular dystrophy.

The primary defect of muscular dystrophy must be some kind of alteration in the DNA, since this disease is genetic. This defect may be responsible for the lack of an essential protein or for an abnormal protein which will be functionally deficient. This protein may either be structural or enzymatic. How and where is this genetic defect

expressed? There are presently three different theories (119) which attempt to relate the genetic disease to its ultimate expression in the muscular tissue.

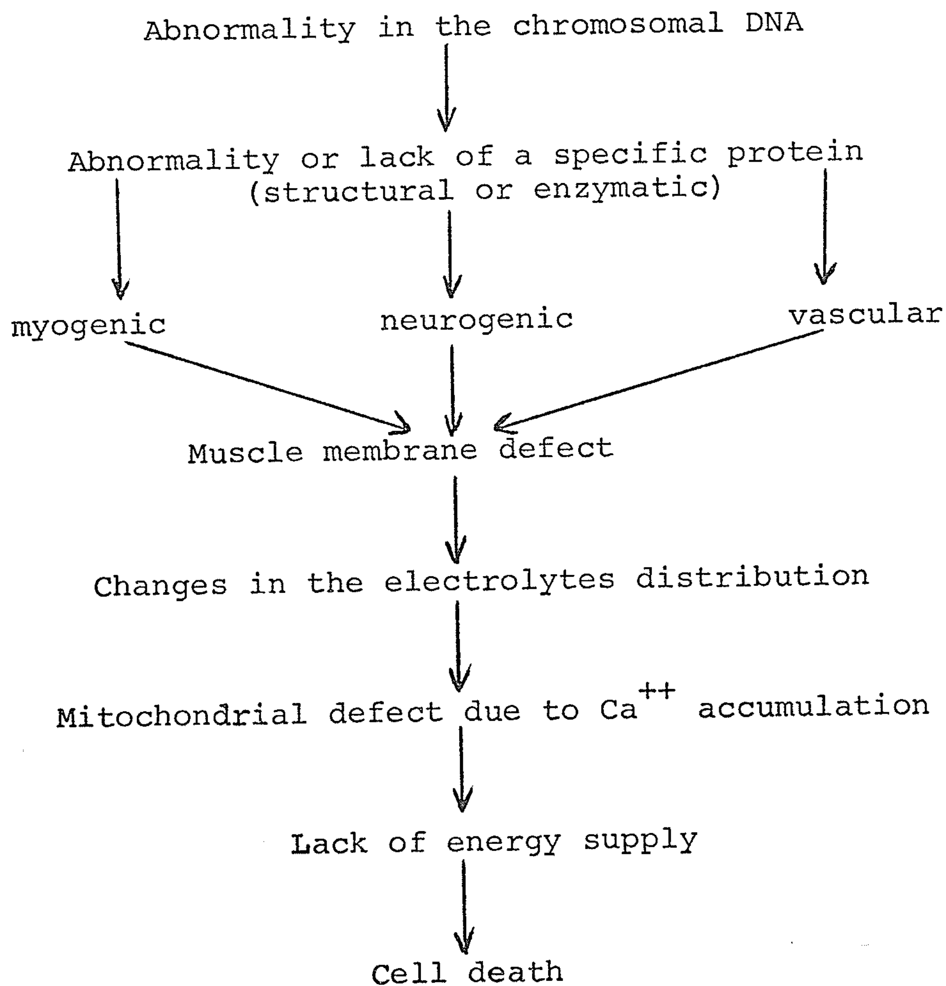
The myogenic theory implies that the primary disorder in muscular dystrophy is manifested directly in a constituent of the muscle fibers (202).

The neurogenic theory proposes that the muscle disorders are associated with aberration(s) within the nervous system where a lack of neuronal trophic factor(s) might disturb the function and survival of the muscle fibers (108, 110, 111, 112, 117, 207).

The vascular theory proposes that a defect in the microcirculation is responsible for a chronic hypoxic state which alters cellular permeability and promotes the escape of sarcoplasmic enzymes from the muscle (208, 209, 210).

The three theories seem to agree that defects appear, sooner or later, in the muscle fibers (see Figure 12). A defect might happen in the permeability of the sarcolemma with subsequent alterations in the electrolyte pattern between extracellular fluid and intracellular milieu. An alteration of the ionic balance would affect the mitochondria and/or the sarcoplasmic reticulum. The high Ca^{++} content in one or two populations of mitochondria might be a response to elevated intracellular Ca^{++} levels. Due to maintenance by the mitochondria of physiological levels of Ca^{++} within

Figure 12: Hypothetical sequence of biochemical disorders occurring in the progress of muscular dystrophy in the BIO-14.6 hamsters.



the cell, this mitochondrial defect is evident even at a very early age of dystrophic hamsters, at least as young as 30 days old. The accumulation of Ca^{++} by the mitochondria impairs oxidative phosphorylation which provides the immediate source of energy for the cell by formation of ATP. This mitochondrial defect might be responsible later for some other structural changes (189) of the muscle fibers. The ultimate result will be cell death.

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