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STUDIES ON THE CONTROL OF PROTEIN BIOSYNTHESIS IN THE SKELETAL MUSCLE OF DIABETIC RATS

A thesis submitted to the University of Glasgow in
candidature for the degree of

Doctor of Philosophy

in the

Faculty of Science

by

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March 1979

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Dedicated to

my parents and my husband

"The seed planted in 1921 has evolved into a great orchard which will bear interesting and valuable fruit for generations."

Rachmiel Levine

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ABBREVIATIONS

Abbreviations used in this thesis are as laid down in the Biochemical Journal Instructions to Authors (Biochem. J. 169 (1978) 1-27), with the following additions:-

POPOP	1,4-Bis-(5-phenyloxazol-2-yl) benzene
PPO	2,5-Diphenyloxazole
PolyA(+)RNA	PolyA-containing RNA
Polyribosomal RNA	Total RNA extracted from the purified ribosome pellet.
1 A_{260} unit	The quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a cell with a 1 cm light path. This is taken to be equivalent to 100 µg of ribosomes or 50 µg of ribosomal RNA

Throughout this thesis, the centrifugal force, "g", on a sample during centrifugation is calculated based on the average radius of rotation of the centrifuge tube.

CONTENTS

Page

TITLE i

DEDICATION ii

ACKNOWLEDGMENTS iv

ABBREVIATIONS v

CONTENTS vi

LIST OF FIGURES xi

LIST OF TABLES xv

SUMMARY xvii

CHAPTER I - INTRODUCTION

1.1 GENERAL EFFECTS OF INSULIN ON METABOLISM 1

1.2 EFFECT OF INSULIN AND DIABETES ON PROTEIN METABOLISM IN SKELETAL MUSCLE 9

1.3 EUKARYOTIC MESSENGER RNA 24

1.4 TRANSLATIONAL CONTROL OF EYKARYOTIC PROTEIN SYNTHESIS 40

CHAPTER 2 - EXPERIMENTAL

2.1 MATERIALS 49

2.1.1 Biological 49

2.1.2 Chemical 49

		page
2.2	CHARACTERIZATION OF EXPERIMENTAL DIABETES	50
2.2.1	Induction of diabetes	50
2.2.2	Enzymatic determination of the concentration of blood glucose	52
2.2.3	Effect of dose of streptozotocin on the induction of diabetes in rats	53
2.3	PREPARATION OF RIBOSOMES FROM RAT SKELETAL MUSCLE	57
2.3.1	Preparation of ribosomes at low ionic strength	57
2.3.2	Preparation of ribosomes at high ionic strength	58
2.3.3	Preparation of 'post-mitochondrial supernatant' by the method of Chen and Young (1968)	60
2.4	EXTRACTION OF RNA FROM RAT SKELETAL MUSCLE	60
2.4.1	Extraction of total RNA	60
2.4.2	Extraction of RNA from purified ribosomes	62
2.4.3	Isolation of polyA(+)RNA by polyU-Sepharose Affinity Chromatography	62
2.5	SUCROSE DENSITY GRADIENT ANALYSIS OF RIBOSOMES AND RNA	63
2.5.1	Sucrose density gradient analysis of ribosomes	63
2.5.2	Sucrose density gradient analysis of rat skeletal muscle 'post-mitochondrial supernatant'	64

page

2.5.3 Sucrose density gradient analysis of RNA 64

2.6 ASSAY OF POLYA(+)RNA BY HYBRIDIZATION WITH (^{3}H) POLYU 65

2.7 ASSAYS FOR CELL-FREE PROTEIN SYNTHESIS 66

2.7.1 Assay of protein synthesis with rat skeletal muscle ribosomes *in vitro* 66

2.7.2 Translation of rat skeletal muscle ribosomes and polyA(+)RNA in a cell-free system from wheat germ 67

2.7.3 Identification of cell-free translation products by polyacrylamide gel electrophoresis in sodium dodecyl sulphate 67

2.8 ASSAY OF RIBONUCLEASE ACTIVITY 69

2.8.1 Assay of ribonuclease activity using yeast RNA as substrate 69

2.8.2 Assay of ribonuclease activity using (^{32}P) rRNA as substrate 71

2.9 DETERMINATION OF PROTEIN 72

2.10 DETERMINATION OF RNA AND DNA 72

2.11 COMPOSITION OF STANDARD BUFFER SOLUTIONS 75

page

CHAPTER 3 - RESULTS 79

3.1 INITIAL CHARACTERIZATION OF RAT SKELETAL MUSCLE RIBOSOMES 79

3.1.1 Comparison of different methods of extraction of ribosomes 79

3.1.2 Optimization of the separation of polysomes and monosomes by sucrose density gradient centrifugation 82

3.1.3 Attempts to optimize the yield and size of polysomes 87

3.2 THE EFFECT OF DIABETES AND INSULIN ON RAT SKELETAL MUSCLE POLYSOMES 93

3.2.1 Effect of diabetes and insulin on the sedimentation profiles of ribosomes 93

3.2.2 Ribonuclease activity of rat skeletal muscle 103

3.3 QUANTITATIVE COMPARISON OF POLYA(+)RNA FROM THE SKELETAL MUSCLE OF NORMAL AND DIABETIC RATS 109

3.3.1 Characterization of the (^{3}H) polyU hybridization assay for polyA(+)RNA 110

3.3.2 Determination of the relative proportions of polyA(+)RNA in various fractions of rat skeletal muscle 115

page

3.4 QUALITATIVE COMPARISON OF POLYA(+)RNA FROM THE SKELETAL MUSCLE OF NORMAL AND DIABETIC RATS 138

3.4.1 Comparison of the size of polyA sequences in skeletal muscle polyribosomal polyA(+)RNA 138

3.4.2 Comparison of the size of polyA(+)RNA from the skeletal muscle of normal and diabetic rats 140

3.4.3 Comparison of template activity of polyA(+)RNA from the skeletal muscle of normal and diabetic rats 147

CHAPTER 4 - DISCUSSION

4.1 CHARACTERISATION OF mRNA FROM ADULT RAT SKELETAL MUSCLE 149

4.2 METABOLISM OF SKELETAL MUSCLE mRNA IN DIABETES 163

4.3 NUCLEASE ACTIVITIES IN THE MUSCLES OF DIABETIC RATS 168

REFERENCES 171

LIST OF FIGURES

		page
1.2.1	Models for polysomes in skeletal muscle	17
1.3.1	Structure of the 5'-terminal 'cap' of eukaryotic mRNAs	29
2.2.1.1	Chemical structure of the two diabetogenic β-cytotoxins	51
2.2.2.1	Standard curve for estimation of blood glucose by the glucose oxidase- peroxidase method	54
2.2.3.1	Effect of streptozotocin on the concentration of glucose in rat blood	56
2.3.1.1	Summary of different fractions obtained during preparation of skeletal muscle ribosomes	59
2.7.3.1	Standard curve for determination of molecular weights of proteins in gels containing sodium dodecyl sulphate	70
2.9.1	Standard curve for estimation of protein by the method of Lowry	73
2.10.1	Standard curve for estimation of RNA by the orcinol method	76
2.10.2	Standard curve for estimation of DNA by the diphenylamine method	77
3.1.1.1	Comparison of the sedimentation characteristics of ribosomes extracted from skeletal muscle in media of different ionic strengths	80
3.1.2.1	Effect of ribonuclease on the sedimentation of ribosomes on sucrose density gradients	83
3.1.2.2.	Analysis of ribosomes extracted at low ionic strength on sucrose density gradients of different ionic composition	84

page

3.1.2.3	Effect of ionic strength and temperature on rat skeletal muscle ribosomes analysed on sucrose density gradients	86
3.1.3.1	Sucrose density gradient sedimentation of ribosomes isolated from rat skeletal muscle in the presence of cycloheximide	88
3.1.3.2	Sucrose density gradient centrifugation of ribosomes isolated from the diaphragm of normal and alloxan-diabetic rats	90
3.1.3.3	Sucrose density gradient sedimentation of ribosomes isolated from rat diaphragm in the presence of cycloheximide	91
3.1.3.4	Sucrose density gradient centrifugation of ribosomes isolated from rat skeletal muscle under conditions designed to reduce possible ribonuclease activity	92
3.2.1.1	Analysis of ribosomes from rat skeletal muscle on sucrose density gradients	94
3.2.1.2	Effect of insulin on the sedimentation of ribosomes isolated from the skeletal muscle of streptozotocin-diabetic rats	96
3.2.1.3	Effect of streptozotocin-diabetes and insulin or rat skeletal muscle ribosomes isolated at low ionic strength	97
3.2.1.4	Effect of diabetes and insulin on the protein synthetic activity of ribosomes from rat skeletal muscle assayed in a cell-free system from rat liver	100
3.2.1.5	Sodium dodecyl sulphate gel electrophoresis of the translation products of rat skeletal muscle ribosomes	102
3.3.1.1	Effect of incubation time on hybridization of (^{3}H) polyU and polyA	111
3.3.1.2	Hybridization of (^{3}H) polyU with different amounts of polyA	112

		page
3.3.1.3	Specificity of (^{3}H) polyU:polyA hybridization assay	113
3.3.1.4	Hybridization of (^{3}H) polyU with polyribosomal RNA from normal rat skeletal muscle	114
3.3.2.1	Summary of different fractions obtained during preparation of skeletal muscle ribosomes	119
3.3.2.2	Summary of polyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation	124
3.3.2.3	Summary of polyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation with a sucrose 'cushion'	127
3.3.2.4	Sucrose density gradient analysis of RNA extracted from the sucrose 'cushion'	131
3.3.2.5	Sucrose density gradient analysis of rapidly sedimenting material from normal and diabetic rat skeletal muscle ribosomes preincubated at 4°C and 37°C	132
3.3.2.6	Sucrose density gradient centrifugation of 'post-mitochondrial supernatants' from rat skeletal muscles extracted at different ionic strengths	134
3.3.2.7	Sucrose density gradient analysis of 'post-mitochondrial supernatants' from skeletal muscle of normal and diabetic rats prepared at 350 mM KCl	136
3.3.2.8	Sedimentation profiles of ribosomes from rat skeletal muscle extracted at 350 mM KCl	137
3.4.1.1	Sedimentation profiles of hybrids between (^{3}H) polyU and polyA tracts from rat skeletal muscle polyribosomal RNA	139

page

3.4.1.2	Sucrose density gradient sedimentation profiles of polyA tracts from rat skeletal muscle polyribosomal RNA	141
3.4.2.1	Sedimentation profiles of hybrids between (^{3}H) polyU and polyA(+)RNA from rat skeletal muscle poly-ribosomal RNA	143
3.4.2.2	Sedimentation profiles of hybrids between (^{3}H) polyU and polyA(+)RNA from rat skeletal muscle poly-ribosomal RNA, analysed under conditions allowing detection of larger species	144
3.4.2.3	Sucrose density gradient sedimentation profiles of polyA(+)RNA from rat skeletal muscle polyribosomal RNA	145
3.4.2.4	Sedimentation profiles of hybrids between (^{3}H) polyU and polyA(+)RNA from unfractionated rat skeletal muscle	146
4.1.1	Comparison of the molecular weights of some proteins with the sedimentation coefficients of their mRNAs	153
4.1.2	Comparison of the molecular weights of some proteins with the number of ribosomes comprising their polysomes	154
4.1.3	Comparison of the number of nucleotides of some mRNAs with the sedimentation coefficients of their mRNAs	158

LIST OF TABLES

		page
2.2.2.1	Concentration of glucose in blood of rats under different physiological conditions	55
3.1.1.1	Effect of ionic strength of extraction medium on the yield of skeletal muscle ribosomes	81
3.2.1.1	Proportion of polysomes in ribosomes from the skeletal muscle of normal and diabetic rats	99
3.2.1.2	Effect of diabetes on the protein synthetic activity of ribosomes from rat skeletal muscle assayed in a cell-free system from wheat germ	101
3.2.2.1	Ribonuclease activity of rat skeletal muscle 'post-mitochondrial supernatants' assayed with non-radioactive yeast RNA as substrate	104
3.2.2.2	Ribonuclease activity of rat skeletal muscle 'post-mitochondrial supernatants' assayed with (^{32}P) rRNA as substrate	106
3.2.2.3	Effect of diabetes and insulin on the amount of RNA, DNA and protein in rat skeletal muscle	107
3.2.2.4	Ribonuclease activity of rat skeletal muscle homogenate assayed with non-radioactive yeast RNA as substrate	108
3.3.1.1	PolyA content of purified ribosomes from normal rat skeletal muscle	116
3.3.2.1	Proportion of polyA(+)RNA to total RNA in unfractionated skeletal muscle from normal and diabetic rats	118
3.3.2.2	Distribution of total RNA and polyA(+)RNA between different fractions of rat skeletal muscle	120

page

3.3.2.3	Comparison of polyA content of purified ribosomes from skeletal muscle of normal and diabetic rats	122
3.3.2.4	PolyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation	125
3.3.2.5	PolyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation with a 70% sucrose 'cushion'	128
3.3.2.6	Comparison of different analyses of the proportion of polyA(+)RNA in skeletal muscle ribosomes from normal and diabetic rats	129
3.4.3.1	Translation of rat skeletal muscle polyA(+)RNA in a cell-free system from wheat germ	148

SUMMARY

The overall aim of the work described in this thesis was to examine the effects of diabetes and insulin on the metabolism of mRNA in rat skeletal muscle. The primary objective was to determine whether insulin stimulates protein synthesis in the skeletal muscle of diabetic rats by promoting the translation of pre-existing mRNA.

Initially the most suitable conditions for the isolation of skeletal muscle ribosomes, and the sedimentation of these on sucrose density gradients were determined. Ionic conditions of 50 mM Tris-HCl, 200 mM KCl, 5 mM $MgCl_2$ (pH 7.6) in the sucrose density gradients were found to be best for decreasing the dimerization of 80S ribosomes which hindered discrimination between monosomes and polysomes. Ribosomes extracted at high ionic strength (which gave superior yields) had similar sedimentation profiles to those isolated at low ionic strength, with about 60% of the total ribosomes in polysomes.

Skeletal muscle polysomes were found to be small in size compared to those from some other cells and tissues, with the most predominant species being those containing three, four and five ribosomes. This small size did not appear to be due to nicking of the mRNA by ribonuclease, for ribosomes prepared under sterile conditions, in the presence of heparin (an inhibitor of ribonuclease) did not give larger polysomes. Neither did addition of cycloheximide, suggesting that the small size of the polysomes was not due to the ribosomes running off the mRNA during isolation. When skeletal muscle ribosomes were translated in a cell-free system from wheat germ, the major products were of molecular weight 15,500 - 17,500, although smaller amounts of species with molecular weights as high as 100,000 could be detected.

Initially diabetes was induced with alloxan or streptozotocin and found to decrease the number of polysomes in rat skeletal muscle ribosomes, as judged by sucrose density gradient analysis. When insulin was administered to either alloxan- or streptozotocin-diabetic rats, and the ribosomes isolated at low ionic strength, the polysomes rapidly reassembled. However, when the ribosomes were extracted at high ionic strength, reassembly of polysomes was only observed with the ribosomes from the rats that had been made diabetic with streptozotocin. These observations imply that alloxan induces a ribonuclease activity that is extracted from another sub-cellular fraction of skeletal muscle by the medium of high ionic strength during isolation, breaking down the polysomes which had reassembled *in vivo* after injection of insulin. Attempts to detect this putative nuclease activity directly using yeast RNA or radioactive HeLa cell ribosomal RNA as a substrate were unsuccessful. The concentration of RNA per gram of DNA in rat skeletal muscle was found to decrease by about 25% from that in normal rats, 3 days after the induction of either alloxan- or streptozotocin-diabetes. Thus the putative ribonuclease, referred to above, cannot be responsible for the decreased skeletal muscle RNA in diabetes.

RNA was prepared by phenol extraction of purified ribosomes (isolated at low ionic strength) and estimated by its ultraviolet absorption. The proportion of mRNA in this RNA was determined as polyA-containing RNA, assayed by hybridization of the total RNA to (^{3}H) polyU followed by digestion with pancreatic ribonuclease and measurement of the amount of (^{3}H) polyU:polyA hybrid retained on glass fibre filters. Using this assay it was found that, on average, 0.108% of skeletal muscle polyribosomal

RNA from normal rats consists of regions of polyA.

The size of polyA-containing RNA in RNA extracted from skeletal muscle polyribosomes was estimated either from the sedimentation of (^{3}H) polyU:polyA-containing RNA hybrids on sucrose density gradients, or by sedimenting the RNA alone and assaying the hybridization of (^{3}H) polyU to the polyA-containing RNA in the different fractions of the gradient. The size of the polyA-containing RNA was found to be small, with a range of sedimentation coefficients from 4-18S and a median value of about 9.5S. The size of the polyA sequences obtained by digestion of the total polyribosomal RNA with pancreatic and T_1 ribonuclease was measured in an analogous way to that of the polyA-containing RNA and a sedimentation coefficient of about 4S was obtained. From these data it was concluded that the polyA region constituted about 20% of the length of skeletal muscle mRNA of adult rats, and that the proportion of mRNA in total polyribosomal RNA was 0.54%

PolyA-containing RNA was isolated from the total RNA on a polyU-Sepharose column, and added to a cell-free translation system from wheat germ. A small stimulation above the endogenous level of translation was obtained, but this was insufficient to establish that the polyA-containing RNA is functional mRNA.

Streptozotocin-diabetes decreased the polysome content of rat skeletal muscle ribosomes by about 50%, and the activity of the ribosomes in a cell-free system for protein synthesis by a comparable value. It did

not appear to affect the qualitative nature of the polypeptide chains synthesized in the cell-free system, as judged by analysis on polyacrylamide gels. Streptozotocin-diabetes did not affect the size of the polyA-containing RNA, nor that of its polyA tract, as judged by sucrose density gradient analysis.

Quantitative measurements of the polyA-containing RNA in the skeletal muscle of diabetic rats by hybridization with (^{3}H) polyU showed that the polyA-containing RNA constituted a similar proportion of the total RNA in both normal and diabetic rats, whether the RNA was extracted from the unfractionated skeletal muscle or from the purified ribosomes. These results showed, for the first time, that in diabetes the amount of muscle mRNA decreases to the same extent as does the total RNA.

When measurements of polyA-containing RNA were made in diabetic rats treated with insulin, it was found that the proportion of polyA-containing RNA in the total polyribosomal RNA was similar to that in diabetic rats. This implies that insulin does not promote protein synthesis by stimulating the transcription of mRNA and therefore must regulate the translation of pre-existing mRNA, present in an inactive form.

Attempts were made to identify this inactive mRNA in sucrose density gradients of ribosomes prepared from the skeletal muscle of diabetic rats, but this RNA pelleted at the bottom of the gradient tube in association with aggregated ribosomes. All attempts to prevent this aggregation were unsuccessful and it was, therefore, not possible to determine in what form the inactive mRNA is stored during diabetes.

CHAPTER 1

INTRODUCTION

1.1 GENERAL EFFECTS OF INSULIN ON METABOLISM

Diabetes is an ancient disease. In one of the Egyptian papyrus, written about 1550 B.C., the directions for the treatment of the disease begin with the words "A medicine to drive away the passing of too much urine". Nearly 2000 years ago, the Greek physician Aretaeus of Cappadocia called it a "mysterious disease". From that time until the early part of this century, the form of the disease which is now known as "juvenile-onset" diabetes mellitus was almost invariably fatal.

In 1889 Minkowski, together with von Mehring, contributed to the discovery of insulin when they observed that pancreatectomy led to the development of diabetes. But it was not until the historic day of July 30, 1921, that a substance extracted from the pancreas was able to reverse this. On that day Banting and Best recorded a steady decline in the blood glucose concentration of a depancreatized dog during the hours following the first injection of a pancreatic extract. The active substance of the pancreas was called "insulin", the name already given to it in 1909 by the Belgian scientist Jean de Meyer, long before the substance itself had been discovered. The discovery of insulin represents one of the major scientific milestones of this century. Although much remains to be learnt about the biochemical action of insulin, considerable progress has been made since 1921, especially during the post-war period. In many of these later studies the β-cytotoxins, alloxan (Lukens, 1948) and streptozotocin (Junod *et al.*, 1969), have been used to induce in small experimental rodents

a form of diabetes which bears some resemblance to the "juvenile-onset" diabetes of humans.

Insulin was the first naturally occurring protein to be completely analysed with respect to its amino acid sequence (Sanger, Thompson and Kitai, 1955; Sanger, 1959). Bovine insulin was shown to be a polypeptide composed of 51 amino acids arranged in two chains (the "A" and the "B" chains) joined to one another by two disulphide bridges. The three-dimensional structure of the insulin molecule has been determined by X-ray diffraction (Hodgkin, 1972). The two chains are compactly arranged with the "A" chain lying above a central helical region of the "B" chain.

Insulin is formed in the β-cells of the islets of Langerhans of the pancreas _via_ the single chain precursor, proinsulin (Steiner _et al._, 1967), with the "A" and "B" chains linked by a connecting peptide, the "C-peptide", of variable length and amino acid composition in different species. Proinsulin is synthesized by the ribosomes attached to the rough endoplasmic reticulum, transferred to the cisternae of the Golgi apparatus in microvesicular structures derived from the rough endoplasmic reticulum, packaged into new secretory granules, and converted by proteolysis to mature insulin (Steiner _et al._, 1967; Steiner _et al._, 1972). Inside the β-granules insulin crystallizes with sequestered zinc ions, the secretory granules migrate to the cell membrane by a process known as exocytosis and, following fusion and dissolution of the two membranes at the point of contact, the contents of the granules are discharged to the outside of the cell (Lacy, 1967). At neutral

pH virtually complete dissociation of zinc-insulin hexamers occurs (Pekar and Frank, 1972), and thus the physiologically active form of insulin in body fluids is considered to be mainly the monomer.

Insulin has profound anabolic effects on the metabolism of carbohydrate, fat and protein, especially in liver, muscle and adipose tissue. In general, it promotes the synthesis of glycogen, protein and lipid while it inhibits the degradation of these substances. Under physiological conditions, the secretion of insulin is stimulated by the metabolic substrates, glucose, amino acids and, perhaps, short-chain fatty acids (Williams and Ensinck, 1971). The major normal stimulus appears to be glucose. It has been proposed (Grodsky, 1972) that this acts by initiating the entry of calcium, which triggers a change in the physical conformation of the microtubules and displacement of the granules to the β-cell surface. Several gastrointestinal hormones (including secretin, pancreozymin and gastrin) stimulate the release of insulin from the pancreas by enhancing the sensitivity of the β-cells to glucose (Kipnis and Permutt, 1972).

Before describing the effects of insulin on metabolism it is important to emphasize that the molecular mechanism by which these effects are elicited is not known. Our present knowledge allows us to distinguish independent effects of the hormone on different metabolic processes and, in some cases, to describe the molecular changes in the target tissue that are responsible for these effects. However we know nothing of the molecular events which link the initial interaction of insulin with the cell (itself subject to some uncertainty) to these molecular changes.

The first effect of insulin to be discovered was its stimulation of the entry of glucose into individual cells (Lundsgaard, 1939; Levine *et al.*, 1949, 1950). Subsequent work has shown that insulin stimulates specific transport systems for sugars, and that these systems are present in muscle (Henderson, Morgan and Park, 1961) and adipose tissue (Renold *et al.*, 1965), but not in liver (Cahill *et al.*, 1958), brain and other nervous tissue (Krahl, 1961). This suggested that the primary site of interaction of insulin with the cell is at the cell membrane. It is now well documented that insulin binds to specific receptor sites in the plasma membrane of its responsive cells, and that these receptor sites are proteins or glycoproteins which are able to recognize and bind insulin (Cuatrecasas, 1974). It is believed that the interaction of insulin with its receptor may result in changes in the reactivity of membrane proteins, such as enzyme and carrier systems; but it is not known whether the signal that leads to activation of transport is solely a membrane phenomenon or whether it involves intracellular loci. Moreover, problems regarding a simple primary interaction were raised by the discovery of other intracellular metabolic effects of insulin, independent of its stimulation of trans-membrane sugar transport. These latter include the inhibition of glycogenolysis and gluconeogenesis in liver (Cahill *et al.*, 1958; Vranic, 1972), lipolysis in adipose tissue (Ball and Jungas, 1964), and the stimulation of glycogenesis in muscle (Villar-Palasi and Larner, 1968). Thus it is not clear whether one or multiple signals are generated, or whether insulin elicits all of its effects from the outside of the cells.

Insulin promotes glycogen synthesis in liver (Bishop *et al.*, 1965) and muscle (Larner *et al.*, 1959) through its rapid increase in the

activity of the enzyme glycogen synthetase(UDPG:glycogen α-1,4-glucosyltransferase). Glycogen synthetase is present in two forms, phosphorylated (inactive) and dephosphorylated (active). In muscle insulin stimulates glycogen synthetase, apparently <u>via</u> cyclic AMP-independent pathways. Probably it either reduces the activity of the protein kinase which converts the active form of glycogen synthetase to the inactive enzyme, or increases the activity of the protein phosphatase to bring about conversion to the active form. Evidence has been presented in support of both possibilities (Nuttall, 1972), but it is not yet clear which is correct (see Nimmo and Cohen, 1977). Besides its rapid effects, insulin also exhibits long-term effects by stimulating enzyme synthesis. In liver, insulin stimulates the synthesis of glucokinase (Pilkis, 1970), thus stimulating glycolysis.

Insulin directly inhibits glucose output by the liver, in contrast to glucagon and adrenalin, which stimulate this process by promoting both glycogenolysis and gluconeogenesis (Mortimore, 1972). These latter hormones increase the intracellular levels of cyclic AMP which is necessary for the activation of glycogen phosphorylase. Although insulin reduces the increased levels of cyclic AMP brought about by low concentrations of glucagon or adrenalin in perfused livers (Mortimore, 1972; Exton and Park, 1972), its effect on hepatocyte glucose output can be dissociated from its effect on intracellular cyclic AMP levels (Davidson and Berliner, 1974). Thus the decrease in phosphorylation of glycogen phosphorylase which is responsible for its inactivation by insulin may be achieved by a more complex mechanism. Insulin stimulates glycolysis by increasing the activity of the key glycolytic enzymes and inhibits gluconeogenesis by suppressing the induction of the key gluco-neogenic enzymes by glucocorticoid hormones (Krahl, 1961; Weber, 1972).

Insulin promotes lipogenesis in liver and adipose tissue in a number of ways. The increased glucose entry and stimulation of glycolysis provides the α-glycerophosphate for the glycerol moiety of triglycerides; it provides acetyl-CoA for the synthesis of fatty acids; and through the partial oxidation of glucose in the pentose-phosphate pathway, it may be the source of reduced pyridine nucleotides (principally NADPH) needed for fatty acid synthesis. Insulin stimulates lipogenesis more directly by activating pyruvate dehydrogenase (Taylor et al., 1973) and possibly acetyl-CoA carboxylase (Halestrap and Denton, 1974); and, over a longer term, by increasing the synthesis of lipogenic enzymes (Nakanishi and Numa, 1970).

The increased insulin secretion that follows high carbohydrate intake, causes the excess formation of triglycerides and very low density lipoproteins in liver (Fredrickson et al., 1967). The enzyme responsible for the uptake of circulating triglycerides is lipoprotein lipase, which requires insulin for its activity in adipose tissue (Robinson and Wing, 1971). This cleaves the triglycerides to yield glycerol and free fatty acids, the latter then being taken up by the adipose tissue and converted into triglycerides.

Insulin also has a direct antilipolytic action on the adipose tissue, by preventing activation of the hormone-sensitive lipase (Ball and Jungas, 1964). This enzyme is activated in adipose tissue through phosphorylation by a cyclic AMP-dependent protein kinase, and its activation is promoted by numerous hormones, including adrenalin, thyroid stimulating hormone, glucagon, adrenocorticotrophic hormone, growth hormone and the glucocorticoids (Robinson and Wing, 1971).

Insulin has been reported to stimulate phosphodiesterase activity when added to intact adipose tissue (Loten and Sneyd, 1970) and this stimulation is dependent on the presence of extracellular calcium ions. Insulin may thus elicit a calcium-dependent activation of phosphodiesterase activity in adipose tissue, which in turn would decrease cyclic AMP levels. As the hormone-sensitive lipase in adipose tissue is regulated by cyclic AMP, it was first suggested that insulin might affect the activity of this enzyme by altering the intracellular levels of this cyclic nucleotide. However, it was found subsequently that insulin had no effect on total cyclic AMP accumulation in fat cells under conditions in which it inhibited lipolysis (Fain et al., 1972). Moreover it appears that the antilipolytic effect of insulin is independent of calcium (Desai and Hollenberg, 1975).

In conditions characterized by insufficiency or lack of endogenous insulin, e.g. starvation or diabetes mellitus, the broad direction of metabolism changes from the deposition of dietary metabolites described above to the mobilization of tissue metabolic reserves. This is appropriate for starvation, but may have pathological consequences in the case of diabetes. Uptake of glucose by adipose tissue and muscle is suppressed and hepatic output of glucose rises; the latter effect being possibly due to glucagon (Marliss et al., 1970) which stimulates glycogenolysis through its activation of glycogen phosphorylase. A second reason for the increased hepatic output of glucose during insulin deficiency is the stimulation of gluconeogenesis by glucagon and glucocorticoid hormones. Removal of the suppressive effect of insulin upon synthesis of the gluconeogenic enzymes leads to increased production of glucose from non-fat substrates, and increased

excretion of nitrogen in the urine. In adipose tissue, metabolism of glucose is reduced and insufficient α-glycerophosphate is generated for the esterification of free fatty acids. In the absence of insulin, the hormone-sensitive lipase activity increases and increased amounts of free fatty acids and glycerol are released into the blood. Some of these are transported to the liver, while others are metabolized by muscle, reducing the oxidation of glucose by this tissue. In the liver, the free fatty acids inhibit conversion of acetyl-CoA to malonyl-CoA by inhibiting acetyl-CoA carboxylase (Majerus and Vagelos, 1967), and so inhibit the synthesis of free fatty acids there. The intermediates involved in synthesis of free fatty acids are then converted to the ketone bodies, aceto-acetic acid and β-hydroxybutyric acid, which are released into the blood. In starvation ketone bodies are an important respiratory fuel for muscle and brain, but in diabetes they build up to pathological levels.

1.2 EFFECT OF INSULIN AND DIABETES ON PROTEIN METABOLISM IN SKELETAL MUSCLE

One effect of metabolic insulin insufficiency mentioned in the previous section was a stimulation of gluconeogenesis in the liver. Under such circumstances the main substrate for this increased gluconeogenesis is amino acids derived from the breakdown of muscle protein (Exton et al., 1970). In the absence of insulin the rate of protein synthesis is depressed to allow a net breakdown of protein to amino acids, a process that is reversed by insulin. The latter stimulates the synthesis of protein and stems the net outflow of amino acids.

An understanding of the stimulation of net protein breakdown in starvation and diabetes has been hampered by our limited knowledge of the actual processes of protein degradation. Skeletal muscle, like many other tissues, contains soluble proteases, though at the moment these are poorly characterized. Some of these proteases have alkaline pH optima and may be involved in the turnover of myofibrils (Koszalka and Miller, 1960 a, b; Noguchi and Kandatsu, 1971; Dahlmann and Reinauer, 1978), while others are group specific and involved in apoenzyme breakdown (Katunuma et al., 1975). An increase in the activity of an alkaline protease has been detected in the skeletal muscle of rats after treatment with glucocorticoids, in alloxan-diabetes, and in rats with hypoproteinaemia (Mayer, Amin and Shafrir, 1974). Treatment of diabetic rats with insulin depresses the increased alkaline proteolytic activity to normal level (Röthig et al., 1978). Other soluble proteases have optimum activity in the neutral pH range. An example of a protease

of this type is the calcium-activated neutral protease that removes the Z bands from skeletal muscle (Kohn, 1969; Reddy et al., 1975). However it is becoming increasingly evident that in many tissues the lysosomal proteases (generally characterized by acidic pH optima) are of major importance in protein catabolism. Recently studies have been directed towards determining the possible participation of lysosomes in the breakdown of intracellular muscle protein during normal protein turnover (Dean, 1975), as well as in conditions of physiological and pathological stress. A possible role for lysosomes in the mobilization of amino acids from skeletal muscle during starvation was suggested by an apparent increase in size of the lysosomes, together with a specific increase in the activity of the protease, cathepsin D, and certain other hydrolases (Canonico and Bird, 1970). Other studies have implicated lysosomes in the loss of muscle protein in muscular dystrophies (Weinstock and Iodice, 1969).

A special problem regarding protein degradation exists in skeletal muscle: this is the question of whether the different proteins of the myofibrils turn over as a unit. This is an attractive idea as it is difficult to envisage in muscle the independent turnover that operates in other cells (Dreyfus, Kruh and Schapira, 1960). Some studies have suggested that the myofibril has a definite life-span, supporting this idea (Zak, Ratkitzis and Rabinowitz, 1971). Other work, however, indicates that the different structural proteins of skeletal muscle turn over separately (Millward, 1970; Koizumi, 1974; Zak et al., 1977).

The way in which insulin regulates protein turnover is of most

relevance to the present work. It has been clearly shown that insulin decreases the release of some amino acids by the perfused rat hemicorpus (Jefferson et al., 1974) and by incubated rat diaphragm (Fulks et al., 1975). However, it is not clear whether the major effect of insulin on protein degradation is direct, or if increased protein synthesis is mainly responsible for the diminished release of amino acids. Millward et al. (1975) have emphasized the importance of the rate of protein synthesis in determining the protein content of muscle. These investigators found that in rat skeletal muscle during rapid growth on a good diet, there is an increased rate of protein synthesis, associated with an increased rate of protein degradation. With age, however, the decline in the growth rate results from a fall in the rate of protein synthesis. This has been demonstrated in some experiments *in vitro*, where the activity of skeletal muscle ribosomes to synthesize protein was reduced with a concomitant decrease in the proportion of polysomes to total ribosomes (Srivastava and Chaudhary, 1969; Nakano and Sidransky, 1978). Starvation or induction of diabetes resulted in a rapid decrease in the rate of protein synthesis (Pain and Garlick, 1974). In these studies the turnover of heart proteins was more rapid than those of skeletal muscle and was affected less markedly by fasting or diabetes.

Other studies have suggested that lack of insulin increases protein degradation *via* lysosomal proteases. This has been shown most clearly in heart muscle (Morgan et al., 1974; Jefferson et al., 1974; Rannels et al., 1975). Rat hearts that had been perfused for 3 hr in the absence of insulin, contained large membrane-limited vacuoles

and showed increased rates of proteolysis. When the activity of the lysosomal enzyme, cathepsin D, was assayed in these studies, an increase in the extra-lysosomal activity was detected, although the total activity did not change (Rannels et al., 1975). This observation suggested that the lack of insulin stimulates the release of this enzyme from the lysosomes. Addition of insulin in physiological concentrations to the perfusate prevented or reversed these changes. Proteolytic effects of insulin were also observed in both perfused rat skeletal muscle and incubated rat diaphragm preparations, although to a lesser extent than in heart muscle (Jefferson et al., 1974; Fulks et al., 1975).

One possible way in which insulin might prevent protein degradation is through an effect on the transport of amino acids into muscle, for it has been reported that the concentration of amino acids - in particular the branched chain amino acids - may regulate protein turnover. Thus, addition to rat diaphragms of branched-chain amino acids, at concentrations five times greater than in plasma, stimulated protein synthesis and reduced protein degradation (Fulks et al., 1975; Buse and Reid, 1975). The mechanism by which the branched-chain amino acids regulate protein turnover is not clear and there is not yet evidence that this type of regulation occurs in vivo.

In the absence of insulin, the net breakdown of muscle protein may be due to the increased influence of other hormones. Thus glucocorticoid hormones have long been known to have net catabolic effects upon protein metabolism (Long et al., 1940), and increases in the levels of free amino acids in the muscle and plasma of animals receiving glucocorticoids have been reported (Friedberg and Greenberg, 1947;

Betheil et al., 1965). Thyroid hormones may play an important role in the regulation of the basal protein turnover in skeletal muscle. Although these hormones have well known effects in promoting protein synthesis in skeletal muscle, it is now clear that under certain circumstances they can also stimulate protein degradation, and may be responsible for the mobilization of body protein during starvation (Goldberg et al., 1977). It was found that thyroidectomy or hypophysectomy decreased protein degradation in rat skeletal muscle. Moreover, treatment of hypophysectomized rats with physiological doses of triiodothyronine or tetraiodothyronine increased protein degradation (Goldberg and Griffin, 1977). Other studies described an increase in the total cellular content of the lysosomal proteases, cathepsin D and cathepsin B, in liver and skeletal muscle of hypophysectomized rats upon treatment with thyroid hormones (De Martino and Goldberg, 1978). Finally it should be mentioned that glucagon may possibly play some part in protein degradation during starvation and diabetes. This hormone has been reported to increase protein degradation in liver (Deter, 1971), but it is not clear whether it has any similar effects on muscle.

The effects of insulin and lack of insulin on protein biosynthesis have so far been discussed with respect to their role in net protein breakdown in muscle, especially in relationship to the overall control of carbohydrate metabolism. However, the effect of insulin on muscle protein synthesis is important both to the overall protein metabolism of the animal and to the question of the mode of action of insulin on a process, the molecular details of which are much better understood than protein degradation (for reviews, see Lengyel, 1974; Weissbach and Pestka, 1977).

The effect of insulin on muscle protein synthesis is well documented. Sinex, MacMullen and Hastings (1952) were the first to demonstrate an effect of insulin upon the synthesis of protein; addition of the hormone to incubation media increasing the incorporation of (^{14}C)-labelled amino acids into the proteins of the isolated rat diaphragm. Similar observations have been reported by other investigators (Krahl, 1953; Manchester and Young, 1958; Wool and Krahl, 1959). This effect of insulin on protein synthesis in vivo and in vitro may be general, for it has been reported to affect - to an equal extent - all the proteins of rat hemidiaphragm which were resolved by discontinuous electrophoresis on non-denaturing polyacrylamide gels (Kurihara and Wool, 1968).

The effect of insulin on protein synthesis in skeletal muscle is independent of its action in facilitating the entry of glucose into the cell, for incorporation of labelled amino acids into muscle proteins is stimulated by insulin in the absence of glucose (Manchester and Young, 1958; Wool and Krahl, 1959). However, insulin also stimulates the entry of amino acids into skeletal muscle; a process which is not secondary to an effect on protein synthesis, for it occurs with non-utilizable amino acids and when protein synthesis is inhibited (Kipnis and Noall, 1958; Manchester and Young, 1960; Wool, Castles and Moyer, 1965). This has led to considerable, and still unresolved, debate whether the effect of insulin on protein synthesis is an indirect consequence of its effect on amino acid transport (see e.g. Wool, 1968). Evidence against the effect of insulin on protein synthesis being secondary to that on amino acid transport was obtained by Manchester and Krahl (1959). They incubated rat diaphragm with a

variety of (^{14}C)-labelled carboxylic acids and bicarbonate in the presence and absence of insulin, and observed that in each case insulin stimulated the incorporation of radioactivity from the several amino acid precursors into protein. As the labelled amino acids in this work would be synthesized intracellularly, the authors concluded that the stimulatory effect of insulin on protein synthesis is independent of its stimulation of the uptake of amino acids. Further evidence in favour of this viewpoint was provided by Kostyo (1964), who used sodium-free medium for his experiments, as this had been found to prevent the effect of insulin on amino acid uptake. He found that insulin stimulated glycine incorporation into protein, even in the absence of sodium ions. Although the evidence seems very strong, Hider, Fern and London (1971) have still argued against the viewpoint that the effect of insulin on protein synthesis is indirect.

The development of cell-free systems for performing protein synthesis *in vitro* was the impetus for attempts to anaylse the effect of insulin on protein synthesis at the molecular level. The first muscle cell-free systems used were from heart muscle, because of the relative ease of homogenization of this tissue (Rampersad and Wool, 1965). However the effect of diabetes on heart muscle protein synthesis is less marked than its effect on skeletal muscle protein synthesis (Wool, Rampersad and Moyer, 1966; Rannels *et al*., 1970), suggesting that the regulation of protein synthesis in these two types of muscle is different. Indeed there are detailed differences between the results obtained by Wool and co-workers in their experiments with cell-free protein synthesis by heart muscle and skeletal muscle ribosomes. Little further work was done on heart muscle, however, and it has been decided to concentrate

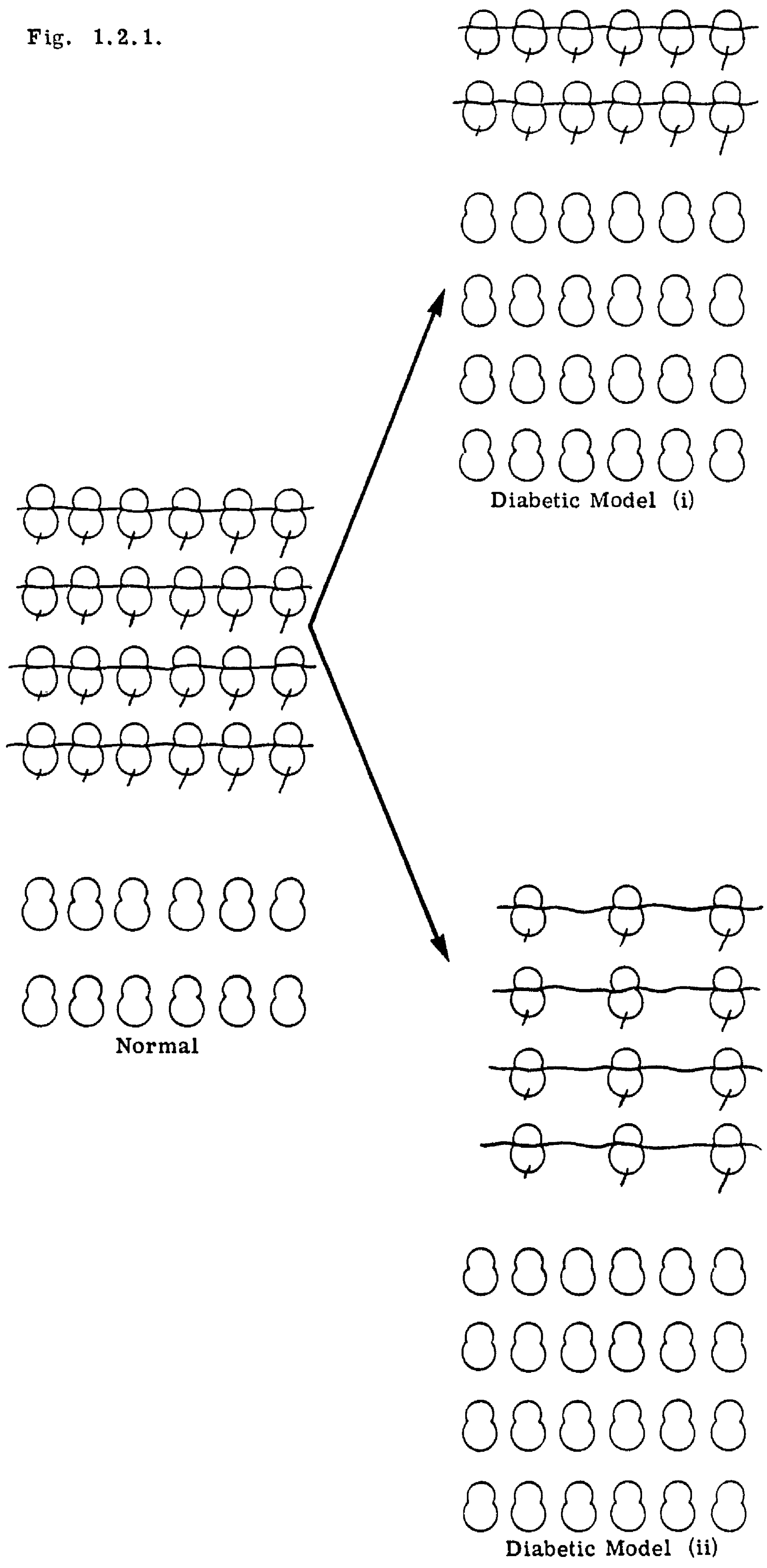
here on reviewing the results obtained with skeletal muscle.

When ribosomes isolated from the skeletal muscle of alloxan-diabetic rats were assayed for their ability to catalyse protein synthesis *in vitro*, they incorporated about 50% less radioactive amino acids into protein than ribosomes derived from normal rats, or from diabetic rats after treatment with insulin (Wool and Cavicchi, 1967). The effect of insulin was seen when it was injected into the animals *in vivo* or added *in vitro* to intact muscle tissue, but insulin did not affect ribosome function when added directly to ribosomes *in vitro*.

These results suggested that the ribosomes from the muscle of normal rats contain more mRNA than those from diabetic rats. Further evidence consistent with this idea came from studies of polysomes. Analysis on sucrose density gradients of ribosomes extracted from the skeletal muscle of normal and diabetic rats revealed that the muscle of diabetic rats contains a smaller proportion of fast sedimenting species (polysomes), and greater proportion of light species (monomers and dimers), compared to the muscle of normal rats (Stirewalt, Wool and Cavicchi, 1967).

It is possible to envisage the difference in polysomes of ribosomes obtained from normal and diabetic rats in two different ways (Fig.1.2.1). The ribosomes in the muscle of diabetic rats could either contain fewer polysomes (and hence less mRNA) than those in normal rats, but with these polysomes being of a similar size to those of the normal (Model (i)); or could contain smaller sized polysomes in which fewer ribosomes are attached to the same number of mRNA molecules as in the normal (Model(ii)). It is not clear which, if any, of these alternatives is correct; although

Fig. 1.2.1.



Models for polysomes in skeletal muscle

the observation of an increase in the number of ribosomal subunits in the skeletal muscle of diabetic rats (Rannels et al., 1970) tends to favour Model (ii), as this is consistent with a block in initiation which would be expected to lead to smaller polysomes (Lodish, 1971).

Model (ii) entails an equal amount of mRNA in the skeletal muscle of normal and diabetic rats, implying that the effect of insulin on protein synthesis is modulated via translational rather than transcriptional control. Although insulin does increase transcription in skeletal muscle (Wool and Munro, 1963), and diabetes does reduce the total RNA content of muscle (Wool, Stirewalt and Moyer, 1968; Pain and Garlick, 1974) and the number of ribosomes therein, there is indirect evidence that translational control is involved in the effect of insulin on the polysome profiles. Thus, administration of insulin to diabetic rats that had been pretreated with actinomycin D, in a concentration sufficient to block RNA transcription (Wool and Moyer, 1964), caused rapid reformation of polysomes and restoration of the activity of cell-free systems for protein synthesis (Stirewalt, Wool and Cavicchi, 1967). The speed with which insulin caused reformation of polysomes and restoration of the activity of cell-free systems for protein synthesis (<5 min with or without actinomycin D) was, in itself, strong circumstantial evidence for translational control. It should be pointed out that model (i) can also accommodate such translational control if there is non-polysomal mRNA sequestered somewhere in the cell.

The point at which the translational control was thought to operate was suggested by further cell-free experiments using added polyuridylic acid (polyU) as template. When polyU was added in vitro to skeletal

muscle ribosomes obtained from either normal or diabetic rats, it stimulated protein synthesis above that directed by the endogenous mRNA on the ribosomes. In the presence of excess polyU, the relative ability of ribosomes from normal and diabetic rats to synthesize polyphenylalanine was critically dependent on the magnesium concentration. At lower magnesium concentrations (below an effective concentration of about 10 mM) ribosomes from the skeletal muscle of diabetic rats incorporated less (^{14}C) phenylalanine than those of normal rats; whereas at higher magnesium concentrations they incorporated more radioactivity than ribosomes from normal rats (Wool et al., 1968).

The relative translation of polyU by the ribosomes at high magnesium concentration was consistent with the idea (previously mentioned) that the ribosomes from diabetic rats contain less mRNA, and hence can bind more polyU, than those from normal rats. However the superiority of the ribosomes from normal rats at low (and presumably more physiological) magnesium concentration was taken to indicate that there was a defect in the ribosomes from diabetic rats, and it was suggested that this defect was responsible for the difference in content of mRNA of the ribosomes, as its reversal by insulin was also rapid and not prevented by actinomycin D (Wool and Cavicchi, 1966).

Wool and co-workers performed a series of experiments to try to elucidate the nature of this defect in ribosomes from diabetic rats for translating polyU at low magnesium concentration. They showed that the defect resided in the 60S subunit. Reconstituted 80S ribosomes or those containing 60S subunits from the skeletal muscle of diabetic rats incorporated less (^{3}H) phenylalanine in the presence of

polyU than reconstituted ribosomes from the skeletal muscle of normal rats at low magnesium concentration (Martin and Wool, 1968). They then proceeded to examine the activity of normal and diabetic ribosomes in the various partial reactions of protein synthesis. They found no difference in the activity of peptidyl transferase (which is located on the 60S subunit) in either 60S or 80S ribosomes from normal and diabetic rats (Stirewalt and Wool, 1970). Nor was there any difference in GTP hydrolysis catalysed by elongation factor-2 (Leader, Wool and Leader, 1970), nor in the non-enzymic binding of turnip-yellow-mosaic virus to the ribosomes (Rolleston, Wool and Martin, 1970).

Further studies examined the polyU-dependent binding of (^{3}H) phenylalanyl-tRNA to reconstituted ribosomes from the 40S and 60S subunits of normal and diabetic rats, both in the absence (Wool and Rolleston, 1971; Castles, Rolleston and Wool, 1971), and in the presence of elongation factor-1 (Leader, Wool and Castles, 1971). In both of these studies it was found that at low magnesium concentration ribosomes from diabetic rats bound less (^{3}H) phe-tRNA than those from normal rats, and in the latter study the difference persisted even in the presence of saturating amounts of elongation factor-1. At high magnesium concentration, on the other hand, the binding of (^{3}H) phe-tRNA was greater in the ribosomes from diabetic rats. However these same studies showed that the superior ability of normal ribosomes to bind (^{3}H) phe-tRNA at low magnesium concentration was not due to an intrinsic difference in ribosomes, but merely to a difference in the amount of pre-existing peptidyl-tRNA on the 60S ribosomal subunits. This emerged from analysis of the products after alkaline hydrolysis of the (^{3}H) species bound to the ribosomes. At the high magnesium concentration the product was, as

expected, mainly (^{3}H) phenylalanine; but at the low magnesium concentration the product was a peptide derived from unlabelled peptidyl-tRNA linked to a C-terminal radioactive phenylalanine residue (Castles, Rolleston and Wool, 1971; Leader, Wool and Castles, 1971). From these studies it appeared that, at low concentrations of magnesium, phe-tRNA binds only to the acceptor site of ribosomes carrying peptidyl-tRNA in the P site, with the subsequent formation of a peptide bond between the two species. This would be because at low magnesium concentrations little or no initiation of the synthesis of peptide chains is possible either non-enzymically or with elongation factor-1. These conclusions were supported by other data. Thus, in these same studies, it was found that puromycin treatment of the ribosomes (to remove the endogenous peptidyl-tRNA) caused a vast reduction in their ability to bind (^{3}H) phe-tRNA and abolished the difference between the binding to ribosomes from normal and diabetic rats. Moreover Stirewalt, Castles and Wool (1971) showed that some peptidyl-tRNA does remain on dissociated 60S ribosomal subunits (consistent with the location of the apparent defect); and a difference in the amount of residual peptidyl-tRNA on ribosomes from the muscle of normal and diabetic rats was also consistent with previous experiments in which ribosomes showed a different capacity to react with (^{3}H) puromycin (Wool and Kurihara, 1967).

These studies, then, showed that the decrease in binding of (^{3}H) phe-tRNA to ribosomes extracted from the skeletal muscle of diabetic rats, at low magnesium concentrations, results from a decreased amount of endogenous peptidyl-tRNA on the ribosomes. The latter obviously reflects the decreased protein synthetic activity occurring on skeletal muscle ribosomes of diabetic rats *in vivo*. Thus the problem of the mechanism of the apparent

translational control of protein synthesis in the skeletal muscle of normal and diabetic rats *in vivo*, still remained. All that had been achieved was the finding of no intrinsic difference in the activity of ribosomes from normal and diabetic rats, consistent with comparison of their RNAs (Wool, Stirewalt and Moyer, 1968) and proteins (Low, Wool and Martin, 1969; Leader, Coia and Fahmy, 1978).

It must, however, be mentioned that, subsequent to the work described above, there have been renewed claims of an intrinsic functional difference in normal and diabetic ribosomes, even when these had been treated with puromycin. It was claimed that ribosomes containing 60S subunits from the muscle of diabetic rats translated mRNA from encephalomyocarditis virus at a slower rate than ribosomes containing 60S subunits from the muscle of normal rats (Wool, 1972). Furthermore 60S subunits from the muscle of diabetic rats were less effective in forming 80S couples, and the 80S ribosomes more susceptible to dissociation by initiation factor-3, than subunits or 80S ribosomes from normal rats (Wettenhall, Nakaya and Wool, 1974; Nakaya, Ranu and Wool, 1974).

In view of the artifactual nature of the previously claimed diabetic ribosomal 'defect', these results should be treated with some caution, especially as they were published in short communications which have not been followed up. Thus, although puromycin releases the peptide from peptidyl-tRNA, it is still possible that deacylated tRNA remains on the ribosomes (Wettenhall, Wool and Sherton, 1973); and this might influence certain of their activities. It is also worth pointing out that in none of these studies was it demonstrated that the supposed

defect was reversed by treatment with insulin.

Whether or not the ribosomes of the skeletal muscle of diabetic rats are modified, the results described earlier in this section provide fairly strong evidence that the, undisputed, alteration in protein synthesis may be modulated by translational control. However, because of the indirect nature of this evidence it seemed important to try to examine the question more directly; and this was the principal objective of the work described in this thesis. The approach adopted was to characterize the mRNA in the skeletal muscle of normal and diabetic rats and diabetic rats treated with insulin. As a background to this, some features of eukaryotic mRNA and the control of its translation are considered in the following sections of this Introduction.

1.3 EUKARYOTIC MESSENGER RNA

In living cells the genetic information encoded in the sequence of bases in the double helix of DNA is transcribed into a complementary sequence of RNA bases to form the molecule known as messenger RNA (mRNA), which is subsequently translated into protein.

The initial discovery of mRNA was made in bacterial cells. Hershey, Dixon and Chase(1953) observed that when E.coli cells were infected with T-even bacteriophages, bacterial DNA transcription stopped and new DNA molecules were formed. Hall and Spiegelman (1961) concluded from hybridization reactions that the RNA synthesized after infection was a T-even specific RNA. The concept that this newly synthesized RNA constituted a messenger between the DNA and the sites of protein synthesis was postulated by Jacob and Monod in 1961. These and other investigators showed that the viral-induced RNA became attached to pre-existing ribosomes but was metabolically much less stable than 16S and 23S rRNA.

The presence or absence of certain inducer molecules in rapidly growing bacteria, causes rapid alterations in the rate of synthesis of certain proteins. This rapid response to environmental changes has been shown to reflect rapid turnover of bacterial mRNAs which have been found to have half-lives of a few seconds to several minutes. In prokaryotic organisms, transcription is tightly coupled to translation, for association of mRNA with ribosomes, synthesis of polypeptide chains and degradation of mRNA may all begin before mRNA synthesis is completed (Adesnik and Levinthal, 1970).

Early attempts to identify mRNA in eukaryotes centred mainly on the nucleus and employed various criteria, none of which was totally satisfactory. For example, rapidity of labelling and relative instability compared to other types of RNA (Penman et al. 1963), heterogeneous sedimentation pattern (Henshaw et al., 1965) and possession of a "DNA-like" base composition (Davidson, 1969), were all taken to be characteristics of mRNA. Progress came when investigations shifted from looking for mRNAs in the nucleus to the polysomes (Warner, Knopf and Rich, 1963) in the cytoplasm, where mRNA directs the incorporation of amino acids into proteins. Although RNA active in stimulating the incorporation of amino acids into protein was found within polysomes (Singer and Leder, 1966; Noll, 1969), unequivocal evidence for a functional mRNA required the translation of the RNA into a specific protein product. This required a heterologous cell-free system for the translation of the mRNA and satisfactory subsequent characterization of the protein product. Perhaps the first such unambiguous translation of a specific eukaryotic mRNA was by Lockard and Lingrel (1969). They translated mouse globin mRNA in a crude lysate from rabbit reticulocytes. The synthesis of mouse globin β-chains was demonstrated by carboxymethyl cellulose chromatography of the cell-free product to distinguish the mouse globin from endogenous rabbit globin.

The reticulocyte lysate was subsequently used for translation of a wide variety of eukaryotic mRNAs such as immunoglobulin light chain (Stavnezer and Huang, 1971), myosin (Rourke and Heywood, 1972) and ovalbumin (Comstock et al., 1972) mRNAs. Although this cell-free system translates exogenous mRNA at a rapid rate, it suffers the presence of large quantities of endogenous (mainly globin) mRNA which compete with exogenous mRNA for

translation and dominate the products obtained.

Several other cell-free systems were therefore developed, the first being the Krebs II ascites tumour cell-free system (Mathews and Korner, 1970). Several mRNAs have been faithfully translated in ascites cell-free systems, e.g. lens crystallin mRNAs (Mathews et al., 1972) and histone mRNAs (Jacobs-Lorena, Baglioni and Borun, 1972). Although this system has a low endogenous activity, and allows high levels of incorporation of radioactivity, it is low in translational efficiency relative to the reticulocyte lysate (Osborn and Mathews, 1974). Furthermore, a major disadvantage of this system is the production of incomplete polypeptide chains either by premature termination or by nuclease cleavage of the mRNA (see, for example, Swan, Aviv and Leder, 1972).

Another cell-free system, which has some advantages over the ascites system, is the wheat germ cell-free system (Marcus, 1972; Roberts and Paterson, 1973). A wide variety of viral and eukaryotic mRNAs have been translated with fidelity in the wheat germ system, and high molecular weight products, such as the rat preproinsulin (Chan, Keim and Steiner, 1976), have been obtained. Unfortunately, the wheat germ system may also suffer from premature termination of translation, especially with longer mRNAs (see, for example, Burstein et al., 1976), and different batches of germ show considerable variation in activity (Marcu and Dudock, 1974).

The use of an intact whole cell, the oocyte of *Xenopus laevis* for translation of exogenous mRNA was pioneered by Gurdon and co-workers

(Lane, Marbaix and Gurdon, 1971). In this system amounts of purified mRNA as low as 1 ng can be injected and efficiently translated, sometimes for several days. Although there is much greater stability of mRNA in this system, it requires considerable technical proficiency to perform the injection, thus limiting its general use.

Recently Pelham and Jackson (1976) described a modification of the reticulocyte lysate system, which eliminates its high endogenous activity allowing easy detection of exogenous mRNA. This involves preincubation of the lysate with micrococcal nuclease (a calcium-requiring enzyme), followed by addition of an excess of a calcium-chelating agent to inactivate the nuclease and allow subsequent translation of exogenous mRNA.

The use of the cell-free systems, described above, to identify eukaryotic mRNAs was the first step in the characterization of the latter, and now much is known of the structure of eukaryotic mRNAs. In contrast to bacterial mRNAs which are polycistronic, eukaryotic mRNAs appear to be monocistronic containing no multiple initiation sites and coding for only one polypeptide. In addition to the coding sequence, eukaryotic mRNAs generally contain extra nucleotides at both the 5'-and 3'-ends of the molecules. Most eukaryotic mRNAs possess at their 3'-end a sequence of 50-200 polyadenylate residues (Brawerman, 1974). Exceptions identified to date include histone mRNAs (Schochetman and Perry, 1972b), about 30% of HeLa cell mRNAs (Milcarek et al., 1974) and certain sea urchin embryo mRNAs (Nemer et al., 1974). In polyA(+)RNAs the polyA region does not have a discrete size, but a range of different sizes are found to exist for a given mRNA. Moreover, no correlation appears to exist between the mRNA size and

the average size of its polyA region. Thus, Mondal et al. (1974) found no relationship between the size of polyA sequences in different sized mRNAs from different size-classes of chick embryonic muscle polysomes.

Sequence analysis has shown that rabbit β-globin mRNA consists of a coding region of 438 nucleotides, flanked by a 5'-non-coding region of 56 nucleotides and by a 3'-non-coding region of 95 nucleotides (Efstratiadis et al., 1977), in addition to the 3'-terminal polyA region. Chicken ovalbumin mRNA is 1,860 residues long, excluding the polyA region; and while the coding sequence is close to the 5'-end, it is 637 nucleotides from the 3'-end (McReynolds et al., 1978). Sequence analysis of nucleotides adjacent to the 3'-terminal polyA regions in six mRNAs (rabbit and human α- and β-globins, mouse immunoglobulin light-chain, and chicken ovalbumin) revealed regions of sequence homology, the most rigidly preserved one being the hexanucleotide AAUAAA at between 14 and 20 nucleotides from the start of the polyA region. The function of this sequence is unknown, but has been suggested to serve as a signal for termination of transcription (Proudfoot, Cheng and Brownlee, 1976).

Most eukaryotic mRNAs possess at their 5'-end a modified 'cap' structure, having the general form $m^7G(5')ppp(5')Xp$ (Fig. 1.3.1), the terminal 7-methylguanosine (m^7G) and the penultimate nucleotide being joined by their 5'-OH groups through a triphosphate bridge (for review, see Shatkin, 1976). Three types of cap structure have been identified: type 0 ($m^7G(5')ppp(5')X$), type 1 ($m^7G(5')ppp(5')XmY$) and type 2 ($m^7G(5')ppp(5')XmYmZ$), differing in the number of 2'-O-methylated penultimate nucleotides. Cap structure type 2 probably arises by a secondary

Fig. 1.3.1.

Structure of the 5'-terminal 'cap' of eukaryotic mRNAs

methylation of the mRNA in the cytoplasm (Perry and Kelley, 1976), whereas types 0 (found in some viruses and lower eukaryotes such as yeast and slime mould) and 1 are thought to arise in the nucleus (see below). In addition to the 5'-methylated nucleotides, internal methyl^{6}adenine residues (m^6Ap) and smaller amounts of methyl^{5}cytosine (m^5Cp) have been demonstrated in mRNAs of some eukaryotes (see, for example, Desrosiers, Friderici and Rottman, 1974).

There is some evidence that eukaryotic mRNAs contain a large degree of secondary structure. Thus globin mRNA normally migrates on polyacrylamide gels as a diffuse band but in the presence of formamide it migrates as a sharp band (Lanyon et al., 1972); and ovalbumin mRNA sediments at about 15S, but migrates on polyacrylamide gels more slowly than 18S rRNA (Palacios et al., 1973).

It is thought that the mRNA in eukaryotes is derived by post-transcriptional modification of larger RNA precursor molecules, known as heterogeneous nuclear RNA, HnRNA, (see, for example, Lewin, 1975). HnRNA molecules are generally larger than cytoplasmic mRNA molecules (between 5,000 and 50,000 nucleotides), with sedimentation values ranging from 10 to 90S (Penman et al., 1968), and constitute about 1% of total cellular RNA in HeLa cells (Darnell, 1968). They are characterized by rapid labelling kinetics, and the majority of these molecules (up to 90%) never enters the cytoplasm. This implies a rapid rate of turnover, and half-lives of 5-60 min have been estimated for HnRNA in the nucleus. Due to the rapid decay rate of HnRNA, and to difficulties with radioactive pulse-chase experiments, it has not been possible to establish a direct precursor-product relationship between

HnRNA and mRNA. However, both types of molecules share some common characteristics which suggest that cytoplasmic mRNA is derived from the nuclear HnRNA. These characteristics include insensitivity of synthesis to low concentrations of actinomycin D, and the presence of a polyA sequence about 200 bases long at the 3'-end of the molecule (Edmonds et al., 1971). Perry et al. (1975a) reported a sequence homology of 20-30% between HnRNA and mRNA in mouse L cells. Melli and Pemberton (1972) have used complementary RNA to purified globin mRNA (prepared in vitro using the enzyme Micrococcus lysodeikticus RNA polymerase) to demonstrate that globin messenger sequences are present in duck reticulocyte HnRNA. Also studies of HnRNA of mouse L cells indicate that these molecules contain 5'-caps (Type 1) and internal N^6 methylated adenine residues (Perry et al., 1975b).

The presence of the 5'-cap structure and the 3'-polyA region in both HnRNA and mRNA posed the problem of how the larger HnRNA molecules could possibly be precursors of the smaller mRNA molecules. An answer to this problem was suggested when it became apparent that non-coding 'intervening sequences' interrupt the coding regions in the genes for several mRNAs (Jeffreys and Flavell, 1977; Brack and Tonegawa, 1977; and Tilghman et al., 1978a). It would clearly be possible to explain the characteristics of the HnRNA, mentioned above, if these intervening sequences were transcribed and only later cleaved from the RNA, which would be re-spliced giving the mRNA. Such a mechanism has recently gained support from the hybridization of the 15S RNA precursor of mouse β-globin mRNA to a mouse β-globin gene containing an intervening sequence of 550 base pairs (Tilghman et al., 1978b).

Only 20-40% of HnRNA molecules contain a polyA segment, and nuclear polyA is not quantitatively converted to cytoplasmic polyA, for some of this

polyA is degraded in the nucleus (Jelinek et al., 1973). In the nucleus polyA synthesis occurs post-transcriptionally by stepwise addition of adenylate residues to HnRNA. Thus, actinomycin D does not inhibit the synthesis of polyA (Darnell et al., 1971), and there is no polydT tract in the DNA sequence of the mouse β-globin gene (Konkel, Tilghman and Leder, 1978). PolyA synthesis can also occur in the cytoplasm by addition of adenylate residues to pre-existing polyA in mRNA. This has been demonstrated in sea urchin embryos immediately after fertilization (Slater et al., 1973).

Heterogeneous nuclear RNA has been found to be complexed with proteins (for review see Van Venrooij and Janssen, 1978). While the exact function of these proteins is not yet clear, it is possible that they may be involved in the protection, stabilization and processing of the RNA during its transport from the nucleus to the cytosplasm. Messenger RNA in the cytoplasm has also been found complexed with proteins in messenger ribonucleoprotein particles, mRNPs (for review see Williamson, 1973) and these are presumed to derive from the complexes of HnRNA and protein. The mRNA in polysomes is also complexed to non-ribosomal proteins, and polysomal mRNP particles can be released by treatment of polysomes with magnesium chelating agents such as EDTA (Huez et al., 1967). Although the role of the protein associated with polysomal mRNA is not yet known, it is possible that it protects the polyA region from ribonuclease digestion (Blobel, 1973).

In contrast to prokaryotic mRNA, eukaryotic mRNA is relatively stable. This can be deduced from the prolonged synthesis of globin in reticulocytes where no functional nucleus exists (Marks et al., 1962), the prolonged

translation of mRNA injected into frog oocytes (Gurdon, 1973) and from the early development of sea urchin embryos where protein synthesis utilizes preformed maternal mRNA (Gross and Cousineau, 1964). The half-life of reticulocyte globin mRNA in the anaemic mouse was estimated to be approx. 15-17 hr (Hunt, 1974), and in HeLa cells two separate classes of mRNAs with half-lives of 7 and 24 hr have been reported (Singer and Penman, 1973), although another study reported a half-life of several days (Murphy and Attardi, 1973).

The functional significance of polyA has not yet been established. It has been suggested that polyA is required for the transport of mRNA from the nucleus to the cytoplasm, for studies with cordycepin showed that inhibition of polyA synthesis also prevents the appearance of mRNA in the cytoplasm (Weinberg, 1973). Clearly the existence of some mRNAs which lack polyA, would argue against this idea unless there were a separate mechanism for the transport of these mRNA molecules from the nucleus to the cytoplasm. Some evidence for such a distinct transport mechanism emerges from the observations that polyA(+)RNA molecules enter into the cytoplasm after a delay of 15-20 min, whereas histone mRNA, which lacks polyA, is rapidly transported to the cytoplasm (Adesnik and Darnell, 1972).

PolyA does not appear necessary for translation of mRNA in cell-free systems, for histone mRNAs are actively translated in Krebs ascites systems (Gross *et al.*, 1973a), and deadenylated rabbit globin mRNA is translated in several cell-free systems with the same efficiency as intact globin mRNA (Sippel *et al.*, 1974). However, polyA may act to stabilize the mRNA molecule by protecting the 3'-terminus from ribonucleolytic attack. Deadenylated globin

mRNA appears to be much less stable than adenylated globin mRNA for translation in Xenopus oocytes (Huez et al., 1974), and reinitiation of translation of deadenylated ovalbumin mRNA was also found to be much less efficient than that of polyadenylated ovalbumin mRNA (Doel and Carey, 1976). In HeLa cells the polyA sequence that initially appears in the cytoplasmic mRNA appears to have the same size (200 nucleotides) as the polyA in HnRNA (Greenberg and Perry, 1972). However, in the cytoplasm the polyA sequence becomes progressively shorter as the mRNA ages (Sheiness and Darnell, 1973). In view of the shortening of the size of polyA with time, it has been suggested that there is a minimum length of polyA that is critical for mRNA stability, and the eventual loss of this would leave the RNA chain vulnerable to exonucleases (Brawerman, 1973). However, it is not yet clear whether the degradation of mRNA proceeds by progressive exonucleolytic attack, or by multiple endonucleolytic cleavage at specific internal sites. Moreover, it has been pointed out that mRNA decays in an exponential fashion, which would imply that every molecule (young and old) is equally susceptible to inactivation (Perry and Kelley, 1973).

The function of the 5'-cap structure is also not completely clear. There is some evidence indicating that it is involved in the formation of mRNA-ribosome complexes and consequently in the initiation of protein synthesis. Cap analogues such as m^7Gp, m^7GTP and $m^7GpppXm$ have been shown to inhibit ribosomal binding and translation of several viral and cellular capped mRNAs in eukaryotic cell-free systems in vitro (see, for example, Canaani, Revel and Groner, 1976; Suzuki, 1976). Shafritz et al. (1976) have reported that m^7Gp inhibits directly the interaction of eukaryotic initiation

factor eIF-4B with capped vesicular stomatitis virus mRNA and HeLa histone mRNA, but not with encephalomyocarditis virus RNA which lacks the 5'-cap structure. On the other hand, it was found that decapping of some viral mRNAs (e.g. vesicular stomatitis virus) and cellular mRNAs (e.g. globin mRNA) did not preclude their translation in cell-free systems (Rose and Lodish, 1976; Held, West and Gallagher, 1977). This - together with the absence of 'cap' from encephalomyocarditis RNA - argues against the necessity of cap for translation. The 5'-cap structure may contribute to the stability of those mRNAs in which it occurs, by protecting the 5'-end of the mRNA from exonucleolytic attack. Thus reovirus mRNAs with blocked 5'-termini were found more stable than unblocked mRNAs when injected into oocytes or when translated in cell-free systems (Furuichi et al., 1977).

The functional significance of the modified methyl^{6}adenosine and methyl^{5}cytidine residues that are found in some eukaryotic mRNAs is much less clear than that of the polyA region and the 5'-cap. It has been suggested that such residues constitute recognition sites for RNA processing and/or splicing enzymes, or that they have a function in translation (Derman and Darnell, 1974); but there is no evidence to support these ideas.

In the last ten years there has been a vast increase in work on mammalian mRNAs. It is therefore worthwhile considering the different preparation methods that have made this possible. A mRNA-rich preparation is usually derived from the RNA extracted from polysomes with phenol, chloroform and a buffer containing sodium dodecyl sulphate. It has been observed that during phenol extraction of polysomes at neutral pH or a relatively high concentration of mono- or divalent cations, the polyA sequences tend to

aggregate non-specifically with the denatured proteins at the interphase layer between the organic and the aqueous phases (Brawerman et al., 1972). Inclusion of chloroform in the extraction medium prevents this aggregation (Perry et al., 1972).

Several procedures have been established for purification of mRNA. One of the earliest involved the sedimentation of the RNA through a sucrose density gradient to separate the mRNA from the rRNA. This technique proved to be useful in isolation of mRNAs from differentiated cell types synthesizing predominantly one protein and having a mRNA with a different S value from the rRNA (e.g. immunoglobulin light chains in myeloma cells, α- and β-haemoglobins in reticulocytes, and α-crystallins in the lens of the eye). Messenger ribonucleoprotein particles (usually released from polysomes by EDTA treatment) often sediment in a sucrose gradient at regions that are distinct from the other RNA species, and have therefore been used as a source of mRNA (see, for example, Lingrel et al. , 1971; Berns and Bloemendal, 1974).

Electrophoresis of RNA in gels containing polyacrylamide or agarose has been used to separate RNA species and estimate their molecular weights (Bishop et al., 1967). More recently electrophoresis in gels containing formamide has been used to separate and recover α- and β-globin mRNAs (Gould and Hamlyn, 1973).

The existence of the polyA sequence at the 3'-end of most eukaryotic mRNAs, however, has provided the most useful and generally applicable tool for the isolation of mRNA from other RNA species. PolyA and polyA-containing mRNA

can be adsorbed on nitrocellulose filters at high ionic strength, and eluted in salt-free buffer (Brawerman et al., 1972). The length of the polyA sequence must be at least 50-60 nucleotides to be adsorbed to the nitrocellulose filters. PolyA can also be adsorbed on glass-fibre filters to which polyU has been coupled by ultraviolet irradiation (Sheldon et al., 1972). However, affinity chromatography on columns prepared by cross-linking synthetic homopolymers to a supporting matrix (e.g. oligo dT-cellulose; polyU-sepharose), has been the most widely used method of isolating polyA-containing mRNAs (see, for example, Aviv and Leder, 1972; Adesnik et al., 1972).

Immunoprecipitation is useful in separating specific mRNAs from a mixture of other mRNAs. Purified antibodies to the protein whose mRNA is to be extracted are added to the isolated polysomes, leading to a specific antigen-antibody reaction and consequent precipitation of the desired polysomes. This method has been adopted in the isolation of catalase - and serum albumin - synthesizing liver polysomes (Uenoyama and Ono, 1972).

Other methods of obtaining preparations which are rich in specific mRNAs are available under certain circumstances. For example, histone mRNAs can be obtained from cultured mammalian cells during the period of DNA synthesis, or from sea urchin embryos in the early embryonic stages where a class of small polysomes engaged in histone synthesis predominates (Robbins and Borun, 1967; Kedes and Gross, 1969). Moreover, the synthesis of some mRNAs can be induced by specific hormones, for example, the synthesis of avidin mRNA in hen oviduct is induced by progesterone (O'Malley et al., 1972), and the synthesis of ovalbumin mRNA in chick oviduct is

induced by oestrogen (Rhoads, McKnight and Schimke, 1971).

The concern of the work described in this thesis is with mRNA from adult skeletal muscle. It is therefore of relevance to consider what is known of muscle mRNAs. Most of the work to date concerned with muscle mRNAs has been conducted on embryonic muscle, particularly chicken, and on embryonic muscle cell cultures. Although the muscle contractile proteins myosin, actin, tropomyosin and troponin are now well characterized, only the mRNA of myosin heavy chain and that of actin have been extensively studied. The successful isolation of myosin heavy chain mRNA relied strongly on its large size, which enabled its separation from the bulk of cellular messengers. Heywood and Nwagwu (1968, 1969) isolated from polysomes of embryonic chick muscle a fraction of RNA sedimenting at 26S, which when added to cell-free systems directed the synthesis of the large subunit of myosin. When this RNA fraction had been separated from rRNA, its migration on gel electrophoresis indicated a sedimentation coefficient of 32S (Morris et al., 1972). Similar results were obtained by Sarkar et al. (1973) and Mondal et al. (1974), who also found that the polyA region of myosin heavy chain mRNA consists of about 170 nucleotides. Later studies (Heywood, Kennedy and Bester, 1975; Bag and Sarkar, 1976) reported the isolation of myosin mRNA from cytoplasmic 'free' ribonucleoprotein particles (not derived from polysomes). The criterion of size could not be exploited to isolate actin mRNA, and so work on this has not involved such pure material as for studies on myosin mRNA. Bag and Sarkar (1975) have found actin mRNA in cytoplasmic non-polysomal mRNP particles in chicken embryonic muscle. Other studies showed that an 8-12S fraction obtained from small polysomes of 19-day embryonic chick red muscle was able to direct the synthesis of myoglobin

in a homologous cell-free system (Thompson, Buzash and Heywood, 1973).

Several experiments suggest that in muscle cells the majority of messengers contain polyA. Kaufman and Gross (1974), using hybridization with (^{3}H) polydT, estimated that most mRNA in the polysomes of fused myotubes contains polyA. Whalen and Gros (1978) have reported that the great majority of mRNA from either prefusion or postfusion cultures of foetal calf myoblasts was polyA(+)RNA; quantitative determinations showing that about 70-90% of the actin mRNA is polyA(+)RNA. It is of interest in this respect to mention that multiple genes have been found for actin (Elzinga _et al._,1976), and Whalen, Butler-Browne and Gros (1976) found that calf embryo muscle cells contain α-(muscle specific), and β- and γ-actins (also found in non-muscle cells). It appears that α- and γ-actin mRNA molecules are poly-adenylated, whereas β-actin mRNA possesses a continuous spectrum of polyA lengths, some of which are very short (Hunter and Garrels, 1977).

1.4 TRANSLATIONAL CONTROL OF EUKARYOTIC PROTEIN SYNTHESIS

In prokaryotes, control of the rate of protein synthesis generally occurs at the stage of transcription, and indeed transcription and translation are tightly coupled. In eukaryotes, however, the messenger RNA is much longer lived (Singer and Penman, 1973) and the sites of transcription and translation are separated by the nuclear membrane. It might therefore be advantageous for the eukaryotic cell to be able to control directly the rate of protein synthesis, and indeed there is evidence for several types of translational control.

There are a number of metabolic circumstances which cause a decrease in the rate of protein synthesis, disaggregation of polysomes and concomitant appearance of inactive monosomes in the cytoplasm. When the cells are returned to normal conditions, the cytoplasmic monosomes re-enter polysomes and protein synthesis resumes. This occurs rapidly, and in the presence of actinomycin D, suggesting that pre-existing mRNA is being used, and that control is being exerted at the translational stage.

The first example of this type of control is when mammalian cells are deprived of one of the essential amino acids, leading to a block in the initiation of protein synthesis (Vaughan et al., 1971), and a breakdown of polysomes (Lee et al., 1971). It has been suggested that amino acid starvation might affect the charging of tRNA which in turn blocks the initiation of protein synthesis. Other examples of this type of control include serum deprivation of cultured cells (Hassell and Engelhardt, 1973), the arrest of protein synthesis at mitosis (Fan and Penman, 1970), the

control of globin synthesis by haemin (Zucker and Schulman, 1968), and the inhibition of the initiation of globin synthesis in reticulocyte lysates by double-stranded RNA (Ehrenfeld and Hunt, 1971) or by oxidized glutathione (Kosower et al., 1972).

Apart from the case of insulin and skeletal muscle, discussed in detail in section 1.2, above, there is some evidence that other hormones may also exert translational control of protein synthesis. Examples are the effects of adrenocorticotrophic hormone on adrenal protein synthesis (Garren et al., 1965); thyroid stimulating hormone on thyroid protein synthesis (Lecocq and Dumont, 1972) and growth hormone on liver protein synthesis (Barden and Korner, 1972).

A common mechanism of controlling protein synthesis has recently been elucidated for some of the above examples. In reticulocyte lysates, incubated without addition of haemin, protein synthesis proceeds for few minutes and then suddenly stops. Fractionation of the reticulocyte supernatant led to the isolation of a protein that is responsible for the inhibition of protein synthesis in the absence of haemin. This is a cyclic AMP-independent protein kinase, the substrate of which is the smallest subunit (mol.wt. 35,000) of the initiation factor, eIF-2 (Farrell et al., 1977; Farrell et al., 1978). The inhibitor is formed from a proinhibitor by an unknown mechanism (Maxwell et al., 1971) and it catalyses the incorporation of one to two phosphate groups into each molecule of eIF-2. In crude systems, it has been shown that the cyclic AMP-independent repressor inhibits the formation of the {eIF-2 . met-tRNA$_f$. GTP . 40S} ribosomal complex in the presence of ATP. Despite extensive

circumstantial evidence that the phosphorylation of eIF-2 was responsible for the inhibition of protein synthesis, phosphorylated eIF-2 was as active as unphosphorylated eIF-2 in cell-free assays for the formation of the 40S initiation complex. Recently, however, another factor has been isolated that stimulates the activity of eIF-2. Phosphorylation of eIF-2 abolishes the effect of the stimulating factor (Ranu et al., 1978; De Haro and Ochoa, 1978), suggesting a molecular explanation for the inhibition of protein synthesis in vivo. A similar mechanism for inhibiting protein synthesis appears to operate in reticulocyte lysates following the addition of double-stranded RNA (Farrell et al., 1977), or oxidized glutathione (Clemens et al., 1975), although distinct protein kinases are involved. This type of control is not restricted to reticulocytes, as it also appears to occur in other cells (Clemens et al., 1976).

There are some other examples of translational control which fall outside the general scheme outlined above. One such example entails an aspect of the antiviral state induced by interferon treatment. Exposure of viral-infected cells to interferon prevents the translation of viral mRNA while the translation of host mRNA is not affected (Yakobson et al., 1977). The mechanism by which interferon exerts this effect has not yet been clarified. There are a number of different effects on the protein synthetic machinery but none of them can easily explain the specificity of interferon against viral protein synthesis. These effects include the deacylation of leu-tRNAs (Sen et al., 1976), the inhibition of mRNA methylation and capping (Sen et al., 1977), the activation of an eIF-2 protein kinase - enhanced by preincubation of the cells with double-stranded RNA - and the phosphorylation of a ribosome-associated protein

of 67,000 mol. wt. (Revel et al., 1977). A heat-stable small molecular weight inhibitor of translation has recently been isolated from the cell sap of interferon-treated cells. This has been identified as an oligonucleotide, pppA(2')p(5')A(2')p(5')A, (Kerr and Brown, 1978) which is synthesized from ATP by a large thermolabile enzyme requiring double-stranded RNA. This small oligonucleotide activates a ribonuclease (Ratner et al., 1977; Schmidt et al., 1978), but this is active against host mRNAs as well as viral mRNAs. Moreover it has been observed that when interferon is given to cells infected with SV40 virus, the translation of the viral mRNA stops and no degradation of the untranslated mRNA occurs (Yakobson et al., 1977). Because attempts, so far, have failed to reveal an interferon-induced inhibitor that is specific for viral protein synthesis in vitro, it has been suggested that there is no such inhibitor, but the very features (whatever these may be) that give viral protein synthesis a selective advantage in vivo also make this more vulnerable than host protein synthesis to the action of the inhibitor(s) induced by interferon (Metz, 1975).

Clearly the inhibitory effects of different viruses on host cell protein synthesis need not, and probably do not, operate by a common mechanism. Attempts to elucidate the mechanism of inhibition in encephalomyocarditis virus infection have had some success. It has been found that the encephalomyocarditis viral RNA out-competes host mRNA for initiation factor eIF-4B in vitro (Golini et al., 1976), and this has been suggested as the mechanism operating in vivo. An alternative mechanism has, however, been proposed. This entails an effect of the virus on the permeability of the host cell membrane to monovalent cations, and a

greater efficiency of translation of viral mRNA under the resulting altered ionic conditions (Carrasco and Smith, 1976). These investigators found that addition of sodium chloride inhibited the translation of globin mRNA or mouse polyA-containing RNA in a cell-free system from ascites cells, while it stimulated the translation of encephalomyocarditis viral mRNA under the same conditions. Moreover, they found that at the onset of viral protein synthesis, the ability of the cell to take up $^{86}Rb^{+}$, a potassium ion analogue, becomes drastically impaired, suggesting a disruption of the normal Na^{+}/K^{+} gradient. The mechanism proposed by Carrasco and Smith is consistent with a previous observation that poliovirus capsid protein is required to produce the inhibition of host protein synthesis (Steiner-Pryor and Cooper, 1973). Egberts et al. (1977), however, have found that the change in ionic permeability in mengovirus-infected cells did not occur until after host protein synthesis had stopped. Moreover, the differential translation of viral and host mRNAs at high ionic strength has not been found with other viruses (Stevely and McGrath, 1978). Thus the generality of the mechanism proposed by Carrasco and Smith is in doubt.

An interesting case of the control of protein synthesis during the S phase of the cell cycle involves the synthesis of histones. Inhibition of DNA synthesis causes rapid disaggregation of polysomes and degradation of histone mRNAs in HeLa cells (Borun et al., 1975); although hybridization studies showed that histone mRNA is still synthesized in the absence of DNA synthesis. This suggests that a regulatory mechanism may exist to degrade histone mRNAs in the cytoplasm in the absence of DNA synthesis (Melli et al., 1977).

Control of protein synthesis also appears to operate in muscle cells during myogenesis. Thus, Buckingham *et al.* (1974, 1976) found that mRNA for myosin heavy chain was present in prefusion myoblasts from primary cultures of foetal calf muscle but was not translated until after fusion. The untranslated prefusion mRNA had a much shorter half-life than the translated postfusion mRNA, but the change in half-life appeared to precede the utilization of the mRNA.

There are a number of examples of translational control involving the utilization of pre-existing mRNA following the fertilization of eggs or during activation of dormant cells. In sea urchin eggs following fertilization, there is an increase in the rate of translation of the stored (maternal) histone mRNAs (Gross *et al.*, 1973b). A similar phenomenon occurs following hydration of *Artemia salina* cysts. In this latter case there is activation and synthesis of initiation factor eIF-2 (Lee-Huang *et al.*, 1977), but this may merely be associated with, rather than responsible for, the utilization of the mRNA.

It appears that the untranslated mRNA is often stored during translational inhibition of protein synthesis in the form of non-polysomal mRNP particles, which appear to be distinct from the mRNP particles that can be generated by treatment of polysomes with EDTA, and from the mRNP particles transporting the mRNA from the nucleus to the cytoplasm. These storage mRNP particles accumulate in many conditions where there is translational inhibition of protein synthesis, and disappear when the rate of protein synthesis is restored (Lee, Krsmanovic and Brawerman, 1971; Schochetman and Perry, 1972a; Terada *et al.*, 1972; Christman *et al.*, 1973; Rudland, 1974; Sonenshein and Brawerman, 1977). It

also appears that mRNA, stored in a dormant form in the cytoplasm of unfertilized eggs, exists as mRNP particles. Thus, maternal 20-40S mRNP particles from unfertilized sea urchin eggs directed the synthesis of histones in a heterologous cell-free system (Gross et al., 1973). Such 'masked' mRNAs were also found in wheat embryos (Marcus, 1969), cotton seedlings(Ihle and Dure, 1972), myoblasts (Heywood, Kennedy and Bester, 1975; Buckingham et al., 1976) and in immature erythroid cells (Marks and Rifkind, 1972).

Although the protein components of mRNP particles might be responsible for keeping the mRNAs in their inactive form, it has been suggested that an RNA species, translational control RNA (tcRNA), has this role. Bester, Kennedy and Heywood (1975) described a tcRNA molecule, obtained from the dialysate of mRNP particles from embryonic chick muscle. This tcRNA molecule contains oligoU sequences sufficient to form small hybrids to synthetic polyA. It has been suggested that this oligoU-rich tcRNA base pairs with the polyA sequence of mRNA in the mRNP particle, thereby inhibiting its translation. More recently, it has been shown that purified tcRNA isolated from myosin mRNP particles from embryonic chick muscle exhibited translational inhibition of myosin as well as globin mRNAs in wheat germ cell-free systems, although the inhibition of globin mRNA translation was less marked (Kennedy, Siegel and Heywood, 1978). A tcRNA molecule of about 20 nucleotides, rich in U and C, has also been described in Artemia salina cysts and has been suggested as a factor in maintaining their dormancy (Lee-Huang et al., 1977). In contrast, a class of small RNA molecules was isolated from a reticulocyte ribosomal wash (Bogdanovsky, Hermann and Schapira, 1973), and from polysomal mRNP particles of embryonic chick muscle (Bester, Kennedy and Heywood, 1975)

that has an apparent stimulatory effect on translation.

It is possible that a mechanism operates within individual cells to produce differential translation of different mRNAs because of different affinities or requirements of these for the various initiation factors. It has already been mentioned that, in extracts from mouse Krebs ascites cells, encephalomyocarditis virus RNA is translated more efficiently than host mRNA, because of a greater affinity for initiation factor eIF-4B. Furthermore, Wigle and Smith (1973) isolated a factor (I_{EMC}) from Krebs ascites cytosol which stimulated the translation of encephalomyocarditis virus RNA but appeared to have no effect on the translation of globin mRNA. This factor, however, is not specific for encephalomyocarditis virus RNA, but is in fact initiation factor eIF-4A, which is also required for the translation of globin mRNA (Staehelin et al., 1975). The apparent specificity was due to the greater requirement of encephalomyocarditis virus RNA for this factor. Such different requirements for initiation factors seem also to occur between different cellular mRNAs. Thus, in rabbit reticulocytes, the synthesis of α- and β-globin chains is maintained at a ratio of 1:1 by different rates of initiation, to compensate for the fact that the amount of α-globin mRNA exceeds that of β-globin mRNA (Lodish, 1971). The observation that initiation factor eIF-4B, and to a lesser extent eIF-4A, relieves the competition between α- and β-globin mRNAs in a purified ribosomal system from reticulocytes (Kabat and Chappell, 1977) suggests that β-globin mRNA has a higher affinity or a lower requirement for the initiation factors than does α-globin mRNA.

In the light of these observations, it is perhaps possible to resolve the

controversy over the claims that specific initiation factors are required for the translation of different mRNAs. In reticulocyte lysate or wheat germ cell-free systems, it has been reported that the translation of myosin heavy chain mRNA from embryonic chick muscle requires the addition of initiation factor eIF-3 prepared from muscle, that from reticulocytes being ineffective. An analogous situation has been found for the translation of globin mRNA, where reticulocyte initiation factor eIF-3 is specifically required (Heywood, 1969; Rourke and Heywood, 1972; Kennedy and Heywood, 1976). However, Przybyla and Strohman (1974), and Mondal *et al.* (1974), by apparently translating chick myosin heavy chain mRNA in a rabbit reticulocyte lysate, have demonstrated that no tissue-specific initiation factor is required to translate this mRNA. It is possible, however, that the observations of Heywood and co-workers may have been due to their eIF-3 preparations being impure and containing different amounts of other contaminating initiation factors, if optimal translation of myosin mRNA requires a different concentration of a given initiation factor than globin mRNA. The cruder but more active cell-free systems used by other workers may have contained sufficient initiation factors to have obscured such differences. It should, however, be pointed out that pure initiation factor eIF-3 comprises about nine polypeptide chains (Schreier *et al.*, 1977). Thus it is possible that this factor has a different complement of subunits in different tissues and that this might modify the affinity of initiation factor eIF-3 towards different mRNAs.

CHAPTER 2

EXPERIMENTAL

2.1 MATERIALS

2.1.1 Biological

The rats used in this study were male albinos of the Wistar strain, weighing approximately 130-150 g. They were housed and bred in the Animal Unit of the Department of Biochemistry and fed on diet 41B (Oxoid Ltd).

2.1.2 Chemical

2-mercaptoethanol, 2,5-diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), 2,5-bis-{2-(5-tert-Butylbenzoxazolyl)}-Thiophene (BBOT), and acrylamide were from Koch-Light Laboratories Ltd., Colnbrook, Bucks: toluene, methoxyethanol, sodium deoxycholate, dithiothreitol, diethylpyrocarbonate, and formamide were from British Drug Houses Chemicals, Poole, Dorset; bovine pancreatic deoxyribonuclease (EC 2.1.4.5 - electrophoretically purified) was purchased from Worthington Biochemical Corporation, Freehold, N.J., USA; cycloheximide, calf thymus DNA, yeast RNA, ribonuclease-free sucrose, dimethylsulphoxide, crystalline bovine pancreatic ribonuclease (EC 2.7.7.16), and alloxan monohydrate (Batch No. A-8128) were from Sigma Chemical Co., St. Louis, Mo., USA; lubrol WX and N-ethylmaleimide were obtained from Grand Island Biological Company N.Y., USA; crystalline bovine plasma albumin was purchased from Armour Pharmaceutical Company, Eastbourne, England; and sodium dodecyl sulphate was from Bio-Rad Laboratories Richmond, California, USA. Glucagon-free insulin (porcine zinc insulin crystals, lot 615-D63-10), assaying 24 units/mg and containing less than 0.003% glucagon, was a gift of Dr. Otto Behrens, Eli Lilly Research Laboratories. Dextrostix and Clinistex reagent strips were purchased from the Ames Company; reagent kits for quantitative determination of

blood glucose and creatine phosphokinase (EC 2.7.3.2) were from Boehringer Mannheim Company; N,N'-methylene bisacrylamide, N,N,N',N', tetramethylethylenediamine (TEMED), ammonium persulphate, bromophenol blue and X-ray film (Kodak X-Omat R) were obtained from Eastman-Kodak Co., Rochester, N.Y., USA; and streptozotocin {2-deoxy-2(3-methyl-3-nitroso ureido)-D-glucopyranose} was kindly donated by the Upjohn International Incorporated , Kalamazoo, Mich., USA. Poly (^{3}H) uridylic acid (specific activity 61.9 μCi/μmole P), in 50% ethanol solution and poly-adenylic acid (K^+ salt) were purchased from Miles Laboratories Inc., Elkhardt, Ind., USA; (^{3}H) phenylalanine (specific activity 5 Ci/mmole) and (^{35}S) methionine (specific activity at the time of use, 1080 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, UK; and Poly U-Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. All other chemicals were purchased from British Drug Houses Ltd. and were, where possible, 'Analar' grade. Solutions were made in glass-distilled, deionized, sterile water. All glassware was acid-washed and baked at 180°C for 12 hr. to destroy any adsorbed ribonuclease.

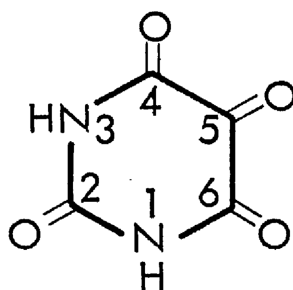
2.2. CHARACTERIZATION OF EXPERIMENTAL DIABETES

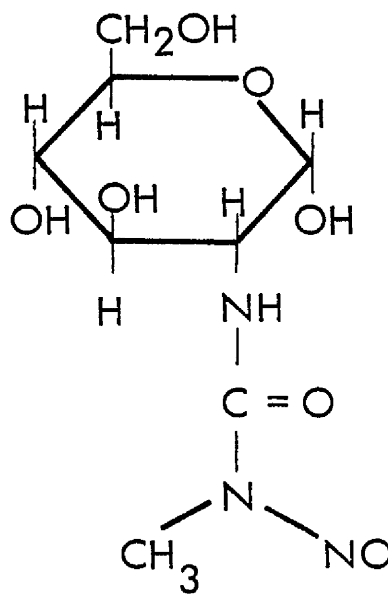
2.2.1 Induction of diabetes

In some experiments diabetes was induced by rapid intravenous injection (o.2 ml) of alloxan monohydrate (60 mg/kg body weight) into rats that had been starved overnight, the drug being dissolved in sterile saline just prior to use. The animals were killed 2 or 3 days later.

Diabetes was also induced by intravenous injection (0.2 ml) of strepto-zotocin (50 mg/kg body weight) into rats that had been starved overnight,

Fig. 2.2.1.1.





Alloxan **α-Streptozotocin**

Chemical structure of the two diabetogenic β-cytotoxins

and the animals were killed 2 or 3 days later. Streptozotocin was dissolved in freshly prepared 100 mM trisodium citrate buffer (pH 4.45) and injected within 15 min of dissolution.

When treated with insulin, each rat was injected intraperitoneally with 5 units of insulin (0.2 mg). For each experiment insulin was freshly dissolved in 0.003 N HCl (1 mg/ml).

For comparison of rats in different physiological conditions (normal, diabetic, and diabetic treated with insulin), processing of their tissues was performed on the same day, and the ribosomes or RNA derived from these were subsequently analysed at the same time.

2.2.2 Enzymatic determination of the concentration of blood glucose

The glucose levels in the urine and blood of diabetic rats and diabetic rats which had been treated with insulin were routinely checked using Clinistix and Dextrostix reagent strips.

For quantitative measurement of the concentration of blood glucose, the method described by Werner *et al.* (1970) was followed, using reagent kits supplied by Boehringer Mannheim Company. Immediately after decapitation of the rats, blood samples were collected in tubes containing heparin. The blood (0.1 ml) was added to 1 ml of a solution of uranyl acetate in saline (1.6 mg/ml), mixed, and centrifuged for 10 min at 2,400 rpm (500 g). The deproteinized supernatant (0.05 ml) was used for glucose estimation and mixed with 5 ml of reagent containing 100 mM phosphate buffer (pH 7.0); 20 μg ($\geq$0.8 U) peroxidase/ml; 180 μg ($\geq$10 U) glucose oxidase/ml; and 1 mg/ml of the chromogen, ABTS {2,2' diazo-bis(3-ethylbenzothiazoline-6-sulphonate)}. Blank and standard solutions were prepared at the same

time. The samples were kept at room temperature for 35 min avoiding exposure to direct sunlight, and the absorbances of samples (A sample) and standards (A standard) were measured at 420 nm against the blank. Fig. 2.2.2.1 shows a standard curve for the assay.

Repeated assays with Dextrostix showed that normal rats had blood glucose concentrations in the range 70-110 mg/100 ml, whereas alloxan-diabetic rats had glucose concentrations >250 mg/100 ml. However, 60 min after injection of insulin the blood glucose concentration was still elevated, at >250 mg/ 100 ml. These results were substantiated quantitatively in a single assay (Table 2.2.2.1) by the glucose oxidase-peroxidase method described above, and are similar to those previously reported by Wool and Cavicchi (1966). In contrast to the results with alloxan-diabetic rats, the Dextrostix measurements showed that insulin injection reversed the elevated blood glucose levels (>250 mg/100 ml) to the normal range (70-110 mg/100 ml) in the case of streptozotocin-diabetic rats. This result was substantiated when more extensive quantitative determinations were performed using the glucose oxidase-peroxidase method (Table 2.2.2.1).

2.2.3 Effect of dose of streptozotocin on the induction of diabetes in rats

Rats were starved for 16 hr, injected (intravenously) with various doses of streptozotocin, killed 2 days later and the blood glucose concentration was determined in each case by the glucose oxidase-peroxidase method described above (section 2.2.2). It is evident from Fig. 2.2.3.1 that 50 mg streptozotocin/kg body weight is the minimal diabetogenic dose and this was used in most of the work described in this thesis.

Fig. 2.2.2.1 Standard curve for estimation of blood glucose by the glucose oxidase-peroxidase method

The assay was performed as described in section 2.2.2, using a standard glucose solution of 0.505 mM. The concentration of glucose (c) in blood was calculated from the following formula:

$$C = 100 \times \frac{\text{A sample}}{\text{A standard}} \quad \text{(mg/100 ml)}$$

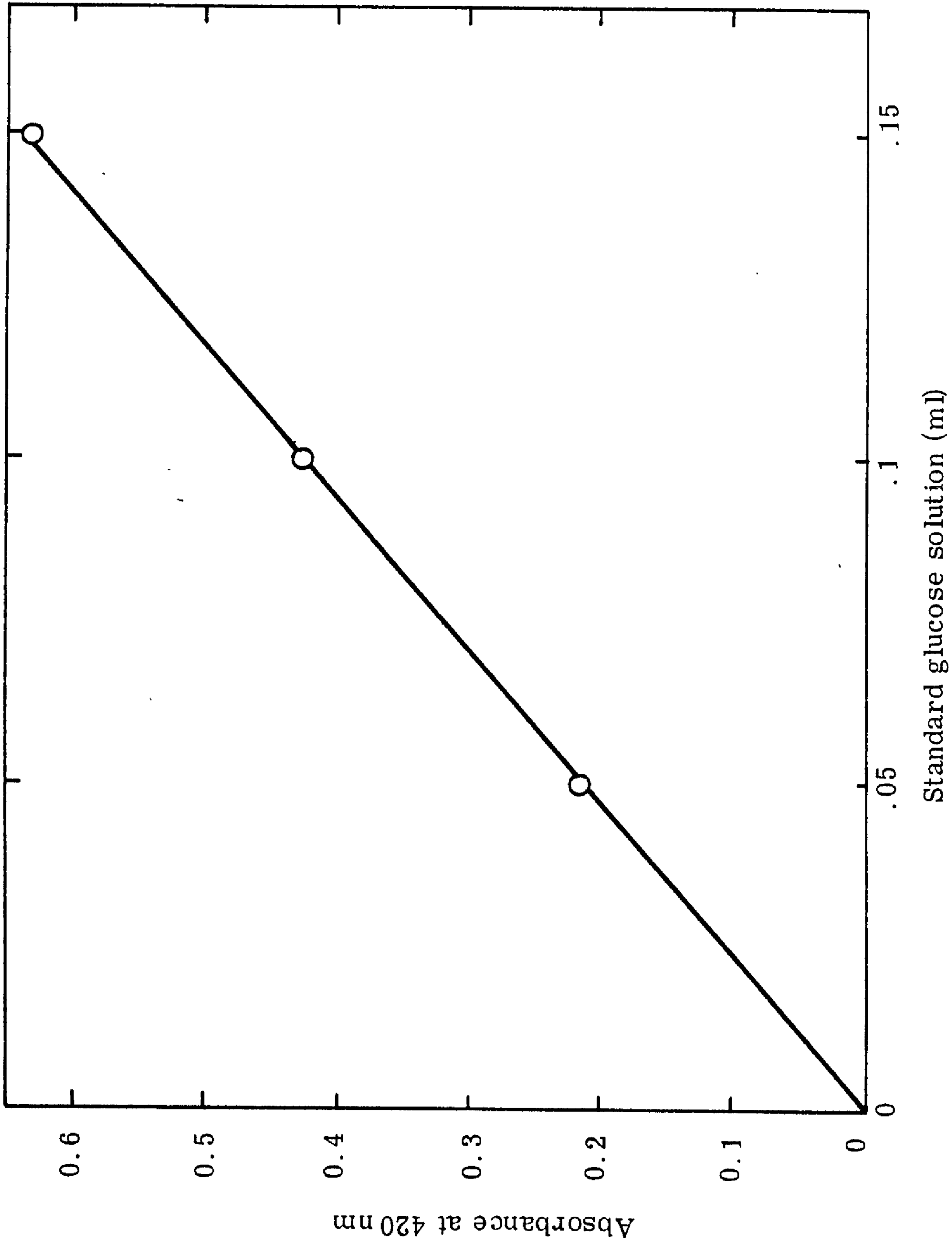


Fig. 2.2.2.1.

Table 2.2.2.1 Concentration of glucose in blood of rats under different physiological conditions

Rats were starved overnight, injected (intravenously) with alloxan-monohydrate (60 mg/kg body weight) or with streptozotocin (65 mg/kg body weight), and killed 3 days later. Some rats were intraperitoneally injected with 5 units of insulin, 60 min before death. The blood glucose concentrations were determined by the glucose oxidase-peroxidase method as described in section 2.2.2.

Values are : Mean + S.E.M. of No. of Determinations.

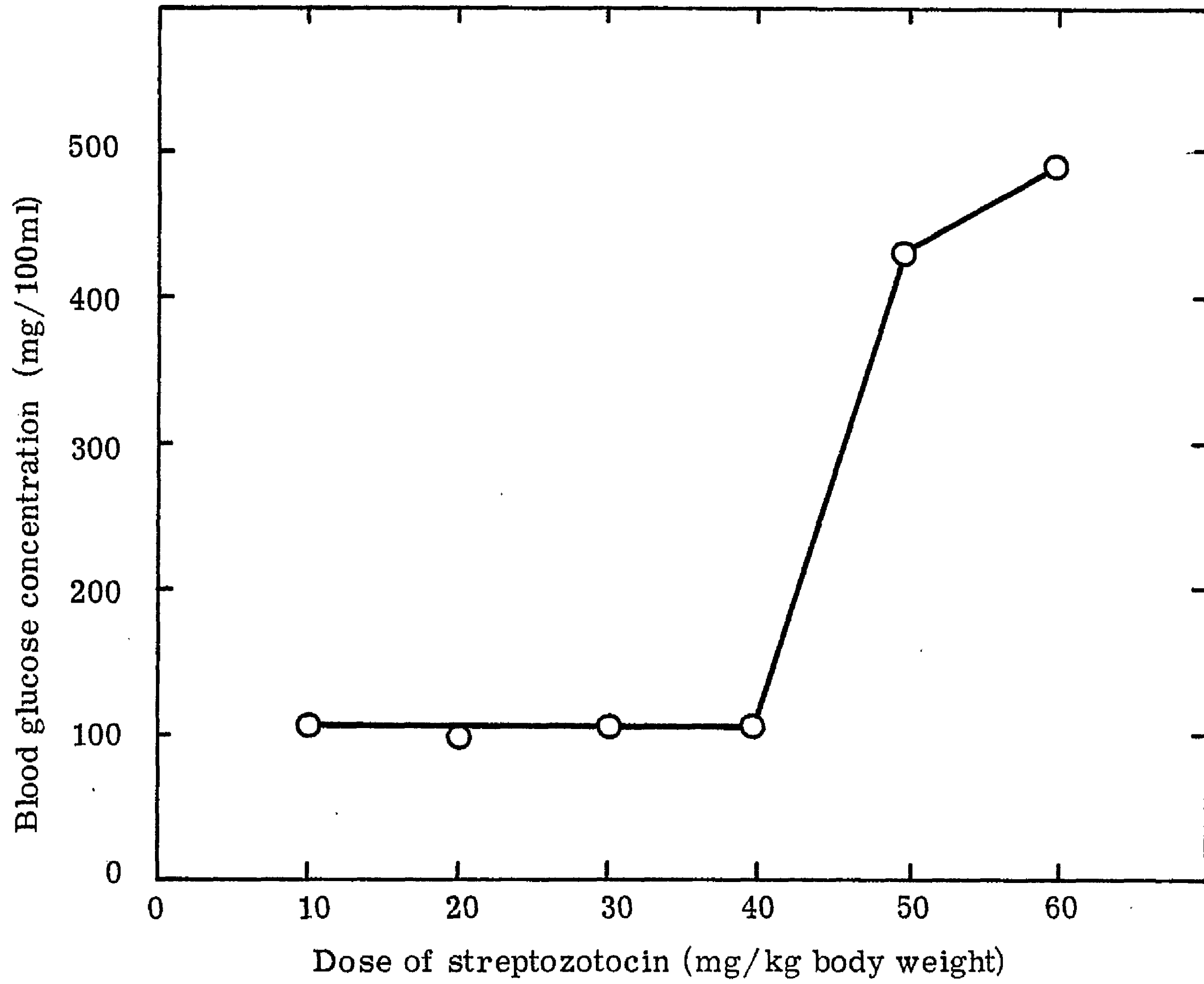
Table 2.2.2.1

Physiological status of rats	Blood glucose concentration (mg/100 ml)	No. of Determinations
Normal	125	1
Alloxan-Diabetic	442	1
Alloxan-Diabetic + Insulin	366	1
Streptozotocin-Diabetic	404 ± 14.4	7
Streptozotocin-Diabetic + Insulin	63 ± 11.6	7

Fig. 2.2.3.1 Effect of streptozotocin on the concentration of glucose in rat blood

Rats were starved overnight, injected (intravenously) with various doses of streptozotocin, killed 2 days later, and the blood glucose concentrations determined by the glucose oxidase-peroxidase method as described in section 2.2.2.

Fig. 2.2.3.1.



2.3 PREPARATION OF RIBOSOMES FROM RAT SKELETAL MUSCLE

2.3.1 Preparation of ribosomes at low ionic strength

This method is that of Florini and Breuer (1966), as modified by Martin *et al.* (1969).

Ribosomes were prepared from thigh, gastrocnemius, and abdominal muscles of male rats. The physical and functional characteristics of ribosomes from these different muscles have been shown to be identical (Wool *et al.*, 1968). All operations after removal of the legs from the rats were conducted in a cold room at $4^{o}C$. Rats were decapitated quickly; the carcasses were separated from the head to prevent flooding of the tissues with hormones from the pituitary. The rear legs were skinned and removed by cutting through the pelvic joint, the abdominal muscle was then removed in one sheet. The muscle was immediately placed in ice and stored until all animals involved in the experiment had been killed. Muscle was then cut away from the bones and tendons, freed of adipose tissue and minced with scissors in small amounts of homogenization buffer, Medium A, containing 50 mM Tris-HCl (pH 7.6), 80 mM KCl, 12.5 mM $MgCl_2$ and 250 mM sucrose. It was then weighed by displacement and homogenized in 2.5 volumes of Medium A for 1 min at the 'high speed' setting of a Virtis "23" homogenizer. The homogenate was centrifuged for 15 min at 12,500 rpm (13,000 g) in the 8 x 50 ML rotor of an MSE 18 centrifuge at $4^{o}C$, sedimenting unfractured cells, mitochondria, nuclei, myofibrils and debris in the 'mitochondrial pellet' fraction. The 'post-mitochondrial supernatant' was filtered through glass wool to remove fat and the filtrate was then centrifuged for 2 hr at 30,000 rpm (78,000 g) in the Type 30 rotor of a Beckman ultracentrifuge at $4^{o}C$. This yielded a 'post-microsomal

supernatant' which was discarded, and a 'microsome pellet'. For further purification, the microsome pellet was resuspended by 12 up-and-down strokes in a homogenizer equipped with a loose-fitting teflon pestle in 30 ml of a mixture of Medium A and the detergents, Lubrol WX (0.5% w/v) and sodium deoxycholate (1% w/v) (This was made up by mixing 25.5 ml of Medium A with 1.5 ml of 10% (w/v) Lubrol WX in 10 mM $MgCl_2$, and 3 ml of freshly prepared 10% (w/v) sodium deoxycholate in distilled water). This was centrifuged for 15 min at 12,500 rpm (13,000 g) in the 8 x 50 ML rotor of an MSE 18 centrifuge at 4^oC, and the supernatant was diluted with Medium A to augment its volume by 15%. The diluted supernatant was layered in 7 ml aliquots over 5 ml portions of Medium B (composition similar to Medium A but with 500 mM sucrose instead of 250 mM sucrose) and centrifuged for 3 hr at 50,000 rpm (165,000 g) in the 50 Ti rotor of a Beckman ultracentrifuge at 4^oC. The 'post-ribosomal supernatant' was then aspirated off; care being taken to avoid contamination of the ribosome pellets with the upper layer, and the tubes were drained and wiped dry. The ribosome pellets were stored at -20^oC. This preparation is illustrated in outline in Fig. 2.3.1.1.

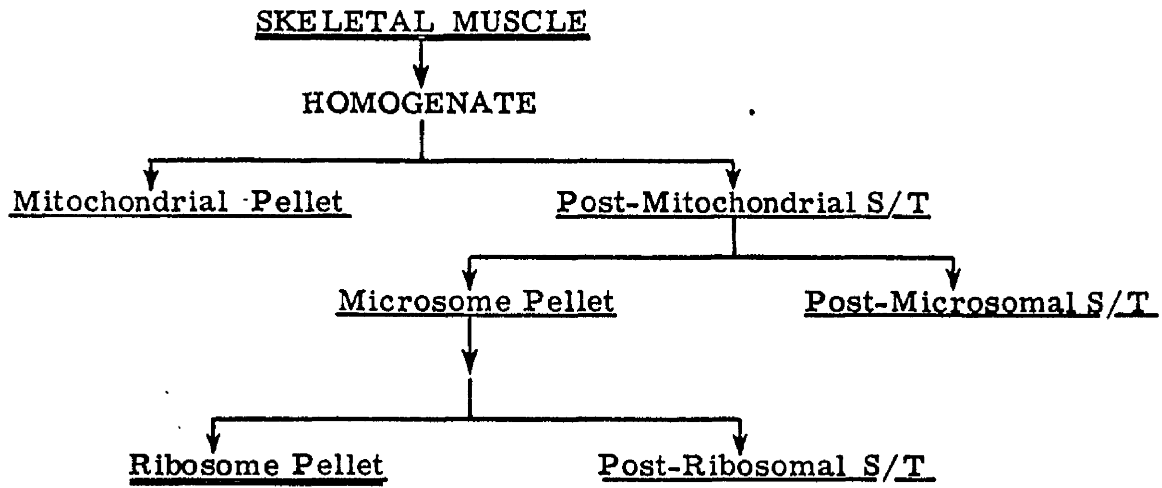
2.3.2 Preparation of ribosomes at high ionic strength

This method (Stirewalt, Castles and Wool, 1971) was similar to that described in section 2.3.1 above (low ionic strength method) except that: (i) the initial homogenization of tissue was carried out in Medium C, containing 50 mM Tris-HCl (pH 7.6), 250 mM KCl, 12.5 mM $MgCL_2$ and 250 mM sucrose; and (ii) the 'mitochondrial pellet' was reextracted with Medium C before centrifugation at 30,000 rpm (78,000 g). The reason for the higher concentration of KCl (250 mM) was to solubilize myosin, which otherwise precipitates in the 'mitochondrial pellet' fraction, trapping

Fig. 2.3.1.1 Summary of different fractions obtained during preparation of skeletal muscle ribosomes

Rat skeletal muscle was homogenized and fractionated as described in section 2.3.1.

Fig. 2.3.1.1.



with it a large number of ribosomes (Heywood, Dowben and Rich, 1967).

2.3.3 Preparation of 'post-mitochrondrial supernatant' by the method of Chen and Young (1968)

The muscle from the rear legs of rats was immersed in chilled Medium D, of the composition: 10 mM Tris-HCl (pH 7.6), 250 mM KCl, and 10 mM $MgCl_2$. The muscle (10 g) was finely minced with scissors at $4^{o}C$ and homogenized in 2 volumes of Medium D for 1 min at the "high speed" setting of a Virtis "23" homogenizer. The homogenate was centrifuged for 15 min at 12,500 rpm (13,000 g) in the 8 x 50 ML rotor of an MSE 18 centrifuge at $4^{o}C$, to sediment the cell debris, mitochondria and nuclei. This 'post-mitochondrial supernatant' was filtered through glass wool and the filtrate was then treated with 10% (w/v) Lubrol WX dissolved in Medium D and an aqueous 10% (w/v) solution of sodium deoxycholate to achieve a final concentration of 0.5% (w/v) Lubrol WX, and 1% (w/v) sodium deoxycholate.

2.4 EXTRACTION OF RNA FROM RAT SKELETAL MUSCLE

2.4.1 Extraction of total RNA

The RNA was extracted according to the method described by Perry *et al.*(1972) which had been found to preserve the integrity of polyA(+)RNA.

RNA was extracted from the unfractionated muscle by quickly freezing about 0.3 g of tissue in liquid nitrogen and pulverizing it in a chilled metallic mortar with a pestle. The powdered tissue was suspended in 10 ml of Medium E containing: 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate in a motor-driven teflon-glass homogenizer at room temperature. One volume of phenol-chloroform (1:1 by volume) was

added to the suspension and the mixture was shaken for 10min. at room temperature in stoppered glass centrifuge tubes. (The phenol was saturated with Medium F, containing 10 mM sodium acetate, 100 mM NaCl, 1 mM EDTA (pH 6.0), just before use). The samples were then centrifuged for 10 min at 2,400 rpm (500 g) at 20°C, and the upper, aqueous, phase was transferred carefully to another centrifuge tube. The lower, phenol, phase and the interphase layers were extracted with 2 volumes of Medium E - chloroform (1:1 by volume) for 5 min, centrifuged as previously, and the upper, aqueous, layer was combined with the previous one. The combined aqueous layers was reextracted with 1 volume of phenol-chloroform (1:1 by volume) for 5 min, centrifuged and the upper, aqueous, phase was removed. RNA and DNA were precipitated from the aqueous phase with 2.5 volumes of 95% ethanol at -20°C for 16 hr, centrifuged for 15 min at 12,500 rpm (13,000 g) at 4°C. The precipitate was then dissolved in 2 ml of DNA-digestion buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$ and 5 mM N-ethylmaleimide. To this solution, 10 µl of deoxyribonuclease solution (2 mg/ml in 150 mM NaCl) was added and the mixture was incubated for 15 min at 37°C. The RNA was precipitated with 2.5 volumes of 95% ethanol at -20°C for 16 hr, centrifuged and dissolved in Medium E and reextracted with phenol and chloroform to denature the deoxyribonuclease, as described previously. To remove any residual phenol, the extracted RNA was precipitated with 2.5 volumes of 95% ethanol at -20°C, centrifuged for 15 min at 12.500 rpm (13,000 g) and ethanol was removed by draining from the inverted tubes for 30 min at 4°C. The RNA was dissolved in 2 ml of Medium F, reprecipitated with ethanol, centrifuged, and dried for 1 hr at 4°C, and finally dissolved in a known volume of Medium H, containing 30 mM trisodium citrate, 300 mM NaCl. After extraction with phenol and chloroform the A_{260}/A_{280} ratio of RNA solution was usually in the range 1.95 - 2.05.

2.4.2 Extraction of RNA from purified ribosomes

To extract RNA from the purified ribosomes of rat skeletal muscle, the ribosome pellet was gently suspended by hand using a small teflon-glass homogenizer in 2 ml of Medium E at room temperature, and the RNA was extracted as previously described for unfractionated muscle (section 2.4.1), except that the DNA digestion step was omitted.

2.4.3 Isolation of polyA(+)RNA by poly U-Sepharose Affinity Chromatography

The method used is described in the booklet "Affinity Chromatography, Principles and Methods", published by Pharmacia Fine Chemicals, Uppsala, Sweden. The polyU-Sepharose (0.3 g) was swollen in a solution of 100 mM NaCl (pH 7.5) for 30 min, washed on a glass filter with the same solution (100 ml per gram dry powder) and poured into a column made from a 5 ml plastic syringe. The column was washed once with 15 ml of buffer containing 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5), 0.2% sodium dodecyl sulphate, then with 5 x 1 ml portions of NETS buffer containing 400 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5), 0.2% sodium dodecyl sulphate; finally equilibrated with 5 x 1 ml portions of a concentrated salt buffer, CSB, prepared in 25% formamide in 700 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 10 mM EDTA. (The formamide had been deionized by stirring with Bio-Rad mixed-bed ion exchange resin AG 501-X8 (D), 20-50 mesh). The RNA extracted from purified rat skeletal muscle ribosomes was dissolved in 1 ml of CSB and heated in a 55°C water bath for 3 min, cooled on ice and applied to the column (2 ml bed volume), the eluate being recycled through the column and retained. The "unbound" RNA was washed off the column by 2 x 1 ml portions and 2 x 2 ml portions of CSB and the washings were combined with the initial eluate. This fraction contained the "unbound" RNA

(predominantly rRNA). The polyA(+)RNA was eluted from the column with 2 x 1 ml portions and 2 x 2 ml portions of elution buffer prepared in 90% formamide (deionized), 10 mM $K_2H\ PO_4$, 10 mM Na_2 EDTA, 0.2% sodium dodecyl sulphate (pH 7.5). The eluate was diluted to 25% formamide with NETS buffer and applied to the column which has been reequilibrated with CSB (20 ml). The 'flow-through' was discarded and the bound polyA(+)RNA was eluted with 5 x 1 ml portions of the elution buffer described above. The RNA was precipitated with 2.5 volumes of 95% ethanol in the presence of 150 mM NaCl. To remove any contaminating formamide, the RNA was suspended in sterile water and applied to a Sephadex G50 'desalting' column. The eluate was collected in 1 ml fractions and the RNA detected by its ultra-violet-absorbance at 260 nm; the peak fractions being collected, lyophilized and kept at $-70^{o}C$ until use.

The amount of RNA eluted as polyA(+)RNA was, on average, 4% of the total applied.

2.5 SUCROSE DENSITY GRADIENT ANALYSIS OF RIBOSOMES AND RNA

2.5.1 Sucrose density gradient analysis of ribosomes

The ribosomes were suspended in the required medium (50 mM Tris-HCl (pH 7.6), 200 mM KCl and 5 mM $MgCl_2$, in most experiments) by gentle manual homogenization in a small teflon-glass homogenizer at $4^{o}C$. A portion (0.2 ml) containing approximately 2 A_{260} units of ribosomes, was then layered onto 5.2 ml of a linear 15-45% (w/v) sucrose density gradient made in the same medium in which the ribosomes were suspended. The gradients were centrifuged for 35 min at 50,000 rpm (234,000 g) in the SW 50.1 rotor

of a Beckman ultracentrifuge at 4°C. After centrifugation, the gradients were analysed for ultraviolet-absorbance at 254 nm using the density-gradient scanner attachment of a Gilford 240 recording spectrophotometer, by upwards displacement with 50% (or 75%) sucrose solution at a rate of 1 ml per min.

2.5.2 Sucrose density gradient analysis of rat skeletal muscle 'post-mitochondrial supernatant'

The 'post-mitochondrial supernatant' from rat skeletal muscle was analysed by layering 1.5 ml on a linear 15-40% (w/v) sucrose gradient (37 ml) prepared in Medium D. The gradient was centrifuged for 3 hr at 27,000 rpm (94,500 g) in the SW 27 rotor of a Beckman ultracentrifuge at 4°C. The ultraviolet-absorbance profiles were measured at 260 nm using a flow-cell with a 5 mm light-path in a Gilford model 240 recording spectrophotometer. The gradients were pumped at a rate of 3 ml per min from the bottom of the tube.

2.5.3 Sucrose density gradient analysis of RNA

Linear, 7-25% (w/v) sucrose density gradients were prepared at 20°C in Medium G consisting of: 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate. About 50 μg of RNA dissolved in 0.2 ml of Medium G was layered onto a 5 ml sucrose gradient. The gradients were centrifuged for 4 hr at 45,000 rpm (189,000 g) in the SW 50.1 rotor of a Beckman ultracentrifuge at 20°C. Gradients were continuously monitored for ultraviolet-absorbance at 254 nm using the density-gradient scanner attachment of a Gilford 240 recording spectrophotometer, as described in section 2.5.1.

When radioactive samples were analysed, fractions of 0.2 ml were collected from the gradients, mixed with 0.3 ml of a solution containing 0.5 M NH_4OH and 0.5 mM EDTA and dried overnight at 60°C in glass scintillation vials. Water (0.3 ml) and 7 ml scintillation liquid (0.4% (w/v) PPO, 0.02% (w/v) POPOP, 40% (v/v) 2-methoxyethanol, 60% (v/v) toluene) were added and the radioactivity was determined in a Beckman scintillation spectrometer with an efficiency of about 15% for ^3H. In some experiments E.coli tRNA (4S) and skeletal muscle ribosomal RNA (18S and 28S) were used as markers on a parallel gradient. A gradient containing (^3H) polyU only was similarly analysed in certain cases.

2.6 ASSAY OF POLY A(+) RNA BY HYBRIDIZATION WITH (^3H) POLYU

The method described by Rudland, Weil, and Hunter (1975) was followed. Hybridization of polyA(+)RNA to (^3H) polyU was accomplished in standard annealing mixtures (0.5 ml) containing Medium H (composed of 30 mM trisodium citrate and 300 mM NaCl), 0.04 μCi (0.25 μg) of (^3H) polyU (Miles Laboratories, specific activity: 61.9 μCi/μmol P), varying amounts of non-radioactive synthetic polyA or varying amounts of polyribosomal RNA. Reactants were incubated at 50°C for 14 hr, allowed to cool to 20°C for 2 hr before digestion with 20 μg pancreatic ribonuclease for 2 hr at 20°C. Samples were then precipitated with 1 ml ice-cold 10% (w/v) trichloroacetic acid after addition of 200 μg of carrier RNA (Yeast RNA dissolved in 200 mM sodium acetate (2 mg/ml)). Variations from these standard conditions are indicated in the figure legends.

After being allowed to stand for 1 hr at 4°C, the precipitates were collected on Whatman GF/C glass fibre discs, washed several times with 5% trichloroacetic acid, transferred into glass scintillation vials, dried at 100°C for 1 hr and cooled. To the vials was added 10 ml of a scintillation fluid

containing 4 g PPO, 0.2 g POPOP per litre of toluene, and the radioactivity was estimated with an efficiency of about 15% for ^{3}H.

2.7 ASSAYS FOR CELL-FREE PROTEIN SYNTHESIS

2.7.1 Assay of protein synthesis with rat skeletal muscle ribosomes in vitro

The assay was carried out in a final volume of 100 μl at 37°C. The assay mixture contained: 20 mM Tris-HCl (pH 7.6), 125 mM KCl, 7.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM ATP (neutralized), 0.1 mM GTP (neutralized), 5 mM creatine phosphate (0.18 mg), 20 μg of creatine phosphokinase, 40 μg of E.coli tRNA (Schwartz-Mann) aminoacylated with (^{3}H) phenylalanine (5 Ci/mmole, Radiochemical Centre, Amersham) and 19 non-radioactive amino acids (Wool and Cavicchi, 1967), and 0.7 mg of rat liver supernatant protein (Rampersad and Wool, 1965). The ribosome fraction was added to start the reaction, and reaction mixtures were incubated on a water bath for 30 min. At the end of incubation period, the proteins were precipitated by adding 5 ml of ice-cold 5% (w/v) trichloroacetic acid, and the reaction mixtures were kept at 0°C for 30 min. The mixtures were then heated at 90-95°C for 20 min. After the samples had been chilled at 0°C for 15 min, protein was collected on glass fibre discs (Whatman GF/C filters, 24 mm diameter), washed with four 10-ml portions of 5% trichloroacetic acid containing 10 mg/ml of non-radioactive phenylalanine. The filter discs were then put into glass vials containing 0.7 ml of 90% formic acid to solubilize the protein on the filters and the radioactivity was measured in 10 ml of scintillation fluid containing, per litre, 500 ml toluene, 500 ml 2-methoxyethanol, and 5 g 2,5-bis-{2-(5-tert-butylbenzoxazolyl)}-thiophene, (BBOT), with an efficiency of about 30% for ^{3}H. Correction was made for a control lacking ribosomes.

2.7.2. Translation of rat skeletal muscle ribosomes and polyA(+)RNA in a cell-free system from wheat germ

The method described by Marcu and Dudock (1974) was adopted.

Standard protein synthesis assay

The assay contained in a final volume of 50 µl: 15 µl of wheat germ S-30 fraction (kindly provided by Mrs. H. Singer), 20 mM N-2-Hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.6, 2 mM dithiothreitol, 1 mM ATP (neutralized), 20 µM GTP, 8 mM creatine phosphate, 0.5 µg creatine phosphokinase, 0.4 mM spermidine, KCl and Mg acetate as indicated, 25 µCi of (^{35}S) methionine (1080 Ci/mmole), ribosomes or polyA(+)RNA and/or water to 50 µl. The wheat germ extract contained sufficient endogenous amino acids and tRNAs to support protein synthesis. Assays were incubated at 25°C for 2 hr. At the end of the incubation 5 µl aliquots were removed for determination of radioactivity incorporated into protein. The aliquots were pipetted onto 2.5 cm discs of Whatman No.1 paper, dried, placed into 10% trichloroacetic acid containing 10^{-5}M non-radioactive methionine for 10 min, washed for 10 min in 5% ice-cold trichloroacetic acid, then boiled in 5% trichloroacetic acid at 90°C for 10 min, rinsed with cold 5% trichloroacetic acid, and dried by sequential rinsing in ethanol, ethanol-diethyl ether (3:1 v/v), and diethyl ether. The filters were then dried in air and then under a heat lamp for 5 min prior to estimation of radioactivity in 10 ml of toluene-based scintillation fluid containing 5% (w/v) PPO with an efficiency of about 85% for ^{35}S.

2.7.3 Identification of cell-free translation products by polyacrylamide gel electrophoresis in sodium dodecyl sulphate

Sodium dodecyl sulphate gels were prepared by the following modification of the method of Laemmli (1970) and samples were in 4 mm tracks on slab of 20 x 25 x 0.15 cm. The separation gel (22 cm deep) contained 10% (w/v)

acrylamide, 0.26% (w/v) bisacrylamide, 0.1% (w/v) sodium dodecyl sulphate, 375 mM Tris-HCl (pH 8.8), 0.03% (v/v) N,N,N',N', tetramethylethylenediamine (TEMED), and 0.1% (w/v) ammonium persulphate.

Stacking gels (3 cm deep) contained 5% (w/v) acrylamide, 0.03% (w/v) bisacrylamide, 0.1% (w/v) sodium dodecyl sulphate, 65 mM Tris-HCl (pH 6.8), 0.1% (v/v) N,N,N',N' tetramethylethylenediamine (TEMED) and 0.1% (w/v) ammonium persulphate.

Prior to electrophoresis, protein precipitates (10 µl of the cell-free assay was precipitated with 5 volumes of acetone) were dissolved each in 50 µl of a reducing mixture containing 100 mM dithiothreitol, 2% (w/v) sodium dodecyl sulphate, 65 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol and 0.001% bromophenol blue tracker. The running buffer contained 25 mM Tris-HCl pH (8.3), 192 mM glycine and 0.1% (w/v) sodium dodecyl sulphate. The gel was electrophoresed overnight at 4°C at 15-20 mA per gel constant current.

Processing of slab gel for fluorography

The method of Bonner and Laskey (1974) was adopted. After electrophoresis, the gel was immersed in three successive baths of dimethyl sulphoxide (500 ml each, for successively 30 min, 30 min, and 45 min) with constant shaking. The gel was then impregnated with PPO by immersion in 200 ml of 20% (w/v) PPO in dimethyl sulphoxide. It was shaken gently for 45 min at room temperature after which the PPO was precipitated in the gel and the dimethyl sulphoxide removed by constant washing with water for a minimum period of 45 min. The gel was then dried onto Whatman 3MM chromatography paper, under vacuum. Exposure of film (Kodak X-Omat R) was at -70°C for 7-28 days.

The molecular weights of the cell-free products were estimated by comparison with parallel samples of proteins of known molecular weight which were immunoglobulin G heavy chain (molecular weight 55,000) and immunoglobulin κ light chain (molecular weight 23,500). The molecular weights of translated proteins were then estimated by the method of Weber and Osborn (1969) as shown in Fig. 2.7.3.1.

2.8 ASSAY OF RIBONUCLEASE ACTIVITY

The ribonuclease activity was determined as described by Barrett (1972). In principle mononucleotides and oligonucleotides are released from RNA by ribonuclease action, the undegraded substrate and large fragments are then precipitated by perchloric acid containing uranyl acetate, and the yield of low molecular weight digestion products is determined in the supernatant, either by measurement of ultraviolet-absorbance at 260 nm, or by estimation of radioactivity.

2.8.1 Assay of ribonuclease activity using yeast RNA as substrate

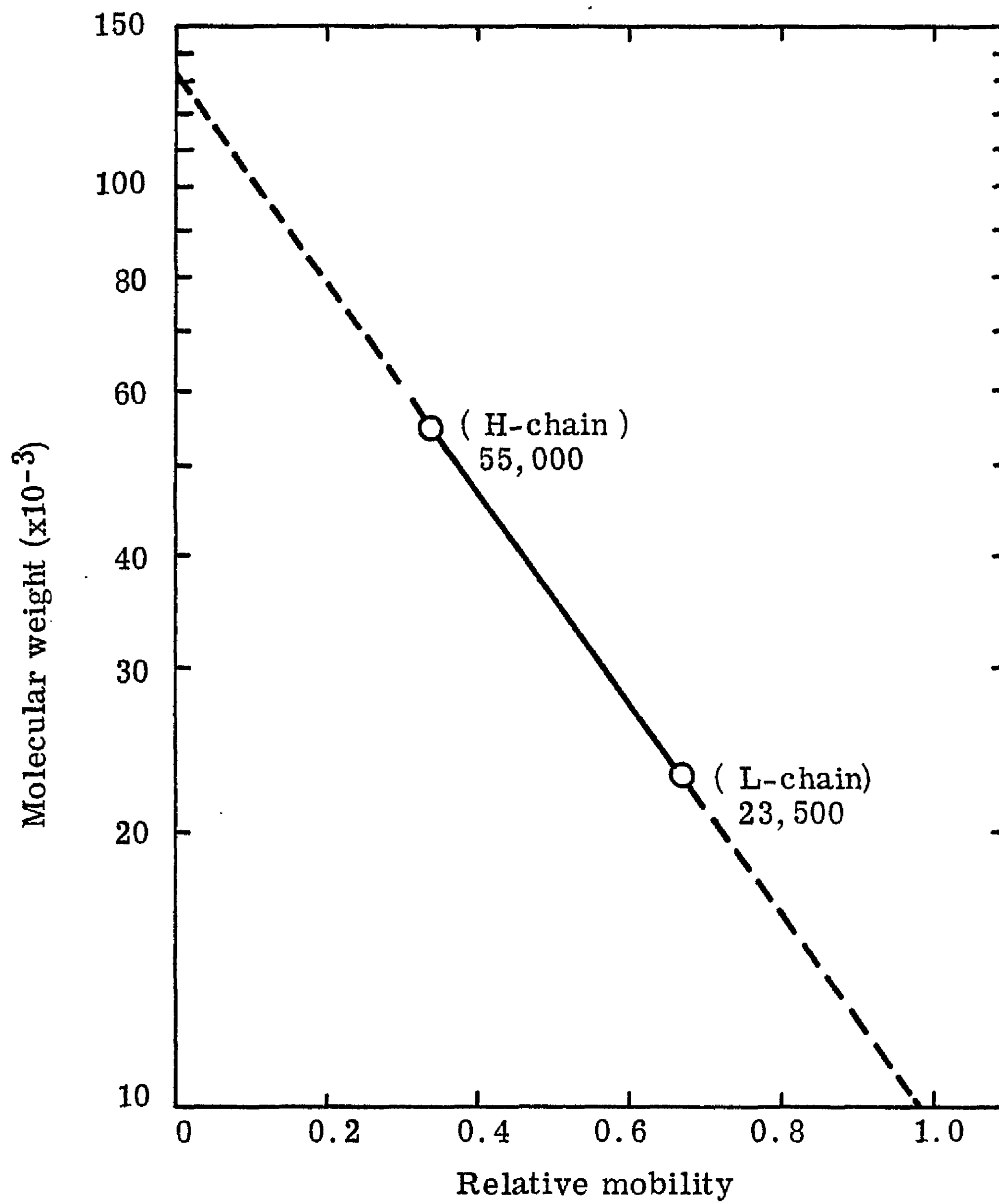
The rat skeletal muscle was homogenized in 3 volumes of either low ionic strength Medium A or high ionic strength Medium C at $4^{o}C$. The homogenate was centrifuged for 15 min at 12,500 rpm (13,000 g) in 8 x 50 ML rotor of an MSE 18 centrifuge at $4^{o}C$, filtered through glass wool and the 'post-mitochondrial supernatant' was used as the source of ribonuclease. To 0.5 ml of the 'post-mitochondrial supernatant', 0.25 ml of buffered substrate (Yeast RNA in 200 mM sodium acetate-acetic acid buffer, pH 7.6, 2 mg/ml) was added to start the reaction and the final volume adjusted to 2 ml in 200 mM sodium acetate (pH 7.6). The mixture was incubated at $37^{o}C$ for 2 hr, and the reaction was stopped by adding 2 ml of ice-cold perchloric

Fig. 2.7.3.1 Standard curve for determination of molecular weights of proteins in gels containing sodium dodecyl sulphate

Mouse immunoglobulin (kappa) light chain and immunoglobulin G heavy chain were electrophoresed on a sodium dodecyl sulphate gel as described in section 2.7.3.

The values derived from this curve are only approximate because of there only being two molecular weight standards. This is especially true in the regions of extrapolation.

Fig. 2.7.3.1.



acid - uranyl acetate reagent (10 g perchloric acid and 0.25 g uranyl acetate per 100 ml water). After being allowed to stand at $4^{o}C$ for 60 min, the samples were centrifuged for 10 min at 2,400 rpm (500 g) at $4^{o}C$, the ultraviolet-absorbance of the supernatant was measured,and the ribonuclease activity expressed as ΔA_{260}/min/mg protein. Control samples were incubated: (i) without added 'post-mitochondrial supernatant', and (ii) without substrate, and the sum of these were subtracted from the experimental values.

In other experiments, the skeletal muscle was homogenized in 3 volumes of Medium C (high ionic strength) at $4^{o}C$ and the homogenate was used as the source of ribonuclease. The substrate yeast RNA in this case was dissolved in 200 mM sodium acetate-acetic acid (pH 5) at a concentration of 2 mg/ml.

2.8.2 Assay of ribonuclease activity using (^{32}P) rRNA as substrate

The method used was as described in section 2.8.1, except that the substrate was (^{32}P) rRNA from HeLa cells, diluted with yeast RNA to a final concentration of 2 mg/ml in 200 mM sodium acetate - acetic acid buffer (pH 7.6). Control incubation was carried out to determine the non-enzymic hydrolysis of RNA. After conducting and terminating the reaction, as before, the post-acidification supernatant (1 ml) was added to 10 ml of Bray's solution: 0.2 g POPOP, 4 g PPO, 60 g naphthalene, 100 ml methanol, 20 ml ethylene glycol, per 1000 ml dioxane (Bray, 1960); and the radioactivity was determined in a Beckman liquid scintillation counter with an efficiency of about 80% for ^{32}P. The ribonuclease activity was measured as (^{32}P) rRNA hydrolysed (counts/min/mg protein).

2.9 DETERMINATION OF PROTEIN

Rat skeletal muscle (0.5 g) was minced and homogenized in 10 ml of 10% trichloroacetic acid in a Virtis "23" homogenizer at the "high speed" setting at 4°C and centrifuged for 10 min at 2,400 rpm (500 g) at 4°C. The precipitate was washed twice with 10 ml ice-cold 0.5 N perchloric acid, centrifuged and the supernatant was discarded. The precipitate (compact volume 0.5 ml) was suspended in 10 ml of 1N NaOH, using a glass rod, and incubated at 37°C for 16 hr. A control containing only 1 N NaOH was similarly incubated. The samples were shaken and an aliquot (10 µl) from each sample was used for protein determination as described by Lowry et al. (1951) using bovine plasma albumin (500µg/ml in distilled water) as a standard.

To standards and samples, adjusted to a final volume of 0.5 ml and a final concentration of 0.1 N NaOH, was added 2 ml of a freshly prepared mixture of 50 parts 2% Na_2CO_3 in 0.1 N NaOH and 1 part 2% Na citrate, 0.4% KI and 1% $Cu\ SO_4 - 5\ H_2O$. These were well mixed and allowed to stand at room temperature for 10 min. To each reaction mixture 0.2 ml of 2-fold diluted Folin phenol reagent (Folin and Ciocalteu, 1927) was added very rapidly and mixed immediately. After 45 min, the absorbance of the samples was determined at 750 nm against a blank in a spectrophotometer. A standard curve for the assay is shown in Fig. 2.9.1.

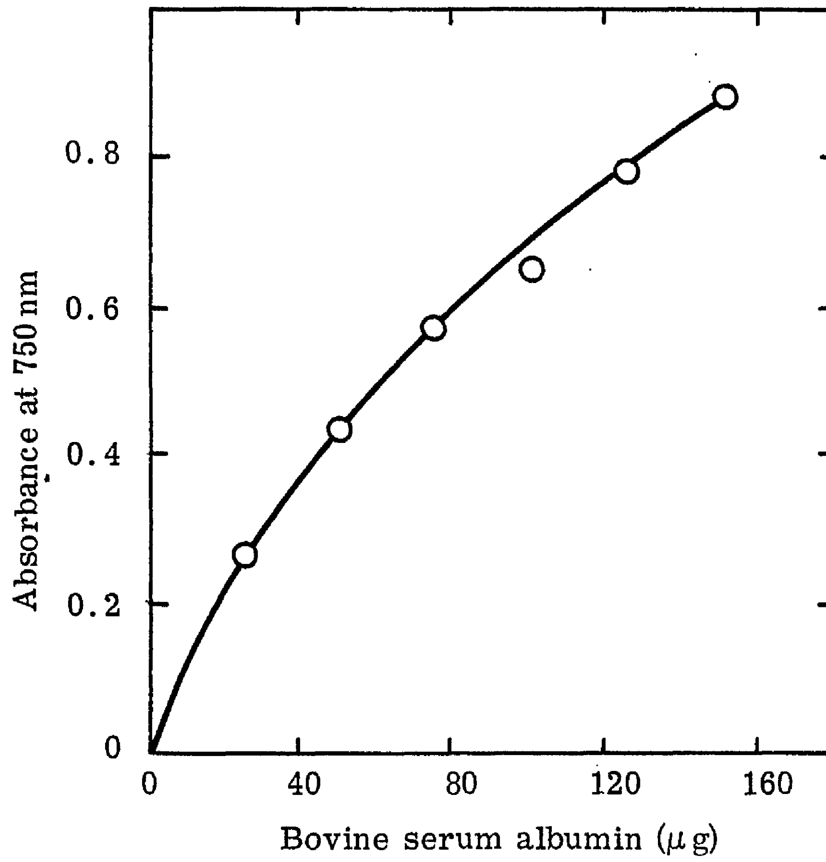
2.10 DETERMINATION OF RNA AND DNA

The skeletal muscle (250 mg) was homogenized in 20 ml of ice-cold 10% trichloroacetic acid for 2 min in a Virtis model "23" homogenizer at the

Fig. 2.9.1 Standard curve for estimation of protein by the method of Lowry

The assay was performed as described in section 2.9 using a standard bovine plasma albumin solution (500 μg/ml). All determinations were carried out in duplicates.

Fig. 2.9.1.



"high speed" setting. Homogenization and subsequent steps were carried out at $4^{o}C$. The homogenate was centrifuged for 10 min at 2,400 rpm (500 g) and the precipitate was washed 4 times with 12 ml of ice-cold 0.2 N perchloric acid.

The skeletal muscle RNA was determined by the method of Fleck and Munro (1962), as modified by Wool, Stirewalt and Moyer (1968). The perchloric acid precipitate was carefully drained of the acid and suspended in 1 ml of 0.3 N NaOH for each 50 mg of muscle, and shaken for 75 min at $37^{o}C$ to hydrolyse the RNA. A control sample containing 0.3 N NaOH was included. The samples were cooled, neutralized, acidified with 1 volume of cold 1 N perchloric acid, allowed to stand at $0^{o}C$ for 10 min to precipitate the DNA, and then centrifuged. The supernatant was decanted and kept aside, the precipitate was washed twice with 4 ml of ice-cold 0.5 N perchloric acid then the supernatant and washings were combined together, diluted with water to make the sample 0.25 N in perchloric acid and the absorbance at 260 and 275 nm was determined using a Beckman spectrophotometer. RNA concentration was calculated according to the formula (Wool, Stirewalt and Moyer, 1968): RNA (μg/ml) = $125\ A_{260} - 109.5\ A_{275}$.

A portion of the diluted supernatant was also used for determination of RNA content by the orcinol method (Mejbaum, 1939), with yeast RNA in 5% trichloroacetic acid (200 μg/ml),as a standard. To samples (1.5 ml in 0.25 N perchloric acid) and standards (1.5 ml in 5% trichloroacetic acid), 1.5 ml of freshly prepared orcinol solution at a concentration of 0.01 g/ml of $FeCl_3$ (0.5 gm/100 ml conc. HCl) was added. Reaction mixtures were placed in a boiling water bath for 20 min (each tube was covered with a marble to prevent evaporation), allowed to cool to room temperature

and the intensity of the green colour was measured at 660 nm against a blank in a spectrophotometer. A standard curve for the assay is shown in Fig. 2.10.1.

The DNA precipitates (above) were heated for 25 min at 70°C in 0.5 N perchloric acid (4 ml), centrifuged for 10 min at 2,400 rpm (500 g) and an aliquot from the supernatant was used for estimation of DNA by the diphenylamine method (Burton, 1956), using calf thymus DNA in 0.5 N perchloric acid (250 μg/ml) as a standard.

The diphenylamine, which was stored in the dark, contained 1.5 g diphenylamine dissolved in 100 ml of glacial acetic acid, to which 1.5 ml of concentrated sulphuric acid was added; and the aqueous acetaldehyde solution contained 0.62 ml of acetaldehyde per 40 ml distilled water (stored at 4°C). The samples and standards (1 ml) in 0.5 N perchloric acid were mixed with 2 ml of freshly prepared diphenylamine reagent (prepared by mixing 20 ml of stock diphenylamine reagent and 0.1 ml of aqueous acetaldehyde) and incubated at 37°C for 16 hr with a control containing 0.5 N perchloric acid only. After incubation, the absorbance was measured at 600 nm against a blank in a spectrophotometer. A standard curve for the assay is shown in Fig. 2.10.2.

2.11 COMPOSITION OF STANDARD BUFFER SOLUTIONS

Medium A: 50 mM Tris-HCl (pH 7.6), 80 mM KCl, 12.5 mM $MgCl_2$ and 250 mM sucrose.

Medium B: 50 mM Tris-HCl (pH 7.6), 80 mM KCl, 12.5 mM $MgCl_2$ and 500 mM sucrose.

Medium C: 50 mM Tris-HCl (pH 7.6), 250 mM KCl, 12.5 mM $MgCl_2$ and 250 mM sucrose.

Fig. 2.10.1 Standard curve for estimation of RNA by the orcinol method

The assay was performed as described in section 2.10 using yeast RNA in 5% trichloroacetic acid (200 μg/ml) as a standard. All determinations were carried out in duplicates.

Fig. 2.10.1.

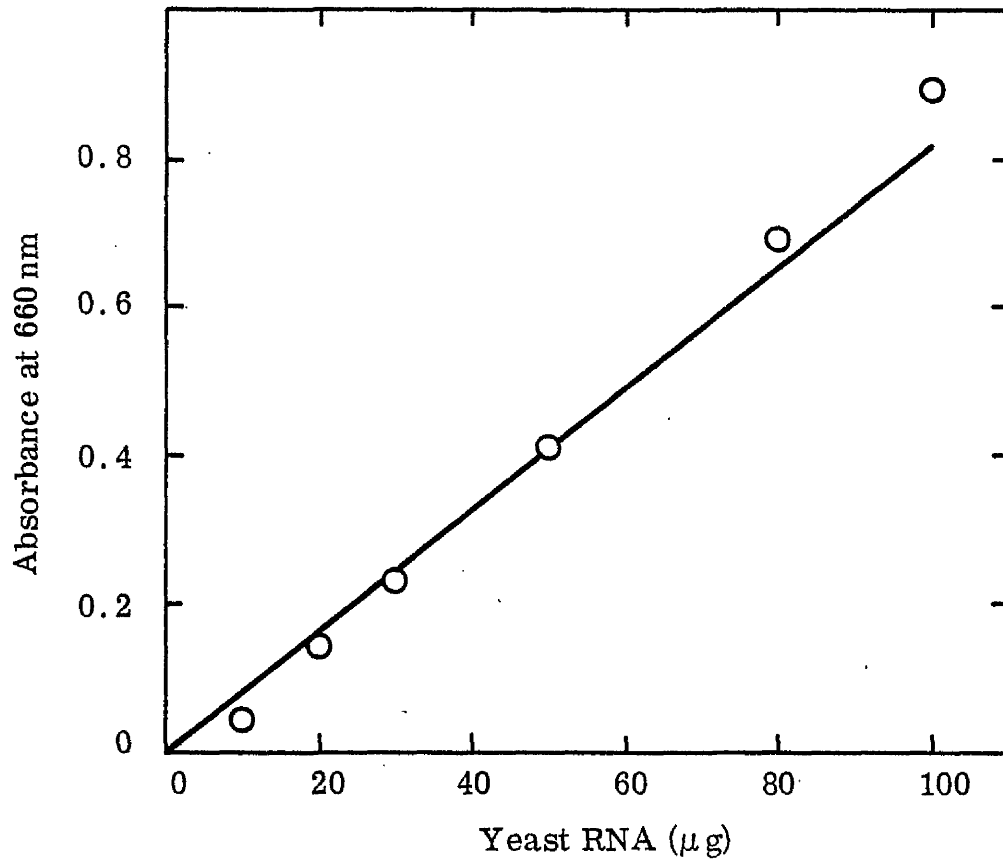
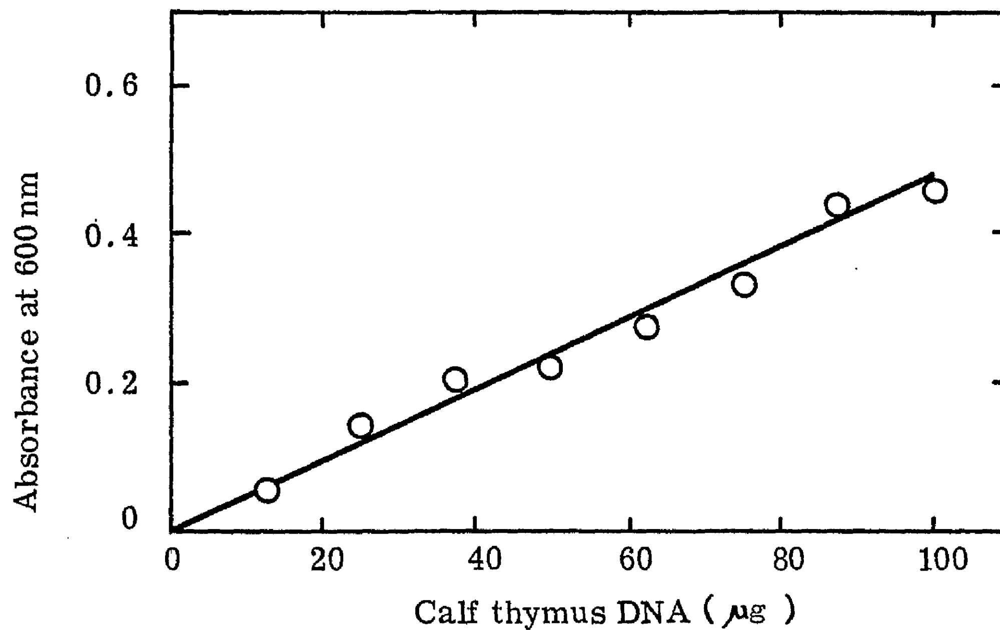


Fig. 2.10.2 Standard curve for estimation of DNA by the diphenylamine method

The assay was performed as described in section 2.10 using calf thymus DNA in 0.5 N perchloric acid (250 µg/ml) as a standard. All determinations were carried out in duplicates.

Fig. 2.10.2.



<u>Medium D:</u> 10 mM Tris-HCl (pH 7.6), 250 mM KCl, and 10 mM $MgCL_2$.

<u>Medium E:</u> 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA and 0.5% SDS.

<u>Medium F:</u> 10 mM Na Acetate, 100 mM NaCl, 1 mM EDTA (pH 6).

<u>Medium G:</u> 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% SDS.

<u>Medium H:</u> 30 mM trisodium citrate, 300 mM NaCl.

CHAPTER 3

RESULTS

3.1 INITIAL CHARACTERIZATION OF RAT SKELETAL MUSCLE RIBOSOMES

Before studying skeletal muscle mRNA it was necessary to determine the best conditions for extraction and fractionation of the polysomes from which this mRNA was to be obtained.

3.1.1 Comparison of different methods of extraction of ribosomes

In the original studies of Wool and co-workers on the effect of diabetes on the activity of skeletal muscle ribosomes (Stirewalt, Wool and Cavicchi, 1967), the ribosomes were isolated using a modification of the method described by Florini and Breuer (1966), in which the initial homogenization of the tissue was in a medium of low ionic strength (80 mM KCl). However, in their later studies (Stirewalt, Castles and Wool, 1971) they used a medium of higher ionic strength (250 mM KCl), as this had been shown to give a greater yield of ribosomes (Heywood, Dowben and Rich, 1967; Earl and Morgan, 1968). However these workers had not published a comparison of the polysomes isolated by the two methods, and it was felt necessary to make such a comparison before proceeding with the study described in this thesis.

Fig. 3.1.1.1 shows that there was a similar proportion of polysomes in the ribosomes extracted at either low or high ionic strength (Experimental; sections 2.3.1 and 2.3.2). As the extraction at high ionic strength resulted in an approximate doubling of the yield of ribosomes (Table 3.1.1.1), it was decided to use this method in subsequent studies.

Fig. 3.1.1.1 Comparison of the sedimentation characteristics of ribosomes extracted from skeletal muscle in media of different ionic strengths

Ribosomes (2 A_{260} units/0.2 ml), extracted at low ionic strength (80 mM KCl), as described in section 2.3.1 (a), or at high ionic strength (250 mM KCl), as described in section 2.3.2 (b), were suspended in medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at 4°C and analysed as described in section 2.5.1.

Fig. 3.1.1.1.

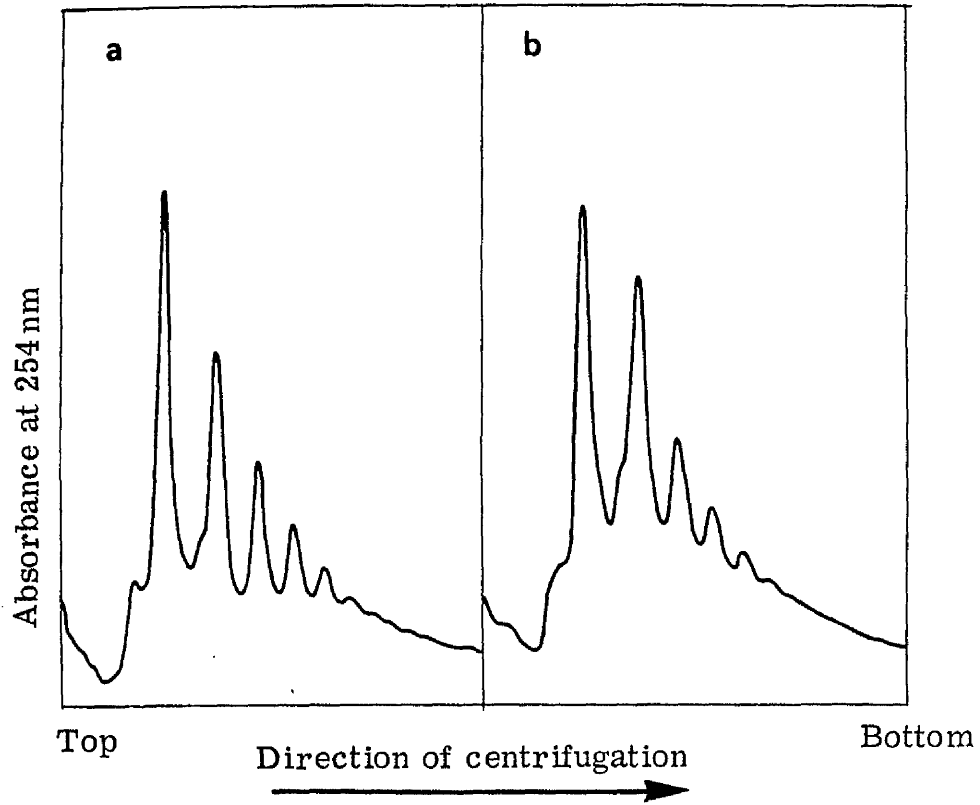


Table 3.1.1.1 Effect of ionic strength of extraction medium on the yield of skeletal muscle ribosomes

Rats were killed, their skeletal muscle quickly weighed and ribosomes were prepared at low ionic strength (80 mM KCl; section 2.3.1) or at high ionic strength (250 mM KCl; section 2.3.2). Diabetic rats had received streptozotocin (50 mg/kg body weight) 2 days before death, and - where indicated - insulin (5 units) 60 min before death. The yields of ribosomes were determined from the ultraviolet-absorbance of the resuspended pellets using the formula:

$$1 \ A_{260} \text{ unit} = 100 \ \mu g \text{ ribosomes}$$

Values are: Mean ± S.D. (No. of determinations)

Table 3.1.1.1

Physiological status of rats	Yield of ribosomes (μg per g wet wt. of tissue)		P
	Extraction with 0.08 M KCl	Extraction with 0.25 M KCl	
Normal	107 ± 61 (10)	215 ± 55 (5)	<0.005
Diabetic	148 ± 72 (9)	262 ± 31 (3)	<0.025
Diabetic + Insulin	89 ± 25 (8)	188 ± 53 (7)	<0.0005

3.1.2 Optimization of the separation of polysomes and monosomes by sucrose density gradient centrifugation

In their studies on the sedimentation on sucrose density gradients of normal skeletal muscle ribosomes extracted at low ionic strength, Stirewalt, Wool and Cavicchi (1967) observed major peaks at 80S, 114S, 145S, 175S and 200S (These assignments were made from parallel measurements in the Beckman Model E analytical ultracentrifuge). The 80S peak was clearly the classical monomeric species and it was concluded that the 145S, 175S and 200S species were polysomes comprising three, four and five ribosomes, respectively. However there was some ambiguity about the '114S' species because, unlike the higher molecular weight species, it was not removed by incubation of the ribosomes with ribonuclease. It was concluded that this was a dimer of the 80S species, stabilized by magnesium ions, and this conclusion is supported by the subsequent finding that reassociated ribosomal subunits can also form such '114S' species (Martin et al.,1969).

In the present work skeletal muscle ribosomes, extracted at high ionic strength, showed a similar peak to the '114S' peak found by Stirewalt et al. (1967) in ribosomes isolated at low ionic strength. This peak was similarly resistant to ribonuclease (Fig. 3.1.2.1). It was still possible, however, that the original '114S' peak (D in b and c) contained dimeric polysomes containing mRNA (disomes), as well as dimers of 80S monomers, lacking mRNA, but stabilized by magnesium ions. It was thought desirable therefore, to try to find conditions to dissociate the dimers.

When muscle ribosomes extracted at low ionic strength (50 mM Tris-HCl, 80 mM KCl, 12.5 mM $MgCl_2$) were analysed on sucrose density gradients at 50 mM Tris-HCl, 200 mM KCl, 5 mM $MgCl_2$ (Experimental; section 2.5.1), it had been found (Fig. 3.1.2.2) that there was a reduction in the proportion

Fig. 3.1.2.1 Effect of ribonuclease on the sedimentation of ribosomes on sucrose density gradients

Ribosomes (2 A_{260} units/0.2 ml), extracted from rat skeletal muscle at high ionic strength (250 mM KCl), were suspended in medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$ and incubated (a) with pancreatic ribonuclease (1 μg/ml) or (b) without pancreatic ribonuclease for 15 min at 37°C; or (c) without pancreatic ribonuclease for 15 min at 0°C. They were then layered onto a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium, centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at 4°C and analysed as described in section 2.5.1.

Fig. 3.1.2.1.

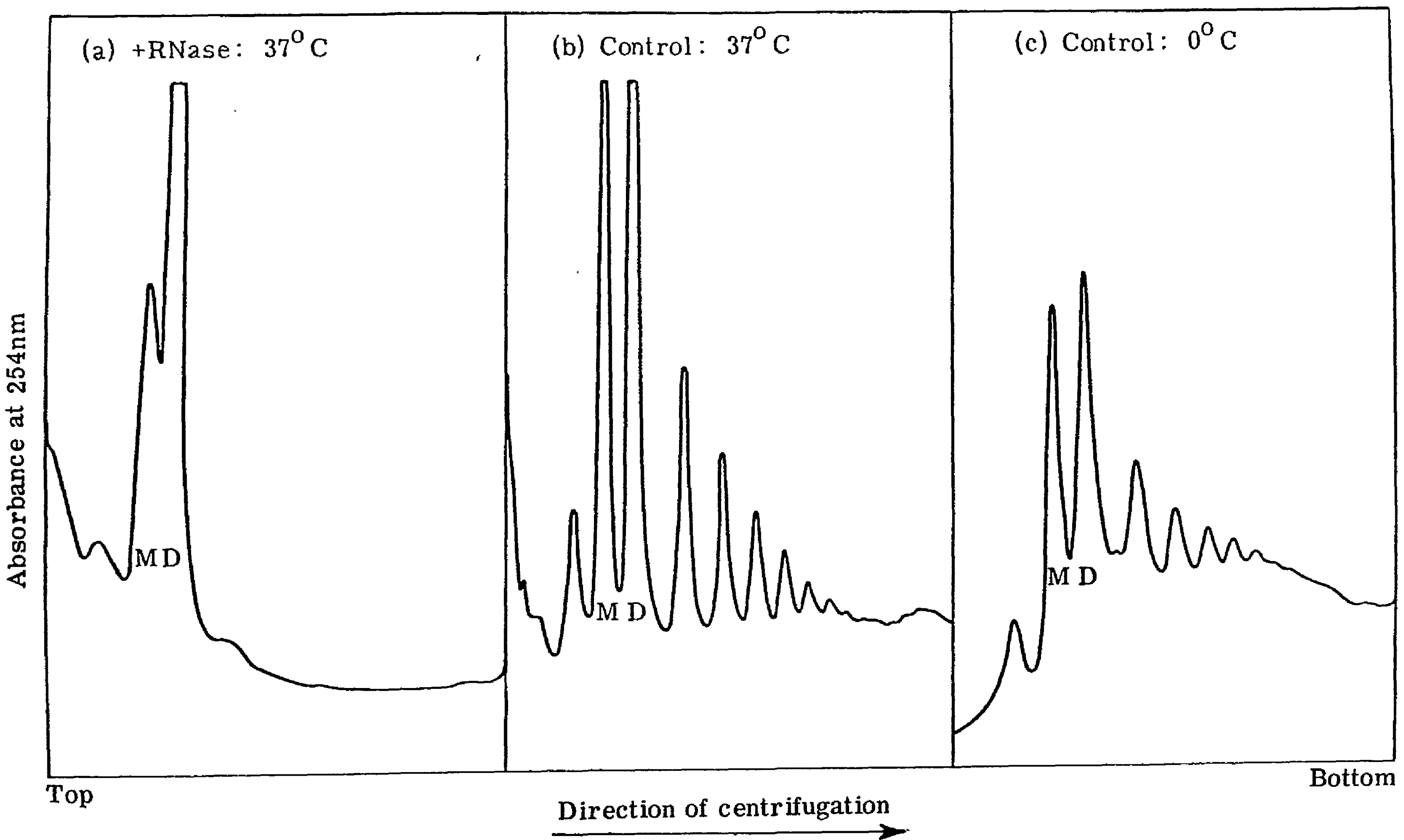
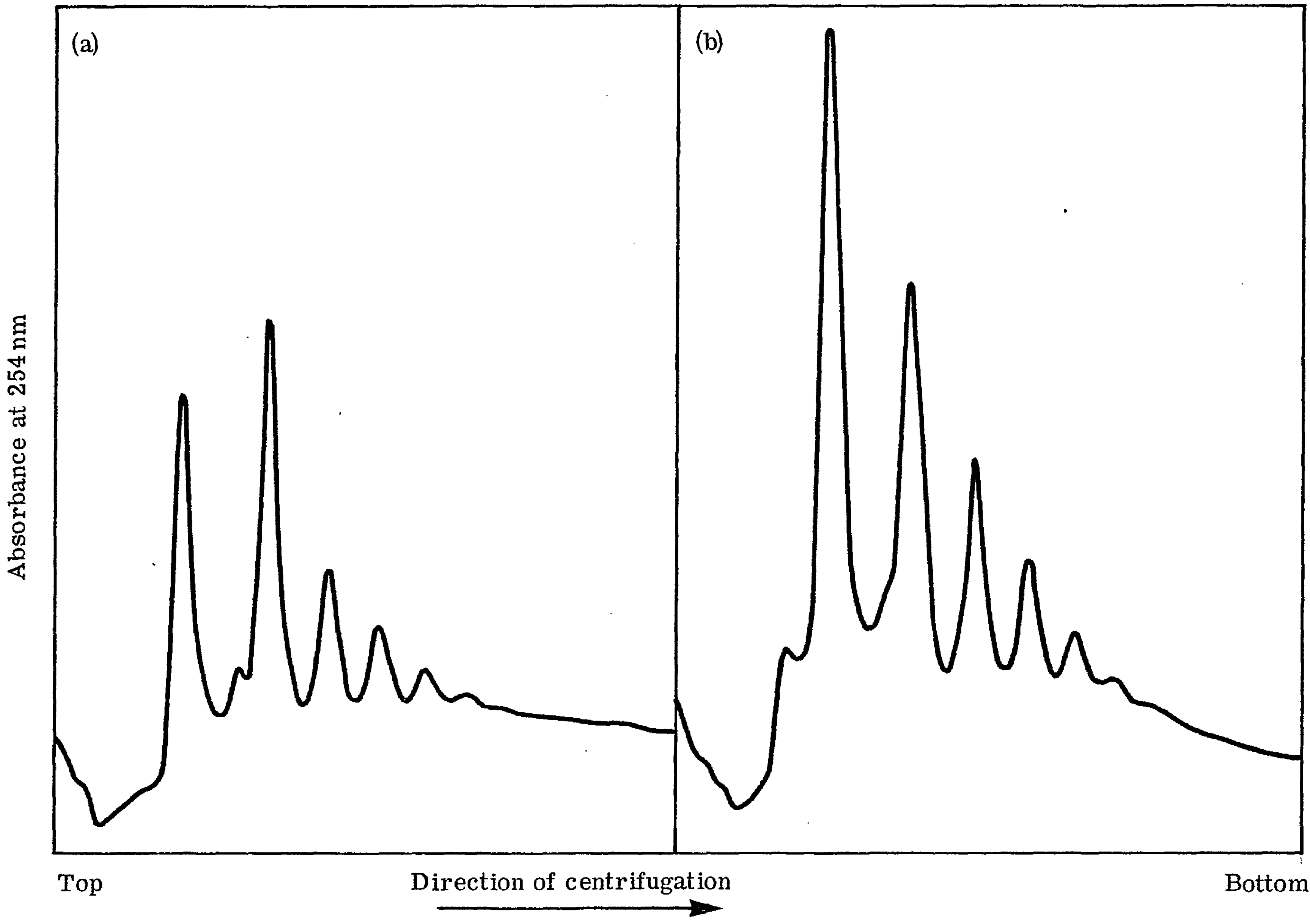


Fig.3.1.2.2 Analysis of ribosomes extracted at low ionic strength on sucrose density gradients of different ionic composition

Ribosomes (2 A_{260} units/0.2 ml), extracted from rat skeletal muscle at low ionic strength (80 mM KCl; section 2.3.1) were suspended and centrifuged in medium containing (a) 50 mM Tris-HCl (pH 7.6), 80 mM KCl, 10 mM $MgCl_2$; or (b) 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$. The 5.4 ml linear gradients of 15-45% (w/v) sucrose were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$, and analysed as described in section 2.5.1.

Fig. 3.1.2.2.



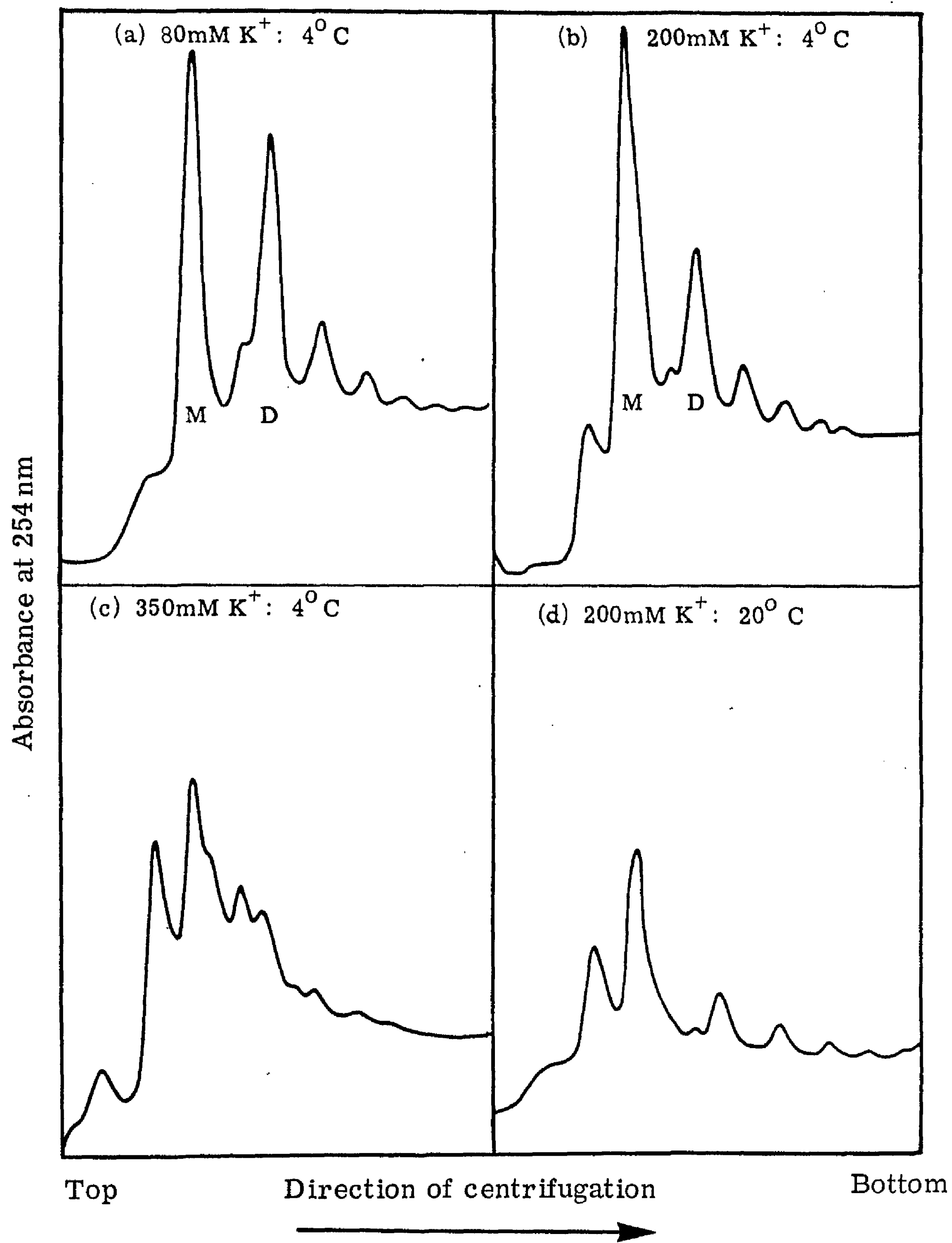
of the '114S' peak (as well as a greater recovery of ribosomes on the gradients). It thus appeared that an increase in the ratio of potassium to magnesium ions might be reducing the formation of dimers of the 80S species. Ribosomes prepared at high ionic strength were therefore analysed on sucrose density gradients at increasing concentration of KCl. Fig. 3.1.2.3 shows that the relative size of the '114S' peak was reduced on gradients containing 200 mM KCl, compared with those containing 80 mM KCl, but that at 350 mM KCl breakdown of polysomes appeared to occur with the simultaneous formation of a peak sedimenting more slowly than 80S, probably representing ribosomal subunits.

In the experiment to determine the effect of ribonuclease on the sedimentation of muscle ribosomes it was observed that the control, incubated at 37°C without ribonuclease, had incidentally increased the resolution of the different polysome species (Fig. 3.1.2.1, cf. b and c). It was thought possible that further disaggregation of the '114S' peak might be achieved by increasing the temperature of centrifugation. However when the centrifugation was performed in medium containing 200 mM KCl at 20°C, the polysome profiles appeared disturbed, and although a reduction of the '114S' peak did occur, there was an increase in species (presumably ribosomal subunits) sedimenting slower than monomers (Fig. 3.1.2.3 d). It was therefore decided to avoid the use of elevated temperature. In subsequent studies a medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl and 5 mM $MgCl_2$ was used for fractionating polysomes on sucrose density gradients and the '114S' peak was always combined with the monomer peak in subsequent fractionation of sucrose density gradients for analysis of messenger RNA.

Fig. 3.1.2.3 Effect of ionic strength and temperature on rat skeletal muscle ribosomes analysed on sucrose density gradients

Ribosomes (2 A_{260} units), extracted from rat skeletal muscle at high ionic strength (250 mM KCl; section 2.3.2), were suspended in 0.2 ml of medium containing (a) 50 mM Tris-HCl (pH 7.6), 80 mM KCl, and 5 mM $MgCl_2$; (b) and (d) 50 mM Tris-HCl (pH 7.6), 200 mM KCl, and 5 mM $MgCl_2$; (c) 50 mM Tris-HCl (pH 7.6), 350 mM KCl, and 5 mM $MgCl_2$; and layered over 5.2 ml linear gradients of 15-45% (w/v) sucrose in the same medium. These were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$ (a, b and c), or for 25 min at $20^{o}C$ (d), and analysed as described in section 2.5.1.

Fig. 3.1.2.3.



3.1.3 Attempts to optimize the yield and size of polysomes

The proportion of polysomes relative to monosomes in the ribosomes obtained in this work was similar to that reported by others working with adult skeletal muscle (Stirewalt, Wool and Cavicchi, 1967; Chen and Young, 1968; Von Der Decken, 1970; Nihei, 1971; Jefferson, Koehler and Morgan, 1972; Manchester, 1974). However this proportion was much less than that obtained from other sources, e.g. cultured cells (Schochetman and Perry, 1972a) or embryonic muscle (Heywood, Dowben and Rich, 1967), where large polysomes predominate under physiological conditions. Although this might reflect a lower protein synthetic activity in adult muscle and, on average, smaller proteins being made; it was also possible that this might not represent the true distribution of polysomes in the cell. It might have resulted from "run-off" of ribosomes (elongation and termination without reinitiation) or from ribonuclease contamination during the preparation of the ribosomes.

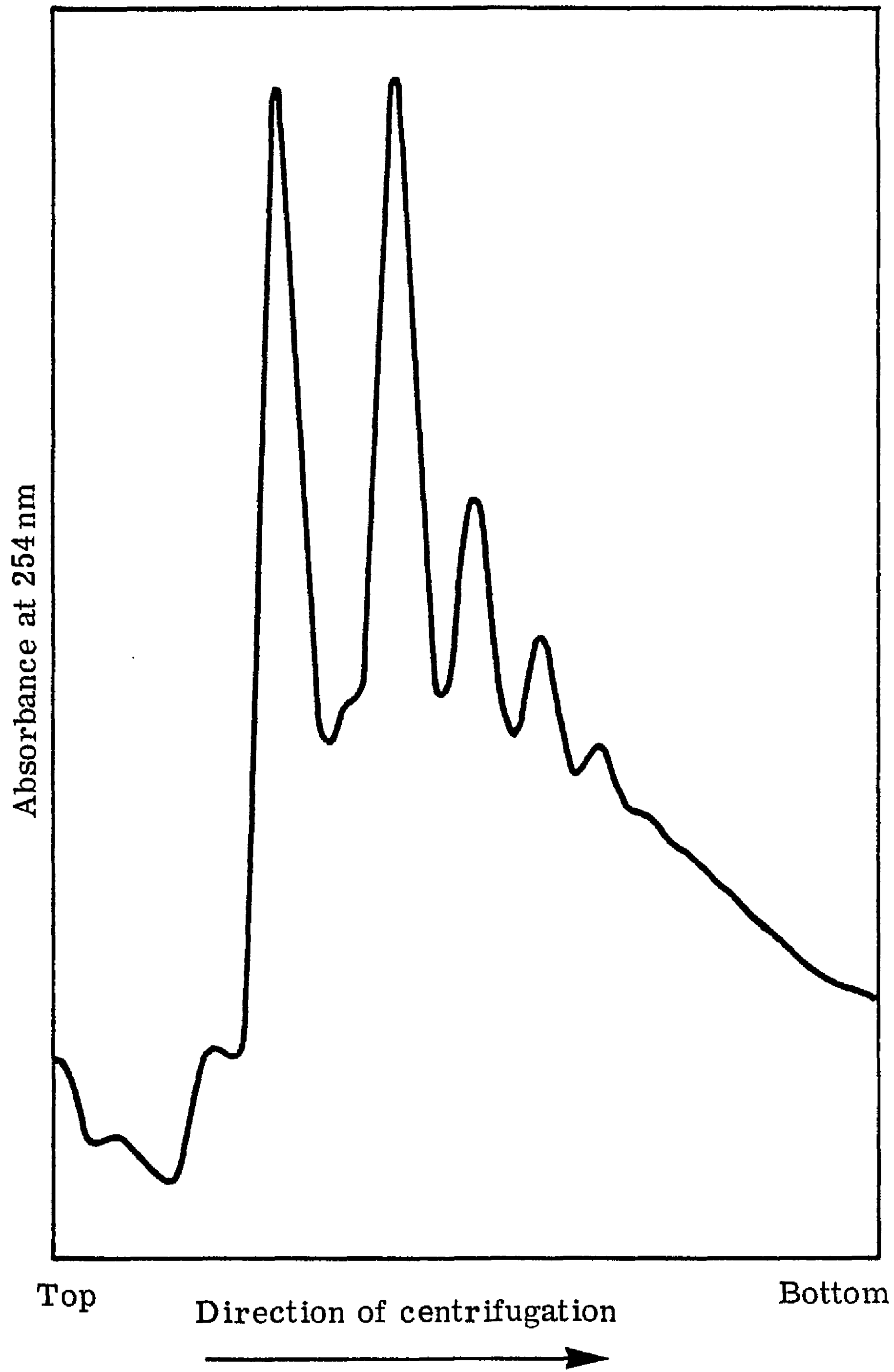
One means of preventing ribosome "run-off" is with cycloheximide which, at suitable concentration, inhibits the elongation of protein synthesis (Baliga, Cohen and Munro, 1970). However, Fig. 3.1.3.1 shows that when skeletal muscle ribosomes were prepared using solutions to which cycloheximide had been added there was no change in the proportion of polysomes compared with Fig. 3.1.1.1.

One possible reason for this result might have been that the skeletal muscle was too thick to allow the cycloheximide to penetrate sufficiently quickly into the majority of the tissue. One type of muscle in which this problem could be overcome was diaphragm, the extreme thinness of which should permit rapid diffusion of the cycloheximide through the tissue. However it had not been shown whether the ribosomes of this type of muscle responded to

Fig. 3.1.3.1 Sucrose density gradient sedimentation of ribosomes isolated from rat skeletal muscle in the presence of cycloheximide

Ribosomes were extracted from rat skeletal muscle at high ionic strength (250 mM KCl; section 2.3.2) in the presence of cycloheximide (added to all solutions to a concentration of 100 μg/ml). The ribosomes (2 A_{260} units) were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$ and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at 4°C and analysed as described in section 2.5.1.

Fig. 3.1.3.1.



diabetes in the same way as those of skeletal muscle; and there was real doubt on this matter, in view of the report that heart muscle does not respond to diabetes with a reduction in the proportion of polysomes (Rannels _et al._, 1970). In fact, Fig. 3.1.3.2 shows that, in diabetes, diaphragm suffers a decline in polysomes, similar to that of thigh and gastrocnemius muscles. When diaphragm ribosomes were isolated in the presence of cycloheximide there was, however, no increase in the proportion of polysomes (Fig. 3.1.3.3). Thus it did not appear that "run-off" of ribosomes was responsible for the relatively low proportion of polysomes.

The second possibility was that limited cleavage of mRNA by ribonuclease was reducing the proportion of polysomes. To try to eliminate ribonuclease, ribosomes were prepared using sterilized glassware and with heparin added to all solutions to inhibit ribonuclease. However this did not increase the proportion of polysomes (Fig. 3.1.3.4). Thus while it was felt prudent to continue to use sterilized glassware for preparation of ribosomes, neither heparin nor cycloheximide were used in subsequent preparations.

Fig. 3.1.3.2 Sucrose density gradient centrifugation of ribosomes isolated from the diaphragm of normal and alloxan-diabetic rats

Ribosomes (2 A_{260} units) were extracted from the diaphragms of (a) normal rats, or (b) rats, 3 days after induction of diabetes with alloxan, at high ionic strength (250 mM KCl; section 2.3.2), suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at 4°C and analysed as described in section 2.5.1.

Fig. 3.1.3.2.

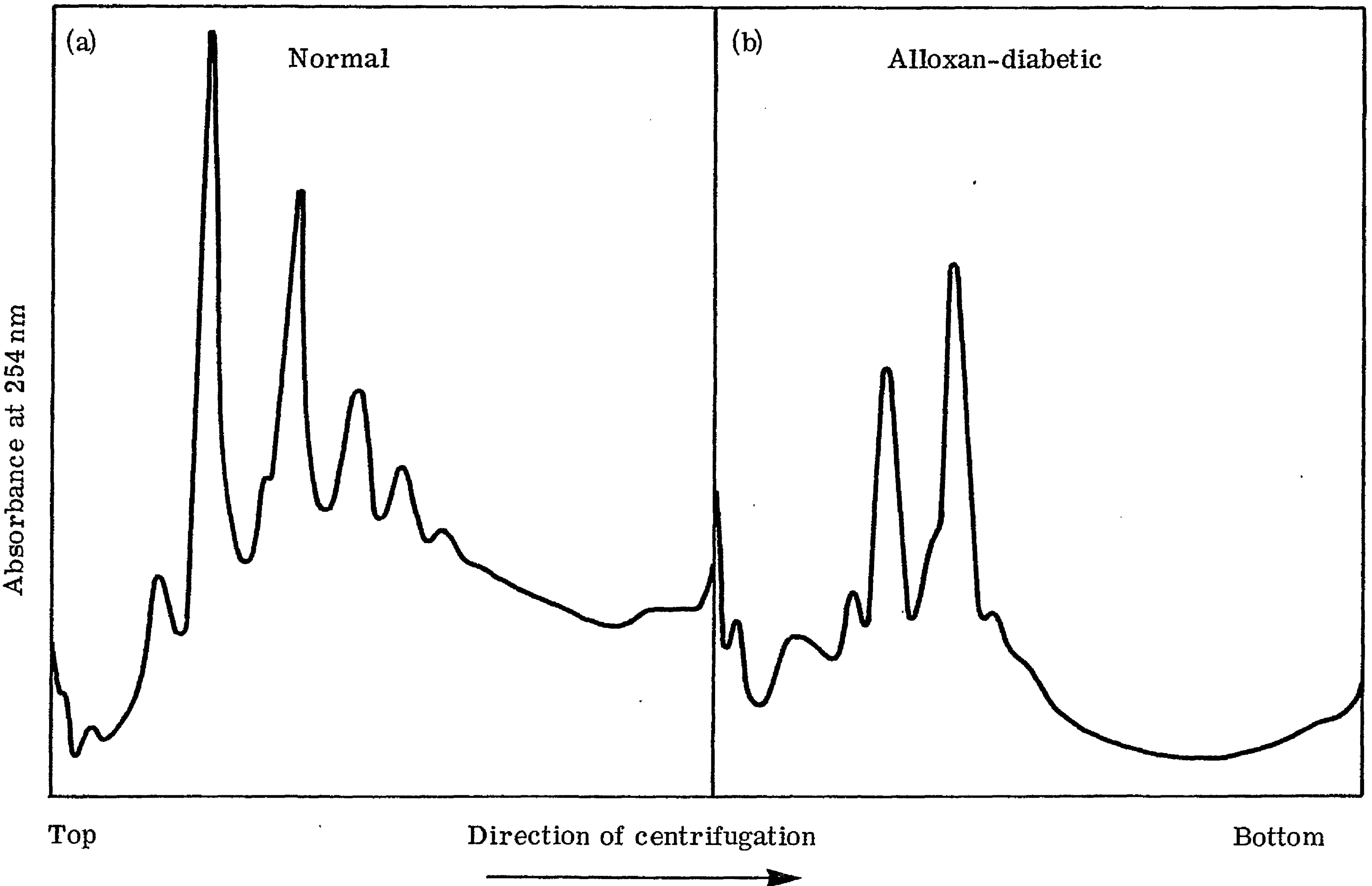


Fig. 3.1.3.3 Sucrose density gradient sedimentation of ribosomes isolated from rat diaphragm in the presence of cycloheximide

Ribosomes were extracted from rat diaphragm at high ionic strength (250 mM KCl; section 2.3.2) in the presence of cycloheximide (added to all solutions to a concentration of 100 µg/ml). The ribosomes (2 A_{260} units) were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$ and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$ and analysed as described in section 2.5.1.

Fig. 3.1.3.3.

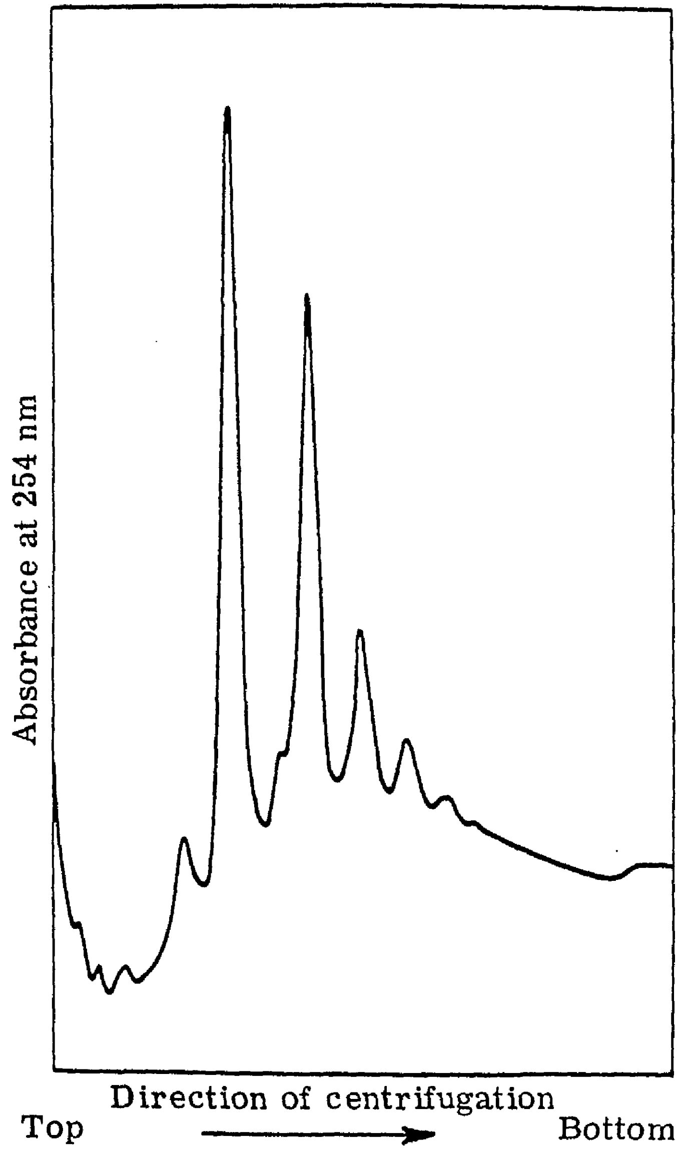
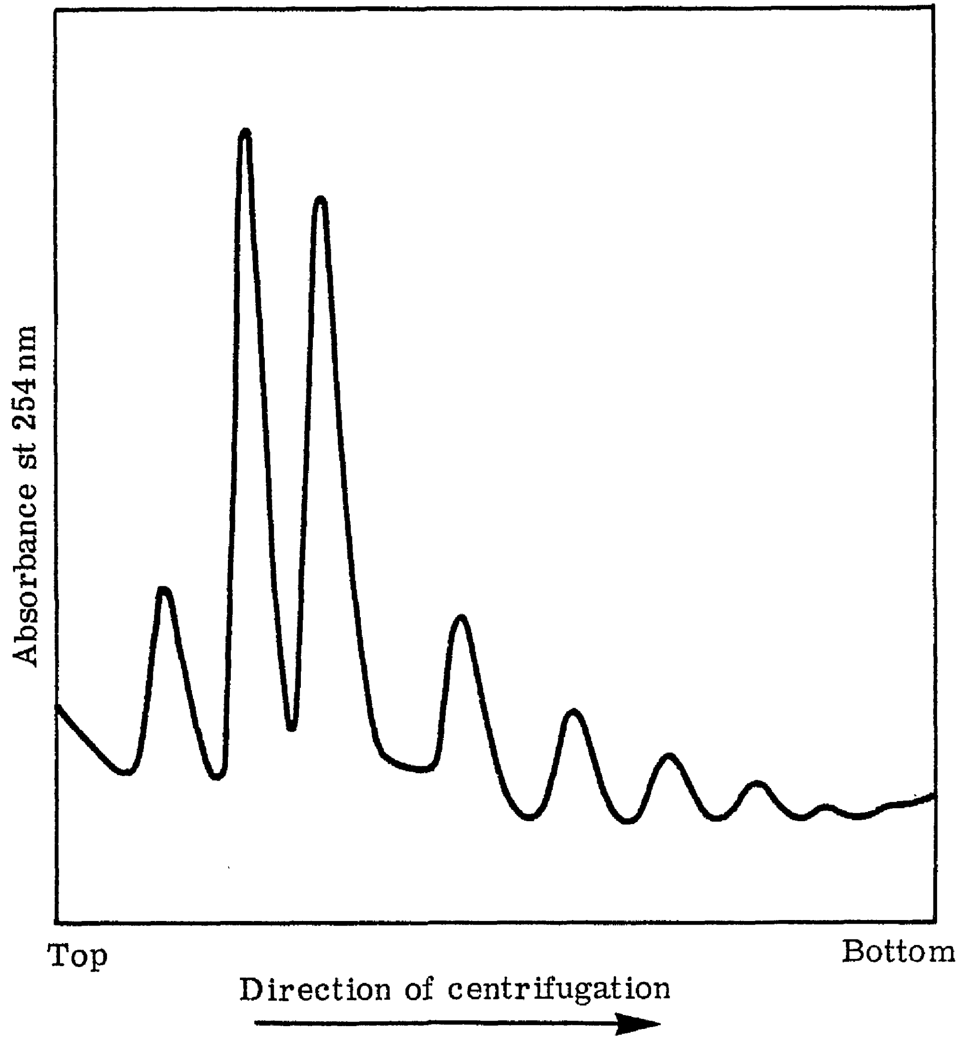


Fig. 3.1.3.4 Sucrose density gradient centrifugation of ribosomes isolated from rat skeletal muscle under conditions designed to reduce possible ribonuclease activity

Ribosomes were extracted from rat skeletal muscle at high ionic strength (250 mM KCl; section 2.3.2) in the presence of heparin (added to all solutions to a concentration of 1 mg/ml). The ribosomes (2 A_{260} units) were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$ and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at 4^oC and analysed as described in section 2.5.1.

Fig. 3.1.3.4.



3.2 THE EFFECT OF DIABETES AND INSULIN ON RAT SKELETAL MUSCLE POLYSOMES

3.2.1 Effect of diabetes and insulin on the sedimentation profiles of ribosomes

Having decided on suitable conditions to isolate ribosomes from rat skeletal muscle, the effect of diabetes and insulin was examined, to determine whether this was similar to that described previously (Stirewalt, Wool and Cavicchi, 1967).

Fig. 3.2.1.1 (a and e) shows that alloxan reduced the proportion of polysomes and increased the proportion of monosomes and other low molecular weight species in skeletal muscle ribosomes extracted at low ionic strength. The effect of diabetes was in agreement with that found by others (Stirewalt, Wool and Cavicchi, 1967). The ribosomes isolated at high ionic strength from the skeletal muscle of rats made diabetic by alloxan also showed a decreased proportion of polysomes and increased proportion of low molecular weight species (c). The detailed shape in (c) is different from that in (e), but this difference was not seen on every occasion.

When insulin was injected into alloxan-diabetic rats, 60 min before death, and ribosomes extracted at high ionic strength, the anticipated reformation of polysomes did not occur (d). This result was most surprising. However, numerous attempts to obtain reformation of polysomes in alloxan-diabetic rats after injection of insulin failed, even though experiments were performed in which the period of diabetes was reduced or younger animals were used. It was decided, however, to examine ribosomes extracted at low ionic strength,

Fig. 3.2.1.1. Analysis of ribosomes from rat skeletal muscle on sucrose density gradients

Ribosomes (1.5 A_{260} units) were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$ and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$, and analysed as described in section 2.5.1.

(a) Normal rat; low ionic strength

(b) Normal rat; high ionic strength

(c) Alloxan-diabetic rat; high ionic strength

(d) Alloxan-diabetic rat + insulin; high ionic strength

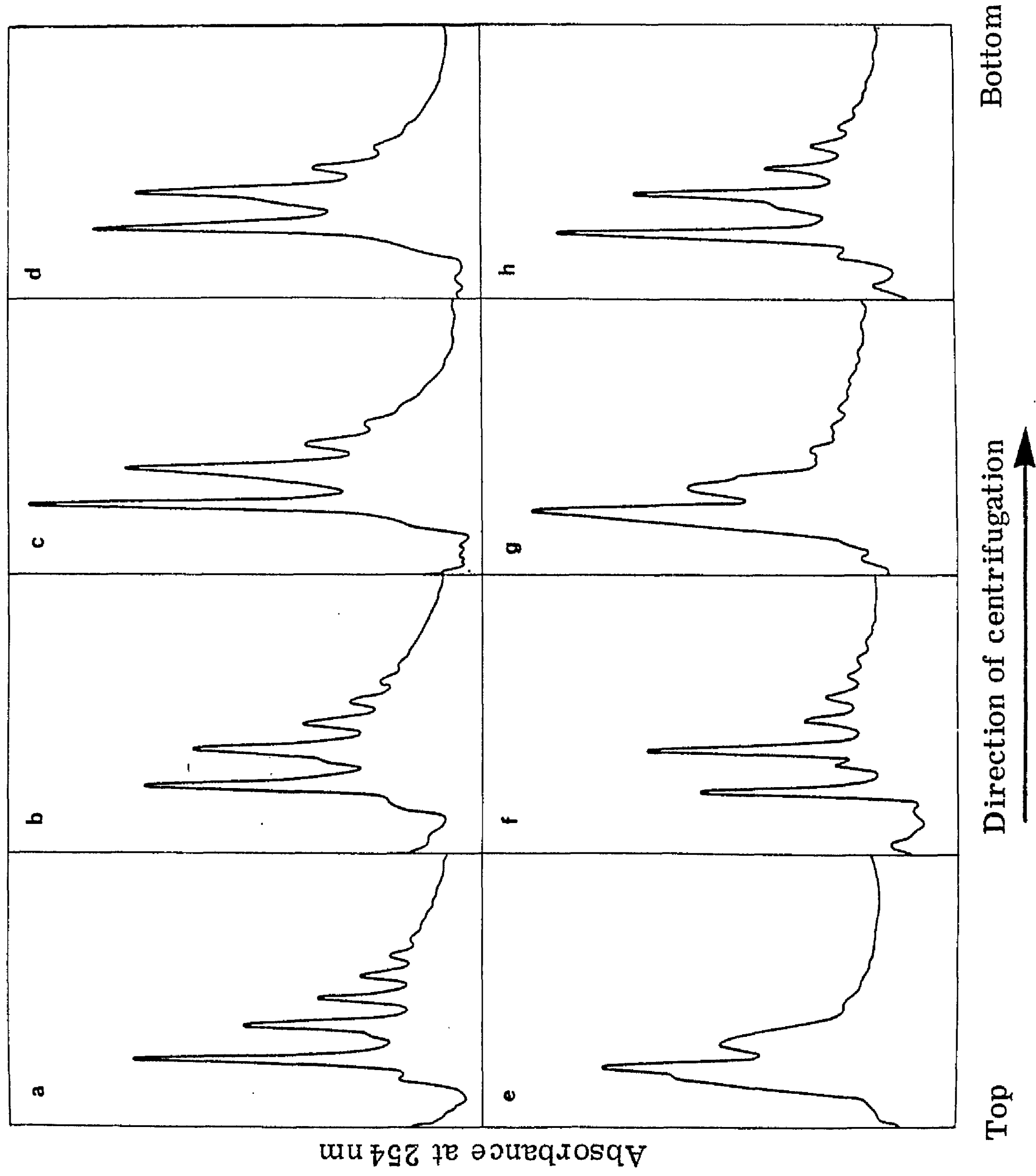
(e) Alloxan-diabetic rat; low ionic strength

(f) Alloxan-diabetic rat + insulin; low ionic strength

(g) Streptozotocin-diabetic rat; high ionic strength

(h) Streptozotocin-diabetic rat + insulin; high ionic strength

Fig. 3.2.1.1.



and in this case the injection of insulin did cause the polysomes to reform (f). Thus the effect of insulin on polysome reformation could be obtained only if the ribosomes were isolated at low ionic strength, but not if they were isolated at high ionic strength. It therefore appeared that the high ionic strength method for extraction of ribosomes produced some artifact which obscured the effect of insulin.

When the other diabetogenic drug, streptozotocin, was used, it was found that insulin caused reformation of the polysomes even when the ribosomes were extracted at high ionic strength (g and h). This effect of insulin was rapid as can be seen from Fig. 3.2.1.2, where a partial effect is evident 10 min after administration of insulin. These results were similar to those of Stirewalt et al. (1967), although those workers found more extensive reformation of polysomes by 5 min. Thus it seems that the artifact associated with ribosomes extracted at high ionic strength from diabetic rats is specific for alloxan.

In the light of these observations, it was decided to select streptozotocin as the diabetogenic drug and this was used at the lowest effective concentration of 50 mg/kg body weight (see Experimental; section 2.2.3). In addition, low ionic strength medium was chosen for extracting the skeletal muscle ribosomes. Though the low ionic strength extraction gives a lower yield of ribosomes than extraction at high ionic strength (Table 3.1.1.1), it seemed prudent to avoid the latter condition. The effects of streptozotocin diabetes and insulin on the sedimentation profiles of ribosomes extracted at low ionic strength are shown in Fig. 3.2.1.3 and were similar to their effects on ribosomes extracted at high ionic strength (Fig. 3.2.1.1).

Fig. 3.2.1.2 Effect of insulin on the sedimentation of ribosomes isolated from the skeletal muscle of streptozotocin-diabetic rats

Ribosomes (1.5 A_{260} units) were extracted at high ionic strength (250 mM KCl; section 2.3.2) from the skeletal muscle of rats 3 days after they had received streptozotocin. They were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. Ribosomes were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$, and analysed as described in section 2.5.1. (a) Ribosomes from rats receiving no insulin. (b) Ribosomes from rats receiving 5 units of insulin 10 min before death. (c) Ribosomes from rats receiving 5 units of insulin 60 min before death.

Fig. 3.2.1.2.

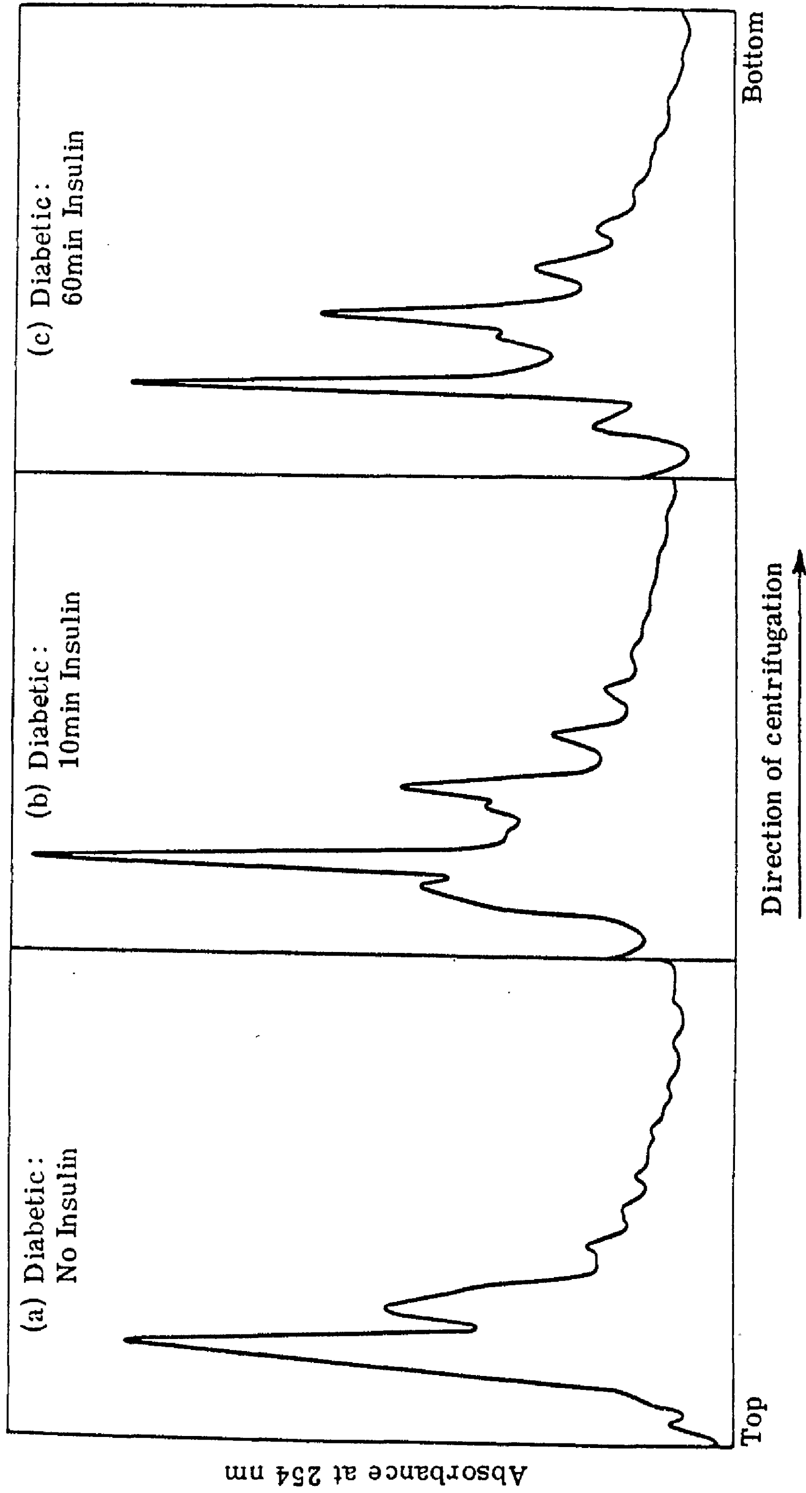
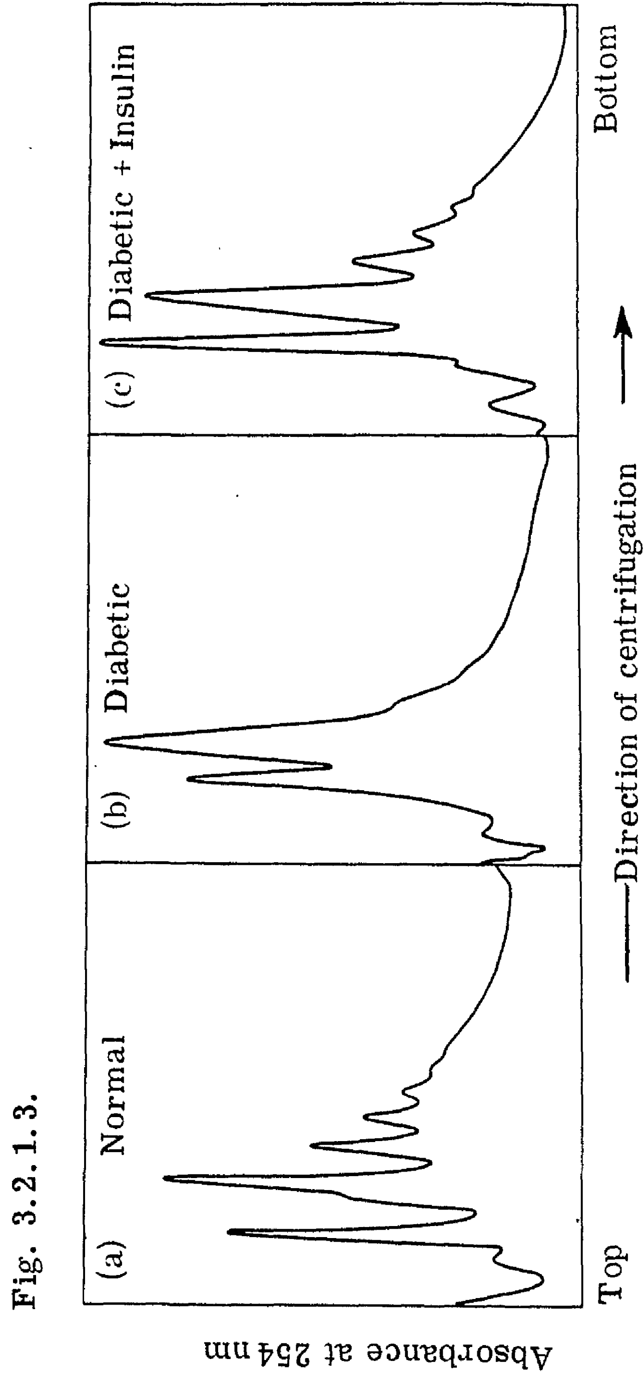


Fig. 3.2.1.3 Effect of streptozotocin-diabetes and insulin on rat skeletal muscle ribosomes isolated at low ionic strength

Ribosomes (1.5 A_{260} units) were extracted from rat skeletal muscle at low ionic strength (80 mM KCl; section 2.3.1), suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$ and analysed as described in section 2.5.1. (a) Ribosomes from normal rats. (b) Ribosomes from rats killed 2 days after having been injected with streptozotocin (50 mg/kg body weight). (c) Ribosomes from streptozotocin-diabetic rats given 5 units of insulin 60 min before death.

Fig. 3.2.1.3.



To quantitate the effect of diabetes on the proportion of polysomes in the total ribosome population , the areas under the monosome + '114S' and polysome peaks were measured by tracing these out and weighing them. It was found that the proportion of polysomes was about 60% in muscle ribosomes from normal rats but was only 30% in muscle ribosomes from diabetic rats (Table 3.2.1.1). Thus the proportion of polysomes in the ribosomes from the diabetic rats was only about 50% of that in the ribosomes from normal rats. This difference in proportion of polysomes was quite similar to the difference in the activity of skeletal muscle ribosomes in cell-free protein synthesis *in vitro*, where the ribosomes from diabetic rats were 40% as active as those from the normal (Fig. 3.2.1.4 and Table 3.2.1.2). When insulin was administered to diabetic rats, the protein synthetic activity of ribosomes was restored to 80% of that of the normal (Fig. 3.2.1.4). These results were in good agreement with those of Wool and Cavicchi (1967). When the products synthesized *in vitro* were analysed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis, the products were predominantly of small molecular weight (15,500 and 17,500), although some species of higher molecular weight were seen on prolonged exposure of the X-ray film (Fig. 3.2.1.5). This is consistent with the small size of the polysomes detected on sucrose density gradients. The polypeptides synthesized by the ribosomes from diabetic rats were in general similar to those synthesized by normal rats, consistent with previous findings using discontinuous electrophoresis on polyacrylamide gels (Kurihara and Wool, 1968). Although some high molecular weight bands (112,000, 104,000, 98,000) can be seen in the products of ribosomes from normal - but not diabetic - rats (Fig. 3.2.1.5), this difference was not always found.

Table 3.2.1.1 Proportion of polysomes in ribosomes from the skeletal muscle of normal and diabetic rats

The rats were made diabetic with streptozotocin (50 mg/kg body weight) and killed 2 days later. The areas under the monomers + '114 S', and under the polysome peaks (of the sucrose gradient profiles) were measured by tracing these out and weighing them.

Figures in parentheses indicate comparison with the normal value of polysomes, set at 100% in each case.

Table 3.2.1.1

Expt.No.	Normal		Diabetic	
	Mono- + Dimers	Polysomes	Mono- + Dimers	Polysomes
1	0.39	0.61 (100%)	0.69	0.31 (51%)
2	0.34	0.66 (100%)	0.71	0.29 (44%)
3	0.36	0.64 (100%)	0.67	0.33 (52%)
4	0.45	0.55 (100%)	0.74	0.26 (47%)
5	0.49	0.51 (100%)	0.71	0.29 (57%)
Mean	0.41	0.59 (100%)	0.70	0.30 (51%)

Fig. 3.2.1.4 Effect of diabetes and insulin on the protein synthetic activity of ribosomes from rat skeletal muscle assayed in a cell-free system from rat liver

The assay mixture (100 µl) contained: 20 mM Tris-HCl (pH 7.6), 125 mM KCl, 7.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM ATP (neutralized), 0.1 mM GTP (neutralized), 5 mM creatine phosphate (0.18 mg), 20 µg of creatine phosphokinase, 40 µg of E.coli tRNA charged with (^3H) phenylalanine (specific activity 5 Ci/mmole) and 19 non-radioactive amino acids, 0.7 mg of rat liver supernatant protein; and various amounts of ribosomes as indicated. Incubation was for 30 min at 37°C, thereafter the radioactivity incorporated into protein was measured as described in section 2.7.1. Diabetic rats had received streptozotocin (50 mg/kg body weight) 2 days before death and, where indicated, 5 units of insulin 60 min before death. Each 100 cpm of radioactivity in protein was the result of incorporation of 0.03 pmole of phenylalanine.

Fig. 3.2.1.4.

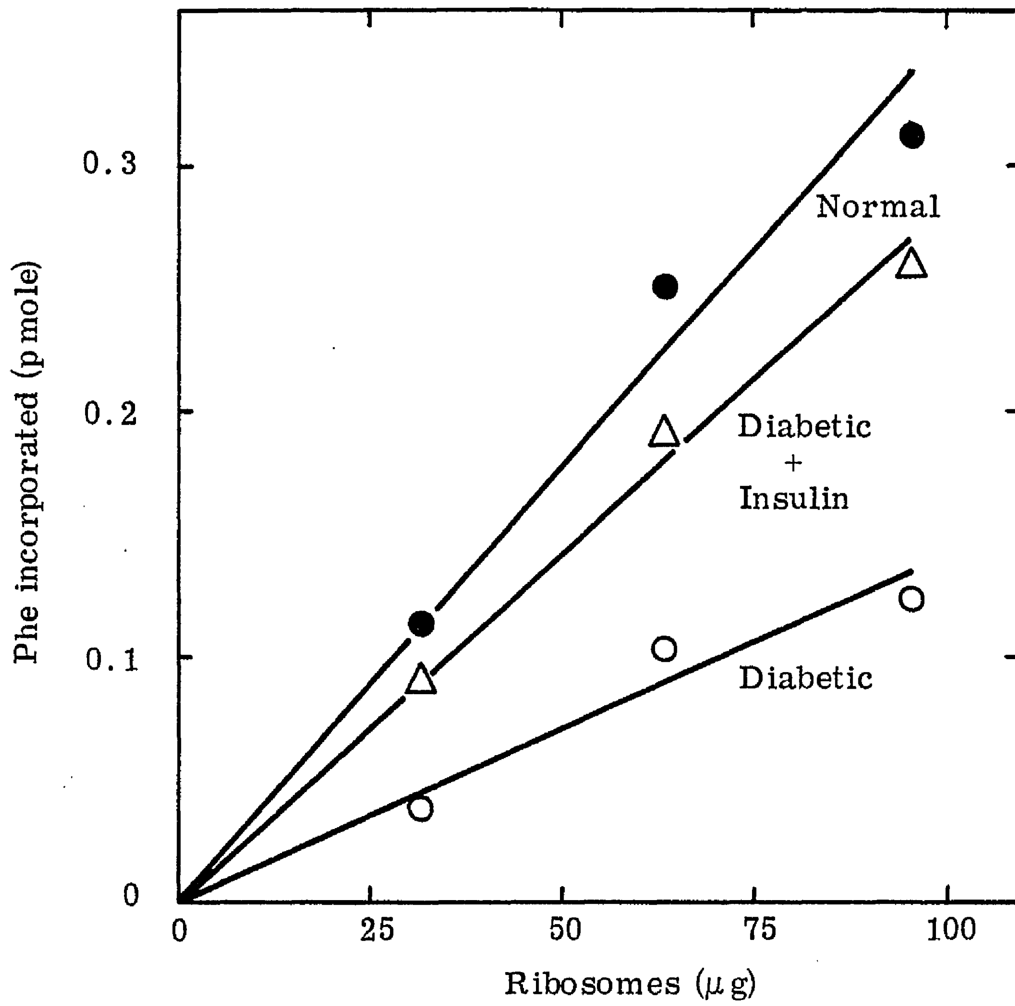


Table 3.2.1.2 Effect of diabetes on the protein synthetic activity of ribosomes from rat skeletal muscle assayed in a cell-free system from wheat germ

The assay mixture (50 µl) contained: 15 µl of wheat germ S-30 fraction, 20 mM HEPES (pH 7.6), 2 mM dithiothreitol, 1 mM ATP (neutralized), 0.02 mM GTP, 8 mM creatine phosphate, 0.5 µg creatine phosphokinase, 0.4 mM spermidine, 180 mM KCl, 2.5 mM Mg acetate, 25 µCi of (^{35}S) methionine (1080 Ci/mmole), 17 µg of ribosomes and/or water to 50 µl. Reaction mixtures were incubated at 25°C for 2 hr, then 5 µl aliquots were removed for determination of radioactivity incorporated into protein as described in section 2.7.2. Diabetic rats had received streptozotocin (50 mg/kg body weight) 2 days before death. (Values were corrected for a background of 23,000 cpm).

Table 3.2.1.2

Source of ribosomes tested	(^{35}S) incorporation (cpm)	% of Normal
Normal rats	148,000	100%
Streptozotocin-diabetic rats	64,000	43%

Fig. 3.2.1.5 Sodium dodecyl sulphate gel electrophoresis of the translation products of rat skeletal muscle ribosomes

The products of translation of skeletal muscle ribosomes in a cell-free system from wheat germ (cf. Table 3.2.1.2) were processed for sodium dodecyl sulphate gel electrophoresis as described in section 2.7.3. Dried gels were subjected to fluorography for (a) 7 days or (b) 28 days.

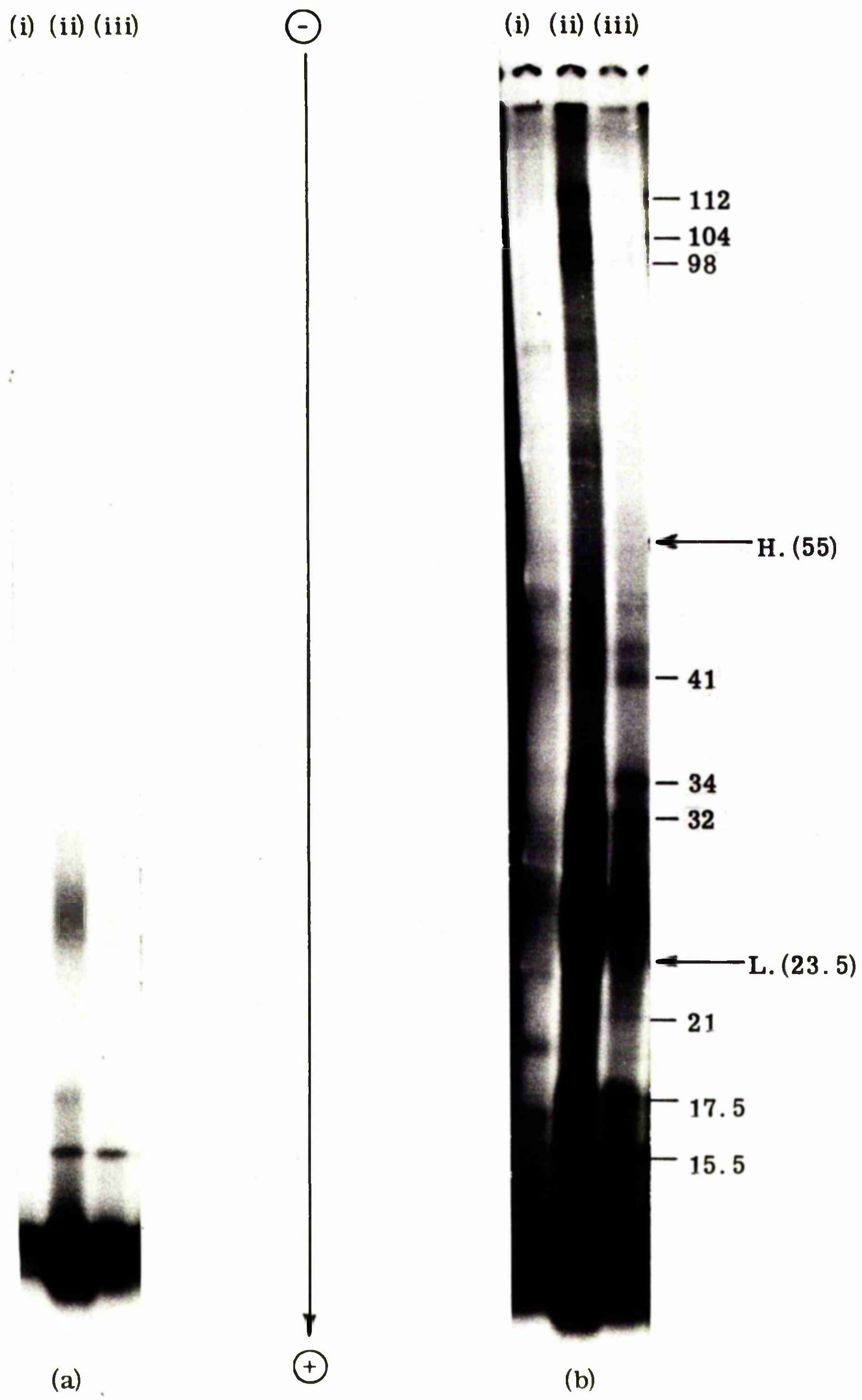
(i) No added ribosomes

(ii) Ribosomes (17 μg) from normal rats

(iii) Ribosomes (17 μg) from streptozotocin-diabetic rats

The molecular weights indicated were calculated from Fig. 2.7.3.1 and are x 10^{-3}. Their accuracy is subject to the limitations noted in the legend to Fig. 2.7.3.1.

Fig. 3.2.1.5.



3.2.2 Ribonuclease activity of rat skeletal muscle

It was desirable to account more precisely for the inability of insulin to reverse the effect of alloxan-diabetes (Fig. 3.2.1.1 c and d) on the polysomes isolated from rat skeletal muscle at high ionic strength. The most obvious explanation was that alloxan causes the induction or activation, in rat skeletal muscle, of a ribonuclease which is only extracted into the 'post-mitochondrial fraction' in a medium of high ionic strength. If this ribonuclease were not inactivated within 60 min of insulin injection, it would then, during extraction of the ribosomes, cause the degradation of polysomes that had reassembled in vivo in response to insulin injection. It is assumed that streptozotocin does not cause the appearance of this putative nuclease, presumably because of its less severe side effects compared with alloxan (Mansford and Opie, 1968).

Attempts were made to detect this putative ribonuclease directly by assaying the ability of 'post-mitochondrial supernatant' to liberate acid-soluble products from added RNA. Initially yeast RNA was used as a substrate, and the liberated nucleotides were detected by their ultraviolet absorbance (Experimental; section 2.8.1). It can be seen from Table 3.2.2.1 that in no case was the ribonuclease activity of the 'post-mitochondrial supernatant' from the muscle of alloxan - or streptozotocin - diabetic rats higher than that from the normal, whether the homogenization of tissue had been made in medium of high or low ionic strength. Because the sensitivity of this method for measurement of ribonuclease activity was relatively low, a second approach was adopted in which a (^{32}P)-labelled rRNA from HeLa cells was used as a substrate (Experimental; section 2.8.2). However, there was also no increase in ribonuclease activity in the 'post-mitochondrial

Table 3.2.2.1 Ribonuclease activity of rat skeletal muscle 'post-mitochondrial supernatants' assayed with non-radioactive yeast RNA as substrate

The 'post-mitochondrial supernatant' (0.5 ml) prepared at low ionic strength (80 mM KCl; section 2.3.1) or at high ionic strength (250 mM KCl; section 2.3.2) together with 0.5 mg of yeast RNA in 200 mM sodium acetate-acetic acid (2 mg/ml), pH 7.6, were adjusted to a final volume of 2 ml in 200 mM sodium acetate (pH 7.6), and incubated at 37°C for 2 hr. Control samples lacking the supernatant or the substrate were similarly incubated. The ribonuclease activity was determined by measuring the ultraviolet-absorbance of the acid-soluble digestion products at 260 nm and expressed as ΔA_{260}/min/mg protein, as described in section 2.8.1.

Table 3.2.2.1

Conditions of homogenization	Ribonuclease Activity ΔA_{260}/min/mg protein		
	Normal	Alloxan-diabetic	Streptozotocin-diabetic
Low ionic strength	0.0069	0.0059	0.0055
High ionic strength	0.0067	0.0060	0.0061

supernatant from the muscle of alloxan-diabetic rats when the conversion of (^{32}P)-labelled rRNA into acid-soluble fragments was followed (Table 3.2.2.2); even though in this case the ribonuclease activity was somewhat higher in the extracts prepared at high ionic strength.

No further attempts were made to pursue this problem as it did not appear to be relevant to the catabolism of skeletal muscle RNA in diabetes. This is evident from measurements of skeletal muscle RNA under various conditions (Table 3.2.2.3), where the reduction in RNA content of muscle (expressed on a DNA basis) was found to be similar in both types of diabetes (74 and 75% of normal for alloxan - and streptozotocin - diabetic rats respectively). It should be noted that this reduction in RNA content of skeletal muscle in diabetes is a separate effect from the reduction in activity of the ribosomes described in section 3.3.2, where equal amounts of ribosomes were always compared. Thus, diabetes reduced the rate of protein synthesis by 60% (Fig. 3.2.1.4), over and above the reduction that would be caused by the 25% decrease in (ribosomal) RNA (Table 3.2.2.3). Moreover insulin causes the reformation of polysomes (Fig. 3.2.1.3) and the restoration in the rate of cell-free protein synthesis (Fig. 3.2.1.4) in less than 60 min, but does not restore the normal ratio of RNA to DNA in this time (Table 3.2.2.3).

It was of interest to see whether the reduction in skeletal muscle RNA in both types of diabetes (Table 3.2.2.3) was associated with an increase in the lysosomal acid ribonuclease, similar to that seen in dystrophic muscle (Abdullah and Pennington, 1968). Therefore homogenates of the skeletal muscle of normal and diabetic rats were assayed for ribonuclease activity with yeast RNA at pH 5 (Table 3.2.2.4). In this case an appreciable release of acid-soluble material was seen (cf. Table 3.2.2.1), but there was no greater ribonuclease activity in the homogenate from the diabetic animal.

Table 3.2.2.2 Ribonuclease activity of rat skeletal muscle 'post-mitochondrial supernatants' assayed with (^{32}P) rRNA as substrate

Reaction conditions were similar to those in Table 3.2.2.1 except that the buffered substrate was (^{32}P) rRNA from HeLa cells. The radioactivity in the post-acidification supernatant was determined as described in section 2.8.2. The ribonuclease activity was measured as (^{32}P) rRNA hydrolysed (counts/min/mg protein).

Table 3.2.2.2

Conditions of homogenization	(^{32}P) rRNA hydrolysed (counts/min/mg protein)		
	Normal	Alloxan-diabetic	Streptozotocin-diabetic
Low ionic strength	453	330	226
High ionic strength	602	581	533

Table 3.2.2.3 Effect of diabetes and insulin on the amount of RNA, DNA and protein in rat skeletal muscle

The assays were performed as described in sections 2.9 and 2.10. Rats were made diabetic by intravenous injection of alloxan (60 mg/kg body weight) or streptozotocin (65 mg/kg body weight) after having been starved overnight, and killed 3 days later. When treated with insulin, each rat was intraperitoneally injected with 5 units of insulin 60 min before death. Figures in parentheses are the percent change of diabetic or diabetic plus insulin compared with normal.

Each figure is the mean of 3 or 4 determinations.

Table 3.2.2.3

Physiological status of rats	Number of determinations	Body weight (g)	Skeletal muscle (g)	DNA (mg/ muscle	RNA (mg/ muscle	Protein (g/ muscle)	RNA/ DNA (g/g)	Protein/ DNA (g/g)
Normal	(4)	146.6	16.6	14.4	19.9	3.17	1.38	220
3 Day Alloxan	(3)	107.2 (-27%)	13.1 (-21%)	15.6 (+8%)	15.9 (-20%)	2.88 (-9%)	1.02 (-26%)	184 (-16%)
3 Day Alloxan + Insulin	(3)	102.7 (-30%)	13.4 (-19%)	12.8 (-11%)	14.8 (-26%)	2.62 (-17%)	1.16 (-16%)	205 (-7%)
3 Day Streptozotocin	(4)	106 (-28%)	13.7 (-18%)	14.4 0%	15.0 (-25%)	2.51 (-21%)	1.04 (-25%)	174 (-21%)

Table 3.2.2.4 Ribonuclease activity of rat skeletal muscle homogenates assayed with non-radioactive yeast RNA as substrate

Rat skeletal muscle homogenate (0.5 ml) prepared at high ionic strength (250 mM KCl, section 2.3.2) together with 0.25 ml (2 mg/ml) of yeast RNA in 200 mM sodium acetate-acetic acid (pH 7.6), were adjusted to a final volume of 2 ml in 200 mM sodium acetate (pH 5), and incubated at 37°C for 2 hr. Control samples lacking the supernatant or the substrate were similarly incubated. The ribonuclease activity was determined by measuring the ultraviolet-absorbance of the acid-soluble digestion products at 260 nm and expressed as ΔA_{260}/min/mg protein, as described in section 2.8.1

Table 3.2.2.4

Condition of rats	Ribonuclease activity (ΔA_{260}/min/mg protein)
Normal	0.045
Alloxan-diabetic	0.043

3.3 QUANTITATIVE COMPARISON OF POLYA(+)RNA FROM THE SKELETAL MUSCLE OF NORMAL AND DIABETIC RATS

Having established suitable conditions for the isolation of skeletal muscle ribosomes from normal and diabetic rats, it was possible to proceed to an analysis of the mRNA in these ribosomes. The amounts of mRNA in cells are small compared with those of other RNA species, and especially compared with rRNA. Thus, although the sedimentation coefficients of mRNAs, generally differ from those of rRNAs, it is difficult to detect mRNA by its ultra-violet absorption when total or polysomal RNA is fractionated by sucrose density gradient centrifugation. For this reason mRNA is often detected by labelling with a short pulse of radioactive RNA-precursor, under conditions where little labelling of rRNA occurs. This is possible in cultured cells and in rapidly metabolizing tissues such as the liver; however in skeletal muscle it is not possible to obtain sufficient incorporation of radioactive precursor into RNA (Wool and Cavicchi, 1966; Florini, 1970). It was therefore necessary to use other means to detect and quantitate the mRNA from this tissue. One way in which this may be done is by hybridizing (^{3}H) polyU with the unlabelled muscle RNA under conditions favouring binding of the homopolynucleotide to the polyA sequence at the 3'-end of the mRNA. The amount of bound (^{3}H) polyU may be determined by digesting the unhybridized species with ribonuclease, and collecting the hybrids on glass fibre filters. The amount of hybrid may then be used as a measure of the amount of mRNA present (Rudland, Weil and Hunter, 1975). Before applying this method to the quantitation of skeletal muscle mRNA, its validity was first confirmed in the experiments described in the following section.

3.3.1 Characterization of the (^{3}H) polyU hybridization assay for polyA(+)RNA

The hybridization assay with (^{3}H) polyU (conducted as described in Experimental; section 2.6) was first characterized with synthetic polyA. When mixtures of polyA and (^{3}H) polyU were incubated for various times (Fig. 3.3.1.1) it was found that there was fairly rapid initial formation of hybrid and a slower increase thereafter. For the convenience of allowing the hybridization to proceed overnight, an incubation time of 14 hr was adopted. It can be seen from Fig. 3.3.1.2 that the amount of (^{3}H) polyU bound was linearly dependent on the amount of polyA in the assay, up to about 0.1 μg polyA. The ratio of polyU to polyA was 2:1, implying hybrids of 2 polyU:1 polyA were formed under these conditions. Such triplexes had been found previously by other workers (Michelson et al., 1967). Thus, it appears from Fig. 3.3.1.1 that hybrids in the ratio 1 polyU:1 polyA were formed in the initial 2 hr hybridization phase.

The specificity of the hybridization reaction was shown by the fact that tRNA from E.coli did not form hybrids with (^{3}H) polyU (Fig. 3.3.1.3).

As the results of the hybridization assay with synthetic polyA appeared satisfactory, the assay was used to determine the polyA(+)RNA in the total RNA extracted from purified skeletal muscle ribosomes. This total RNA extracted from the purified ribosome pellet (Experimental; section 2.4.2) is predominantly ribosomal RNA (28S, 18S and 5S species), but it also contains the mRNA which is, of course, associated with the polysomes (and, as will be seen later, with other smaller ribosomal species, to a certain extent). For convenience this extracted RNA will be referred to as 'polyribosomal RNA'. It can be seen from Fig. 3.3.1.4 that the amount of

Fig. 3.3.1.1 Effect of incubation time on hybridization of (^{3}H) polyU and polyA

Each incubation mixture contained 0.042 μg polyA and 0.25 μg (^{3}H) polyU in 0.5 ml of solution containing 30 mM trisodium citrate and 300 mM NaCl. Mixtures were incubated at 50°C for different periods, cooled and digested with pancreatic ribonuclease (20 μg). The hybrids resistant to ribonuclease digestion were precipitated with ice-cold 10% trichloroacetic acid and 200 μg of carrier yeast RNA, collected and the radioactivity was measured as described in section 2.6.

Fig. 3.3.1.1.

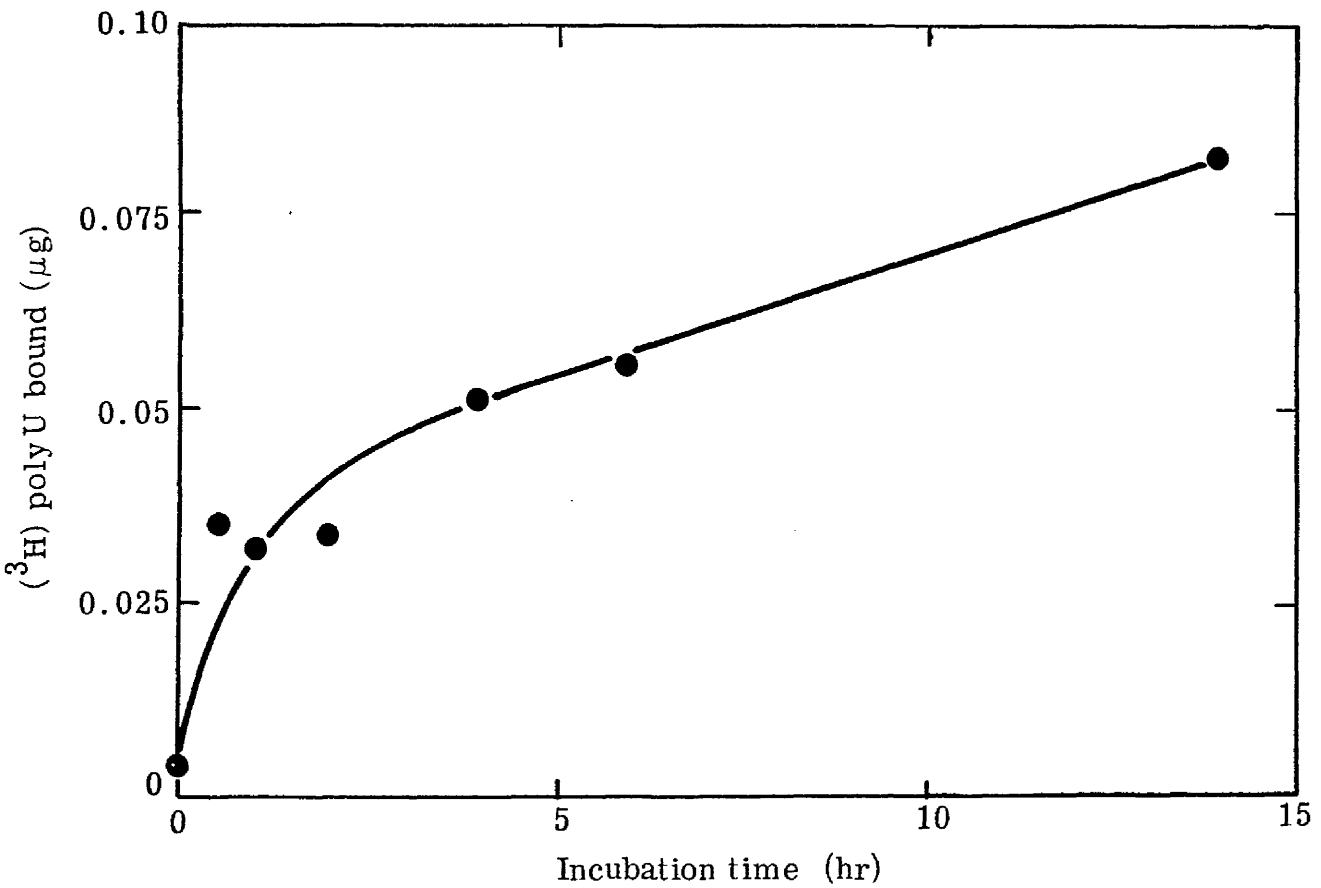


Fig. 3.3.1.2 Hybridization of (^{3}H) polyU with different amounts of polyA

Each incubation mixture contained 0.25 μg (^{3}H) polyU and different amounts of synthetic polyA in 0.5 ml of solution containing 30 mM trisodium citrate and 300 mM NaCl. Mixtures were incubated at 50°C for 14 hr, cooled and digested with pancreatic ribonuclease (20 μg). The hybrids resistant to ribonuclease digestion were precipitated with ice-cold 10% trichloroacetic acid and 200 μg of carrier yeast RNA, collected and the radioactivity was determined as described in section 2.6.

Fig. 3.3.1.2.

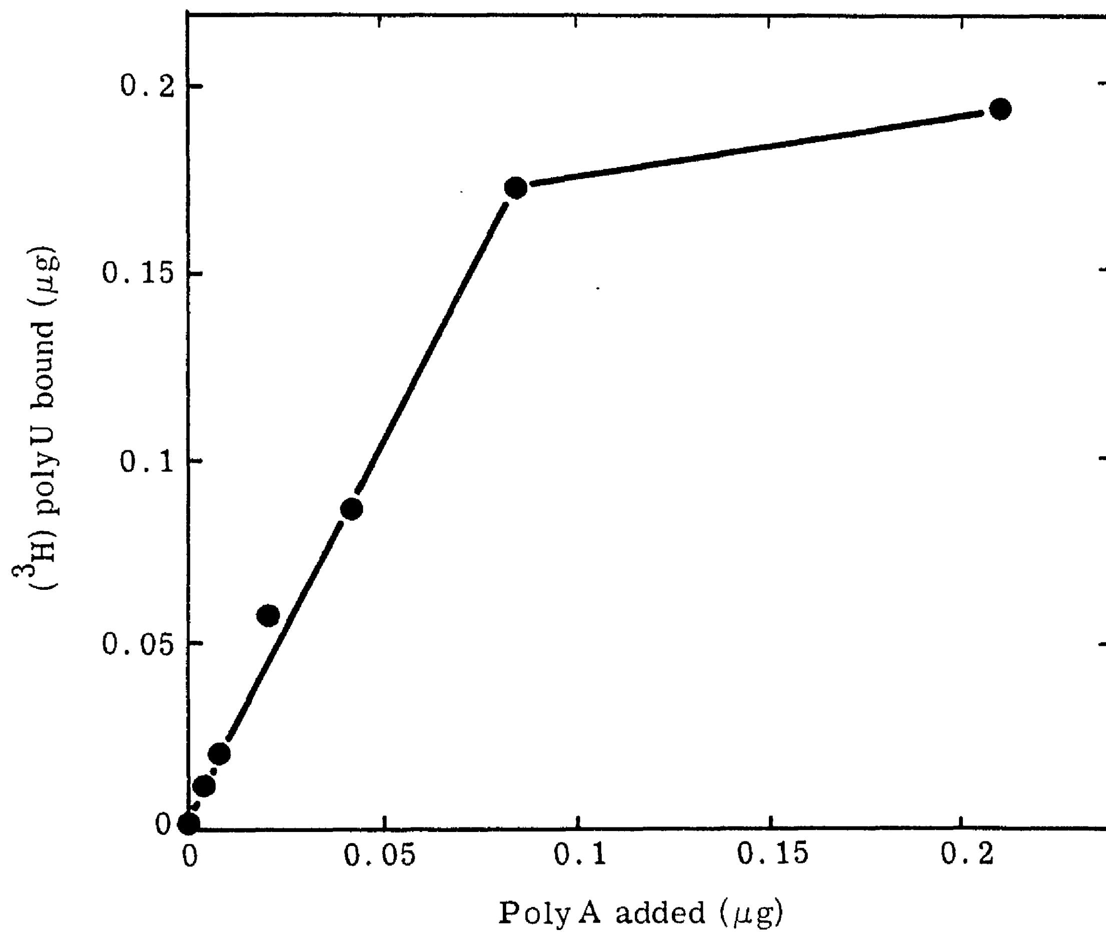


Fig. 3.3.1.3 Specificity of (^{3}H) polyU: polyA hybridization assay

Each incubation mixture contained 0.25 μg (^{3}H) polyU and different amounts of E.coli tRNA or synthetic polyA in 0.5 ml of solution containing 30 mM trisodium citrate and 300 mM NaCl. Mixtures were incubated at 50°C for 4 hr, cooled and digested with pancreatic ribonuclease (20 μg). The hybrids resistant to ribonuclease digestion were precipitated with ice-cold 10% trichloroacetic acid and 200 μg of carrier yeast RNA, collected and the radioactivity was measured as described in section 2.6.

Fig. 3.3.1.3.

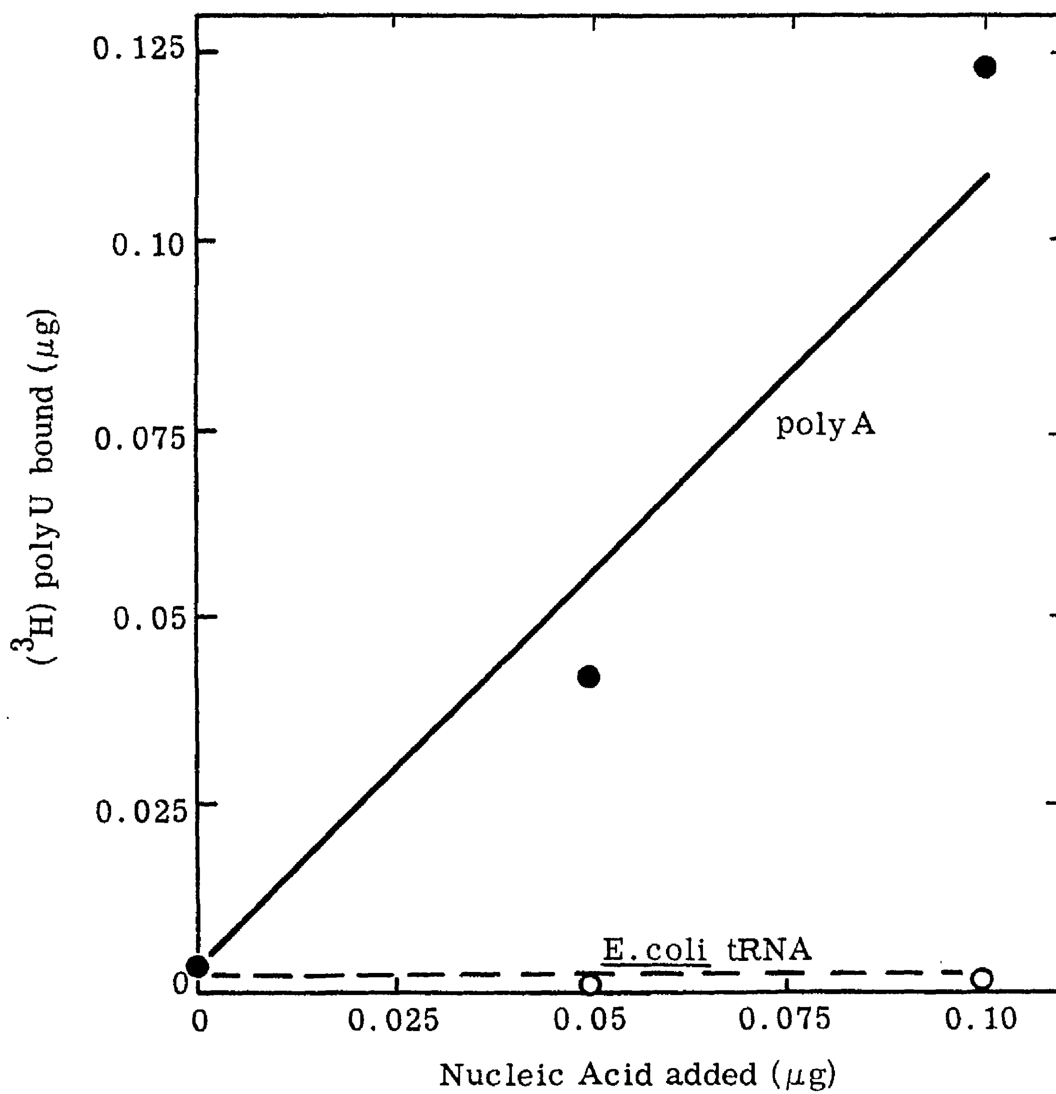
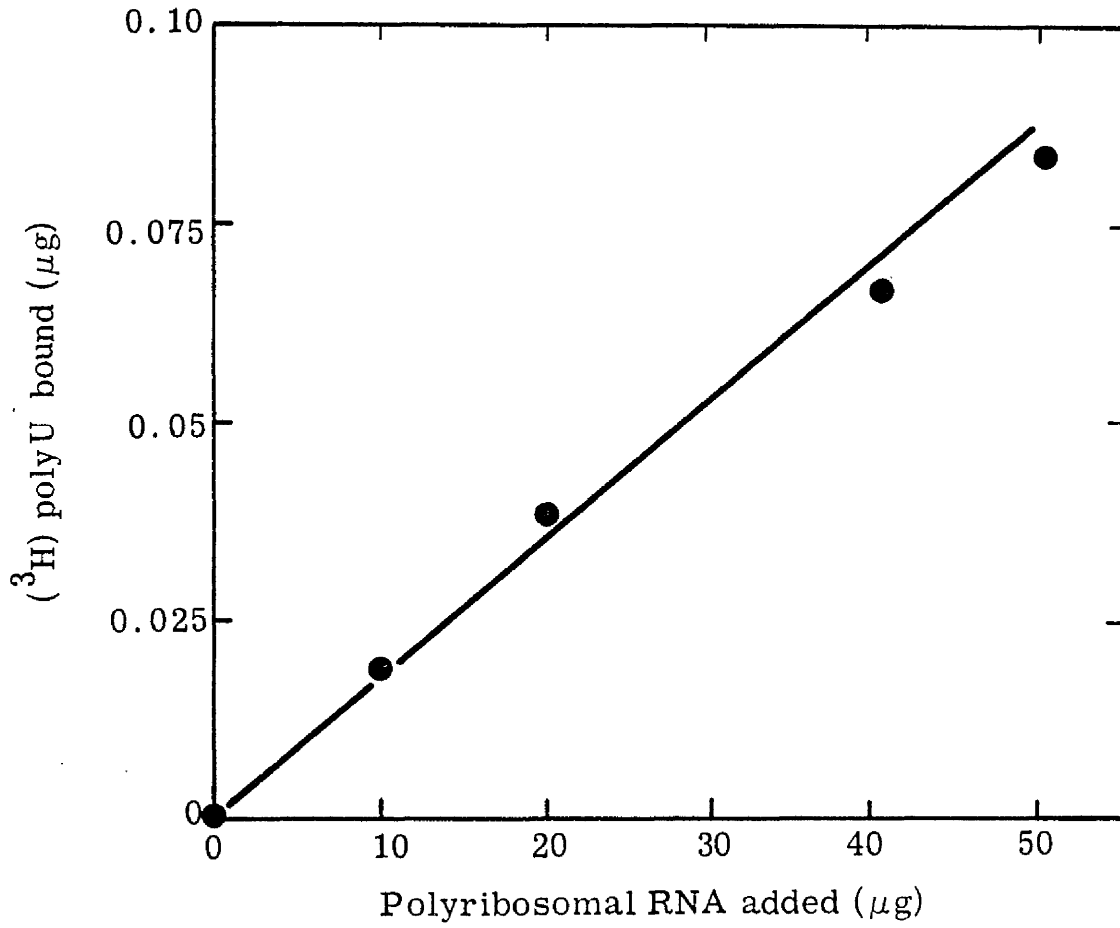


Fig. 3.3.1.4 Hybridization of (^3H) polyU with polyribosomal RNA from normal rat skeletal muscle

Each incubation mixture contained 0.25 μg (^3H) polyU and different amounts of polyribosomal RNA in 0.5 ml of solution containing 30 mM trisodium citrate and 300 mM NaCl. Mixtures were incubated at 50°C for 14 hr, cooled and digested with pancreatic ribonuclease (20 μg). The hybrids resistant to ribonuclease digestion were precipitated with ice-cold 10% trichloroacetic acid and 200 μg of carrier yeast RNA, collected and the radioactivity was measured as described in section 2.6.

Fig. 3.3.1.4.



hybrid formed was linearly dependent on the amount of this polyribosomal RNA added. The results of this experiment showed that 50 μg polyribosomal RNA bound approximately 0.1 μg (^{3}H) polyU. As the hybrids formed are triplexes composed of 2 polyU:1 polyA, 50 μg polyribosomal RNA contains approximately 0.05 μg polyA. Thus about 0.1% of the muscle polyribosomal RNA consists of regions of polyA - seven experiments giving an average value of 0.108% (Table 3.3.1.1). This compared with a value of 0.3% for the polyA content of total cytoplasmic RNA from mouse fibroblasts (Rudland, Weil and Hunter, 1975), suggesting that the values for muscle mRNA given by the hybridization reaction were not unreasonable. From detailed considerations to be described in Discussion (section 4.1), this implies that about 1% of the total polyribosomal RNA is polyA(+)RNA.

3.3.2 Determination of the relative proportions of polyA(+)RNA in various fractions of rat skeletal muscle

As mentioned in the Introduction, one objective of this work was to investigate the possibility of translational control in rat skeletal muscle by examining the relative amounts and locations of mRNA in the muscle of diabetic rats before and after injection of insulin. If there is translational control, one would expect a similar proportion of polyA(+)RNA to total RNA in both of these condidions. However, such anticipated similarity between the muscle RNA in diabetic rats and in diabetic rats treated with insulin could occur irrespective of differences or similarities between the proportion of polyA(+)RNA in the normal and diabetic rats. Thus, even if there were a reduction in the proportion of mRNA to total RNA in the muscle of rats following induction of diabetes, there might be sufficient non-polysomal mRNA to account for all the mRNA in the polysomes that reform upon injection of insulin (Fig. 3.2.1.3).

Table 3.3.1.1 PolyA content of purified ribosomes from normal rat skeletal muscle

In each experiment the amount of polyribosomal RNA (extracted from the purified ribosomes) was determined by its ultraviolet-absorbance at 260 nm, and its polyA content was measured by hybridization with (^{3}H) polyU as described in sections 2.4.2 and 2.6.

Table 3.3.1.1

Expt. No	PolyA (μg per μg total RNA)
1	0.00086
2	0.00068
3	0.00108
4	0.00154
5	0.00130
6	0.00100
7	0.00112
Mean	0.00108

Nevertheless, it was of interest to see whether during diabetes there was a change in the amount of skeletal muscle mRNA and, if so, whether it was similar to or different from that found for total RNA (Table 3.2.2.3) and hence for rRNA (cf. also the results of Wool, Stirewalt and Moyer, 1968). Therefore the polyA(+)RNA content of intact skeletal muscle was examined.

This was done by extracting the RNA from the intact tissue (see Experimental; section 2.4.1) and determining the ratio of polyA(+)RNA (determined from the (^{3}H) polyU hybridization assay) to total RNA (determined from measurement of ultraviolet absorbance at 260 nm). Table 3.3.2.1 summarizes the results obtained from five separate experiments using skeletal muscle of normal, streptozotocin - diabetic and streptozotocin - diabetic rats after treatment with insulin for 60 min. It is clear that the polyA(+)RNA constitutes a similar proportion of the total muscle RNA in both normal and diabetic rats. Thus in diabetes it appears that the amount of muscle mRNA decreases to the same extent as does the total RNA (which has declined by about 25% with respect to DNA, 3 days after induction of diabetes by streptozotocin - Table 3.2.2.3).

Table 3.3.2.1 also shows that the ratio of polyA(+)RNA to total RNA is similar in both the diabetic rats and the diabetic rats treated with insulin for 60 min. This, then,was consistent with the idea of translational control. However it was possible that, in extracting the total tissue polyA(+)RNA, measurements of cytoplasmic mRNA were obscured by the polyA(+)RNA in heterogeneous nuclear RNA, also being extracted. Therefore the polyA(+)RNA content of the different subfractions of skeletal muscle (see Fig. 3.3.2.1) were examined (Table 3.3.2.2). Unfortunately the

Table 3.3.2.1 Proportion of polyA(+)RNA to total RNA in unfractionated skeletal muscle from normal and diabetic rats

Diabetic rats had received streptozotocin (50 mg/kg body weight) 2 days before death and, where indicated, 5 units of insulin 60 min before death. The RNA (extracted from the unfractionated muscle as described in section 2.4.1) was determined by its ultraviolet-absorbance at 260 nm, and its polyA content was measured by hybridization with (^{3}H) polyU as described in section 2.6. Figures in parentheses indicate comparison with a normal value set at 100%.

Table 3.3.2.1

	PolyA/Total RNA		
	Normal	Diabetic	Diabetic + Insulin
Expt. 1	0.00166 (100)	0.00115 (69)	0.00147 (88)
Expt. 2	0.00104 (100)	0.00099 (95)	0.00109 (105)
Expt. 3	0.00108 (100)	0.00095 (88)	0.00087 (81)
Expt. 4	0.00123 (100)	0.00169 (137)	-
Expt. 5	0.00100 (100)	0.00097 (97)	-
Mean	0.00120 (100)	0.00115 (96)	0.00114 (95)

Fig. 3.3.2.1 Summary of different fractions obtained during preparation of skeletal muscle ribosomes

Rat skeletal muscle was homogenized and fractionated as described in section 2.3.1. (This figure is identical to Fig. 2.3.1.1).

Fig. 3.3.2.1.

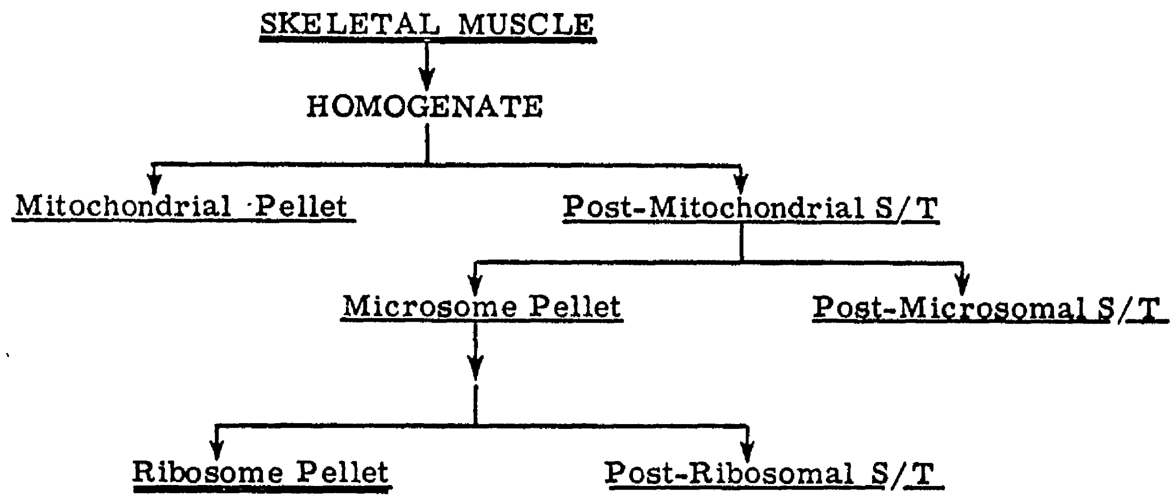


Table 3.3.2.2 Distribution of total RNA and polyA(+)RNA between different fractions of rat skeletal muscle

A part of the intact rat skeletal muscle was directly extracted with phenol as described in section 2.4.1 and the rest of muscle was homogenized at low ionic strength (80 mM KCl; section 2.3.1). A portion of each fraction was used to determine the RNA content in that fraction and the rest was used for further fractionation. The RNA in each case was extracted with phenol as described in section 2.4.2, and its ultraviolet-absorbance at 260 nm was determined. The polyA content was measured by hybridization with (^{3}H) polyU as described in section 2.6. (In the case of supernatant fractions, a portion of each supernatant was precipitated with 95% ethanol in the presence of 100 mM NaCl at -20°C for 16 hr, and the precipitates were extracted with phenol thereafter). The values of RNA content in different fractions were normalized to 100 μg RNA as the initial amount, and to 100% recovery at each stage.

Table 3.3.2.2.

Tissue fraction	Normal			Diabetic			Diabetic/ Normal (%)
	RNA (μg)	PolyA (μg)	PolyA/RNA	RNA (μg)	PolyA (μg)	PolyA/RNA	PolyA/RNA
Intact muscle	100	0.1240	0.00124	100	0.1700	0.00170	137%
'Mitochondrial pellet'	71	0.1306	0.00184	71	0.1257	0.00177	96%
'Post-mitochondrial S/T'	29	0.0238	0.00082	29	0.0210	0.00072	88%
'Microsome pellet'	17	0.0221	0.00130	16	0.0180	0.00112	86%
'Post-microsome S/T'	12	0.0036	0.00030	13	0.0036	0.00028	93%
'Ribosome pellet'	15.4	0.0166	0.00108	14.7	0.0140	0.00095	88%
'Post-ribosomal S/T'	1.6	0.0032	0.00203	1.3	0.0024	0.00185	91%

comparative values of polyA(+)RNA to total RNA for the intact tissue are atypical in this experiment (that from the diabetic being 137% of that from the normal), as can be seen by comparison with Table 3.3.2.1. Moreover there were some inconsistencies between the total polyA(+)RNA in individual fractions and the subfractions derived from them (e.g. cf. the 'microsomal pellet' with the sum of the 'ribosome pellet' and 'post-ribosomal supernatant'). But despite these deficiencies, this experiment was important, for it indicated that there was no stage in the purification of ribosomes where the ribosome-containing fraction derived from the diabetic animal lost polyA(+)RNA relative to that from the normal, with the polyA(+)RNA appearing in a non-ribosomal fraction. Thus it appeared that the ribosome pellets derived from the muscles of the normal and diabetic rats contained similar proportions of polyA(+)RNA, although this conclusion was not completely unequivocal because the polyA(+)RNA content of the original intact muscle was higher in the diabetic rat than in the normal. However this conclusion was confirmed by the results of subsequent measurements on the purified ribosome pellets (Table 3. 3.2.3). These results also showed a similar proportion of polyA(+)RNA for the diabetic rats and the diabetic rats after treatment with insulin, confirming the conclusion of translational control by insulin, suggested by Tables 3.3.2.1 and 3.3.2.2.

The finding of similar proportion of polyA(+)RNA in ribosomes from normal and diabetic rats was of some significance, for the proportion of polysomes in the sucrose density gradient sedimentation profiles of ribosomes from normal and diabetic rats was different (Fig. 3.2.1.3), implying a different content of polysomal mRNA. This suggested that in the ribosome pellet from the diabetic rat there was non-polysomal mRNA co-sedimenting with the

Table 3.3.2.3 Comparison of polyA content of purified ribosomes from skeletal muscle of normal and diabetic rats

Diabetic rats had received streptozotocin (50 mg/kg body weight) 2 days before death and, where indicated, 5 units of insulin 60 min before death. The purified ribosomes prepared at low ionic strength (section 2.3.1) were extracted with phenol as described in section 2.4.2. The total RNA was determined by its ultraviolet-absorbance at 260 nm and the polyA by hybridization with (^{3}H) polyU as described in section 2.6. Figures in parentheses indicate comparison with a normal value set at 100%.

N.D. = not determined.

Table 3.3.2.3

Expt. No.	PolyA (μg per μg total RNA)		
	Normal	Diabetic	Diabetic + Insulin
1	0.00086 (100)	0.00079 (92)	0.00110 (128)
2	0.00108 (100)	0.00104 (96)	0.00125 (116)
3	0.00112 (100)	0.0087 (78)	N.D.
Mean (% of Normal)	0.00102 (100)	0.00090 (88)	0.00118 (116)

ribosomes, perhaps as messenger ribonucleoprotein particles.

To determine whether this was the case, equal amounts of ribosomes (containing similar amounts of polyA(+)RNA)from normal and diabetic rats were applied to sucrose density gradients,and fractions collected as shown in Fig. 3.3.2.2. In each fraction, the ribosomes were precipitated with 95% ethanol in the presence of 100 mM NaCl, dissolved in sodium dodecyl sulphate-containing Medium E and extracted with phenol and chloroform as described in Experimental (section 2.4.2). The total RNA recovered was determined by its ultraviolet absorbance at 260 nm, and the polyA(+)RNA by hybridization with (^{3}H) polyU. It can be seen from Table 3.3.2.4 that, as expected, most of the polyA(+)RNA was within the polysome fraction (3) from the ribosomes of normal rats and diabetic rats treated with insulin. This polyA(+)RNA disappeared from the diabetic fraction, in proportion to the decline in polysomes (Fig. 3.3.2.2). However, surprisingly, no other fraction of the gradient of the ribosomes from the diabetic rats contained an amount of polyA(+)RNA greater than in the fraction of the sucrose density gradient of the ribosomes from the normal rats; in contrast to what would have been expected from the fact that similar amounts of polyA(+)RNA were applied to each gradient. This can be seen most easily from the bar diagram in the lower part of Fig. 3.3.2.2, which is taken from the more detailed analysis in Table 3.3.2.4. Inspection of the latter shows that incomplete recovery of polyA(+)RNA was obtained in all cases. However, whereas the recoveries for the RNA from normal rats and the diabetic rats treated with insulin were 56% and 61% respectively that for the RNA from the diabetic rats was only 25%

It was thought possible that these losses of polyA(+)RNA might be in the

Fig. 3.3.2.2. Summary of polyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation

Ribosomes (4 A_{260} units) from (a) normal, (b) streptozotocin-diabetic rats and (c) streptozotocin-diabetic rats treated with insulin 60 min before death, were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$, and analysed as described in section 2.5.1. Three fractions were collected from each gradient as indicated by the dotted lines. The RNA in each fraction was precipitated with 95% ethanol in the presence of 100 mM NaCl at $-20^{o}C$ for 16 hr, and the precipitates were extracted with phenol as described in section 2.4.2. The RNA was determined by its ultraviolet absorbance at 260 nm and the polyA by hybridization with (^{3}H) polyU as described in section 2.6.

The bar diagram in the lower panel represents the polyA recovered in the different fractions.

Fig. 3.3.2.2.

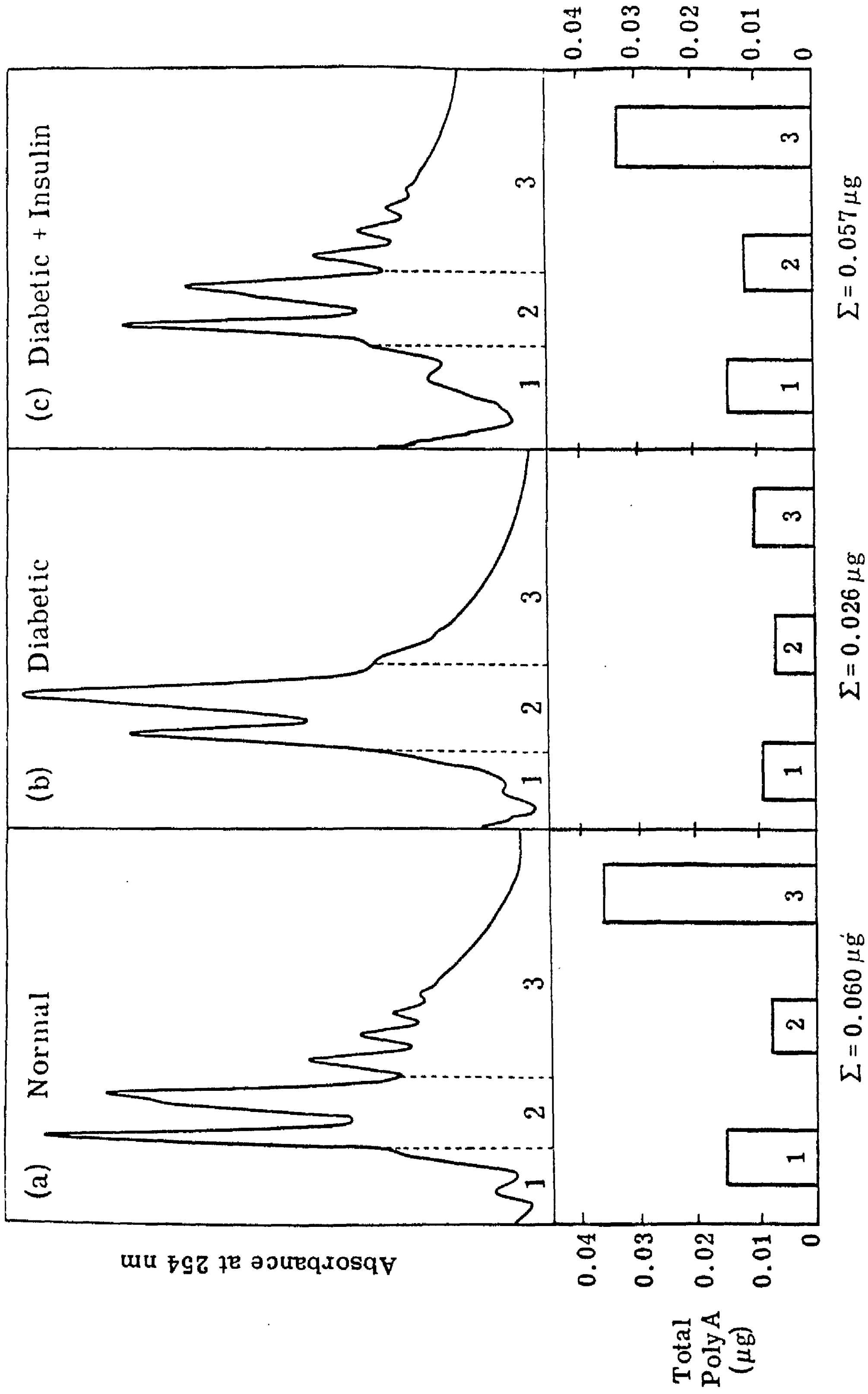


Table 3.3.2.4 PolyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation

This table presents the detailed figures for the experiment described in the legend to Fig. 3.3.2.2.

*These values were corrected to 100% recoveries of the RNA applied to the gradients, this later being normalized to 100 μg. The original amounts of ribosomal RNA applied to the sucrose gradients were 200 μg from normal, 200 μg from diabetic rats, and 181 μg from diabetic rats treated with insulin. The recoveries were 67.5%, 81.9% and 67.7% in RNA from normal, diabetic, and diabetic rats treated with insulin, respectively. The values for 'Total applied' RNA were obtained by direct extraction of a portion of the same ribosome pellets.

Table 3.3.2.4

	Normal			Diabetic			Diabetic + Insulin		
	RNA (μg)	polyA (μg)	polyA/RNA	RNA (μg)	polyA (μg)	polyA/RNA	RNA (μg)	polyA (μg)	polyA/RNA
1. 'Sub-monosomes'	18	0.016	0.00091	12	0.009	0.00079	18	0.014	0.00076
2. Monosomes + dimers	36	0.008	0.00022	60	0.007	0.00012	43	0.011	0.00026
3. Polysomes	46	0.036	0.00078	28	0.010	0.00035	39	0.032	0.00082
Total	100	0.060	0.00060	100	0.026	0.00026	100	0.057	0.00057
Total applied	100	0.108	0.00108	100	0.104	0.00104	100	0.094	0.00094

very high molecular weight material (probably aggregated) which was observed as small pellets at the bottom of the tubes after centrifugation. Because of the difficulty in recovering these pellets after piercing the bottom of the tubes for fractionation, it was decided to try to prevent the pelleting of the high molecular weight aggregates by layering a 'cushion' of 70% sucrose at the bottom of the tubes.

It can be seen from Fig. 3.3.2.3 that a considerable amount of ultraviolet absorbing material was found on this sucrose 'cushion'. When the polyA(+)RNA of these sucrose 'cushion' fractions (3) was analysed, it was found that that from the ribosomes of the diabetic rats was greater than that from the ribosomes of the normal rats. Moreover this excess approximately compensated for the extent to which the polyA(+)RNA was greater in polysome and subpolysome fractions from ribosomes derived from normal rats, compared with those from ribosomes derived from diabetic rats (lower part of Fig. 3.3.2.3 and Table 3.3.2.5).

The recovery on this fractionated gradient of much of the polyA(+)RNA, previously lost from the ribosomes of diabetic rats, was a result of prime importance in this work. Because of the possibility of errors in analysis of the subfractions from which the totals were summed in Table 3.3.2.5, the total RNA from a similar, but unfractionated, gradient was also analysed in the same experiment, and similar values for the proportion of polyA(+)RNA in ribosomes from normal and diabetic rats were observed (Table 3.3.2.6).

Although the ultraviolet-absorbing material collected on the sucrose 'cushion' (fraction 3 of Fig. 3.3.2.3) was relatively rich in polyA(+)RNA (Table 3.3.2.5), it contained predominantly non-polyA(+)RNA. Hence it

Fig. 3.3.2.3 Summary of polyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation with a sucrose 'cushion'

Ribosomes (4 A_{260} units) from (a) normal and (b) streptozotocin-diabetic rats were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium with 0.3 ml of 70% sucrose at the bottom. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at 4^oC, and analysed as described in section 2.5.1. Three fractions were collected from each gradient as indicated by the dotted lines, the RNA in each fraction was precipitated with 95% ethanol in the presence of 100 mM NaCl at -20^oC for 16 hr, and extracted with phenol (section 2.4.2). In each fraction, the RNA was determined by its ultraviolet-absorbance at 260 nm and the polyA by hybridization with (^3H) polyU. The bar diagram in the lower panel represents the polyA recovered in each fraction.

Fig. 3.3.2.3.

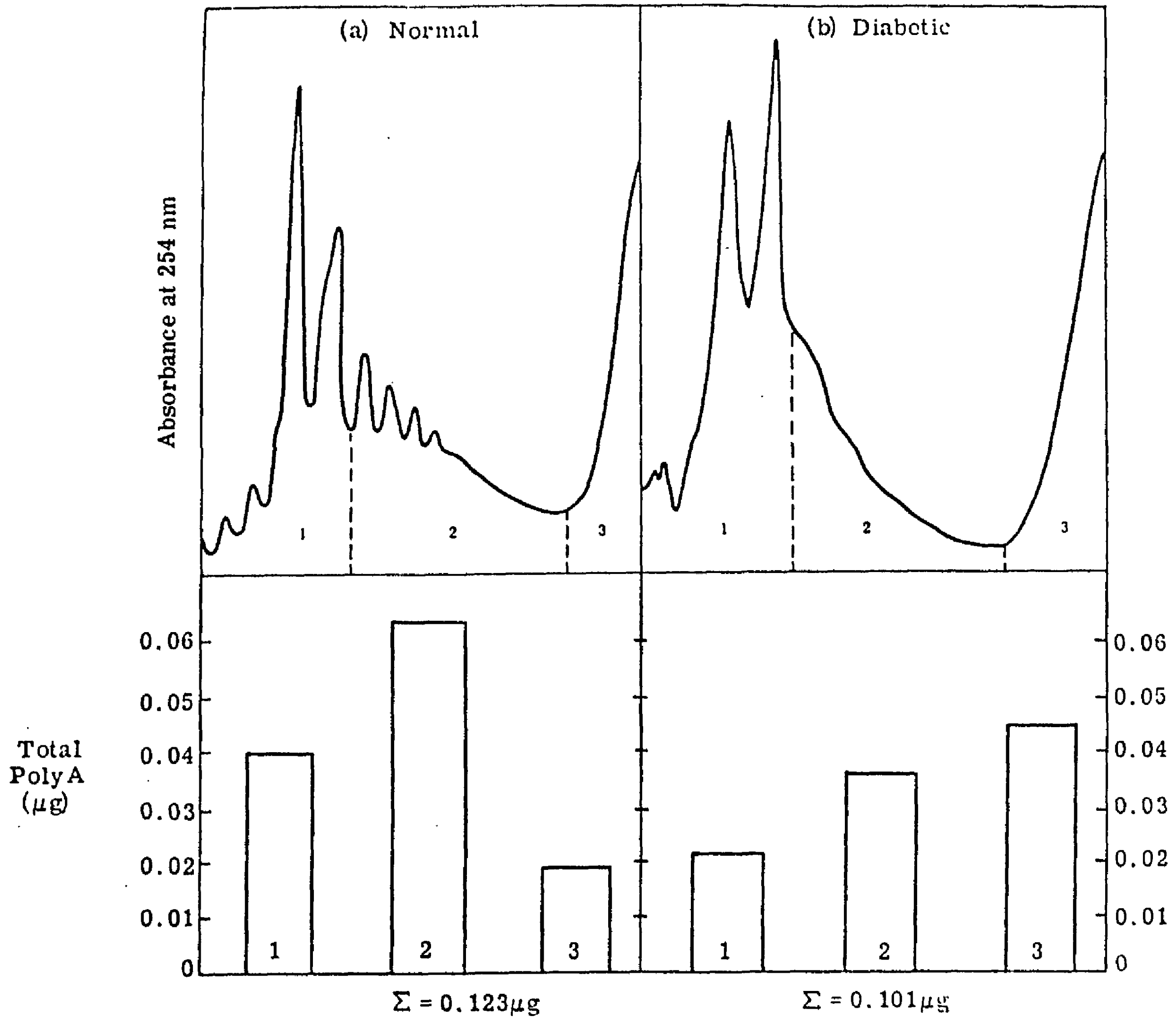


Table 3.3.2.5 PolyA(+)RNA in different fractions of normal and diabetic rat skeletal muscle ribosomes analysed by sucrose density gradient centrifugation with a 70% sucrose 'cushion'

This table presents the detailed figures for the experiment described in the legend to Fig. 3.3.2.3.

*These values were corrected to 100% recoveries of the RNA applied to the gradients, this later being normalized to 100 μg. The original amounts of ribosomal RNA applied to the sucrose gradients were 200 μg from normal, and 148.8 μg from the diabetic rats. The recoveries were 46.9% and 62.2% in RNA from normal and diabetic rats respectively.

The values for 'Total applied' RNA were obtained by direct extraction of a portion of the same ribosome pellets.

Table 3.3.2.5

	Normal			Diabetic			$\frac{Diabetic}{Normal}$ %
	RNA (μg)	polyA (μg)	$\frac{polyA}{RNA}$	RNA (μg)	polyA (μg)	$\frac{polyA}{RNA}$	polyA
1. Monosomes + dimers	31	0.040	0.00129	42	0.021	0.00050	53%
2. Polysomes	51	0.064	0.00125	42	0.036	0.00086	56%
3. Cushion	18	0.019	0.00105	16	0.044	0.00275	231%
Total	100	0.123	0.00123	100	0.101	0.00101	82%
Total Applied	100	0.112	0.00112	100	0.087	0.00087	78%

Table 3.3.2.6 Comparison of different analyses of the proportion of polyA(+)RNA in skeletal muscle ribosomes from normal and diabetic rats

The ratio of polyA(+)RNA to total RNA in a single preparation of ribosomes was determined either by direct extraction (cf. legend to Table 3.3.2.3); in the sucrose gradient fractions indicated in Fig. 3.3.2.3; or in a similar gradient to Fig. 3.3.2.3 that was not fractionated.

Table 3.3.2.6

Source of RNA	PolyA/Total RNA		
	Normal	Diabetic	$\frac{\text{Diabetic}}{\text{Normal}}$ %
Ribosome pellet	0.00112	0.00087	78%
Fractionated sucrose gradient	0.00123	0.00101	82%
Unfractionated sucrose gradient	0.00095	0.00075	79%

seemed likely that this ultraviolet-absorbing material was predominantly ribosomes (presumably in an aggregated form). This would be consistent with the incomplete recovery of RNA applied to the gradient (Table 3.3.2.4). To determine whether this was in fact so, the RNA extracted from this 'cushion' fraction was subjected to sucrose density gradient centrifugation in sodium dodecyl sulphate-containing Medium G as described in Experimental (section 2.5.3). Fig. 3.3.2.4 shows that this RNA was predominantly 18S and 28S, which is consistent with the ultraviolet absorbing material of the sucrose 'cushion' fraction being predominantly ribosomes.

It appeared that the extra polyA(+)RNA in the sucrose 'cushion' from ribosomes of diabetic rats (Fig. 3.3.2.3 - Table 3.3.2.5) might be in messenger ribonucleoprotein particles, trapped with the aggregated ribosomes. Attempts were therefore made to obtain conditions which would prevent the putative messenger ribonucleoprotein particles being precipitated in this way, allowing their true sedimentation value to be investigated.

Elevated temperature had been found to enhance the resolution of different polysome species on sucrose density gradient analysis (Fig. 3.1.2.1) and therefore it was thought that preincubating the ribosomes at 37°C might possibly reduce the aggregation of ribosomes, and hence allow the polyA(+)RNA to sediment in its authentic form. However Fig. 3.3.2.5 shows that there was still a large amount of ultraviolet-absorbing material on the sucrose 'cushion' when the gradients were performed under these modified conditions. Hence the problem of aggregation could not be overcome by these means.

The second approach to this problem was rather different. Most analyses of messenger ribonucleoprotein particles have been performed on 'post-mitochondrial

Fig. 3.3.2.4 Sucrose density gradient analysis of RNA extracted from the sucrose 'cushion'

The RNA in the sucrose 'cushion' fractions of Fig. 3.3.2.3 was precipitated by 95% ethanol in the presence of 100 mM NaCl at -20°C for 16 hr. The precipitates were extracted with phenol (section 2.4.2), reprecipitated, and 25 μg RNA from (a) normal and (b) diabetic rats was resuspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate and applied to a 5 ml linear gradient of 7-25% (w/v) sucrose in the same medium. They were centrifuged for 2.5 hr at 45,000 rpm (189,000 g) in a Beckman SW 50.1 rotor at 20°C, and analysed as described in section 2.5.3.

The first peak in (b) is probably machine 'noise'. The S values are nominal.

Fig. 3.3.2.4.

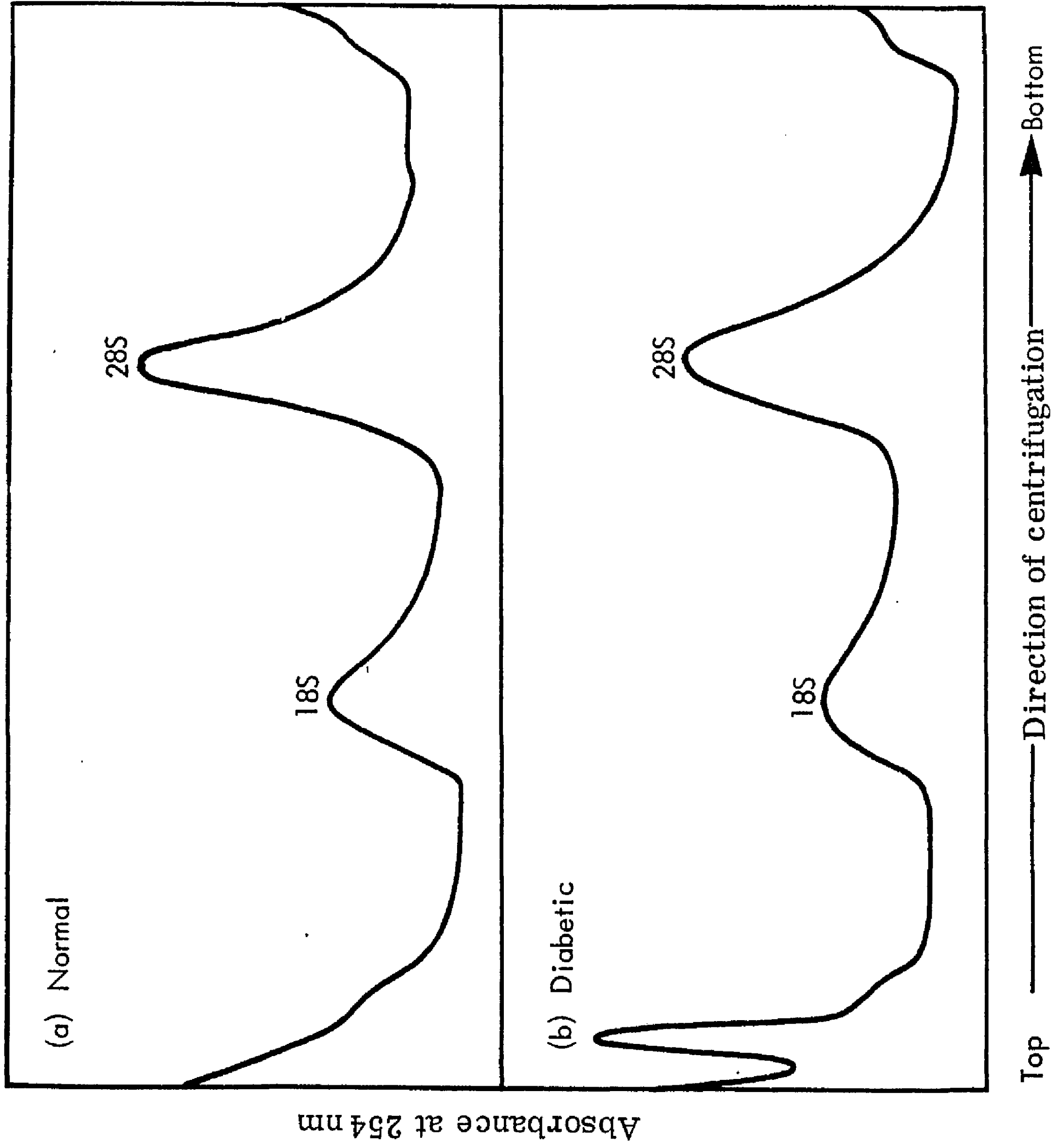
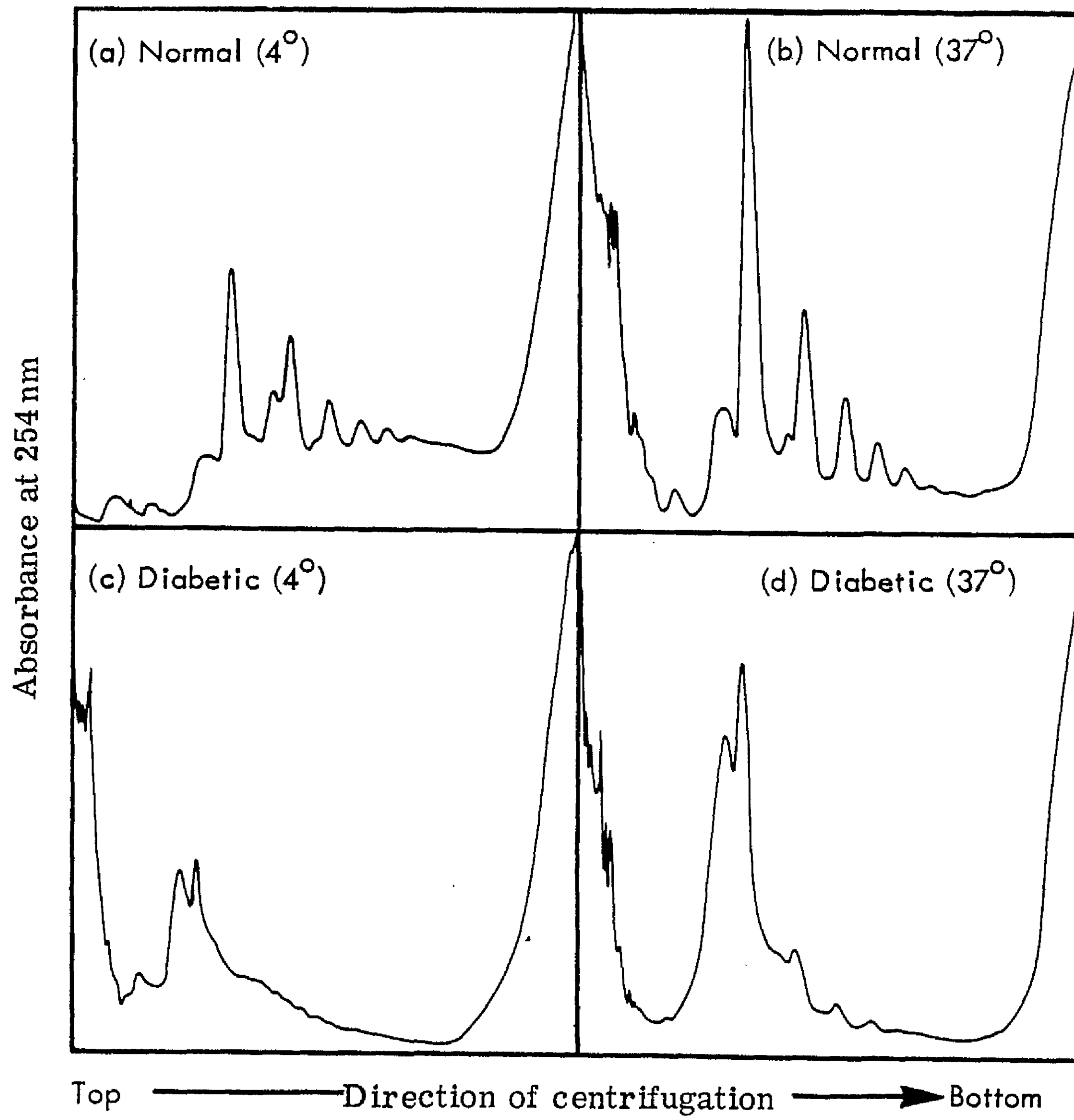


Fig. 3.3.2.5 Sucrose density gradient analysis of rapidly sedimenting material from normal and diabetic rat skeletal muscle ribosomes pre-incubated at 4^oC and 37^oC

Ribosomes (1.5 A_{260} units) from the skeletal muscle of normal (a and b) and diabetic (c and d) rats were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, incubated for 15 min at 4^oC (a and c) or 37^oC (b and d), and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium with 0.3 ml of 70% sucrose at the bottom. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at 4^oC, and analysed as described in section 2.5.1.

Fig. 3.3.2.5.



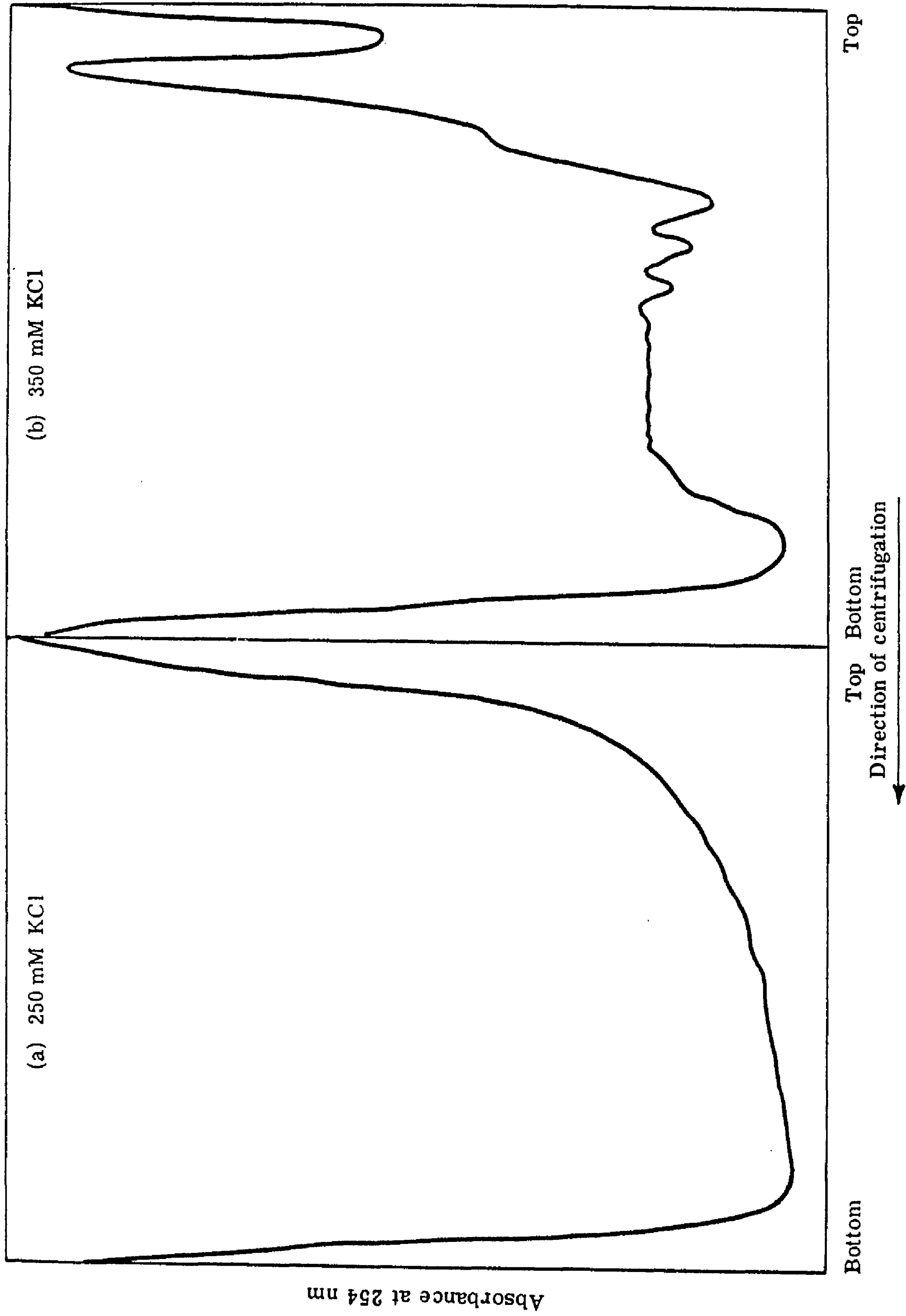
supernatants' rather than purified ribosomes (e.g. Jacobs-Lorena and Baglioni, 1972; Bester, Kennedy and Heywood, 1975). Towards the end of this work it was discovered that pelleting of messenger ribonucleoprotein particles can cause their aggregation (M.E. Buckingham, personal communication), although other reports do not indicate this (Huynh-Van-Tan and Schapira, 1978). Clearly it was desirable therefore to analyse the polyA(+)RNA in the 'post-mitochondrial supernatant' from rat skeletal muscle. However in initial studies when the 'post-mitochondrial supernatant' (prepared as described in Experimental; section 2.3.1) was displayed on sucrose density gradients, little ultraviolet-absorbing material could be detected and most of the ribosomes were sedimented to the bottom of the centrifuge tube under the conditions used. The latter result was observed even when the concentration of KCl in the homogenizing medium was increased to the 250 mM used in the preparation of ribosomes in this work (section 2.3.2), or in the work of Chen and Young (1968) for the analysis of the 'post-mitochondrial supernatant' of rat skeletal muscle by sucrose density gradient centrifugation (Fig. 3.3.2.6 a). Indeed, it was for this reason that most of the studies already described in this work had been concentrated on the purified ribosome fraction. However at this stage it was felt important to make further attempts to display the 'post-mitochondrial supernatant' on a sucrose density gradient. This was achieved using the procedure of Chen and Young (1968), modified so that the concentration of KCl, in the homogenizing medium was 350 mM (section 2.3.3). Even under these conditions, however, the majority of the ribosomes were still aggregated and sedimented to the bottom of the tubes. (Fig. 3.3.2.6 b).

Although this preparation of 'post-mitochondrial supernatant' did not prevent the aggregation of ribosomes, it did appear that in the case of the

Fig. 3.3.2.6 Sucrose density gradient centrifugation of 'post-mitochondrial supernatants' from rat skeletal muscles extracted at different ionic strengths

A 'post-mitochondrial supernatant' (1.5 ml) prepared as described in section 2.3.3 either (a) in medium containing 10 mM Tris-HCl (pH 7.6), 250 mM KCl, 10 mM $MgCl_2$ or (b) in medium containing 10 mM Tris, 350 mM KCl, 10 mM $MgCl_2$, was applied to a 37 ml linear gradient of 15-40% (w/v) sucrose made in the same medium. They were centrifuged for 3 hr at 27,000 rpm (94,500 g) in a Beckman SW 27 rotor at 4^oC, and analysed as described in section 2.5.2.

Fig. 3.3.2.6.



normal rat, there were relatively more large polysomes than had been observed in previous analyses of purified ribosomes (Fig. 3.3.2.6 b - cf. with Fig. 3.1.1.1), although the resolution of the individual peaks was poor. Because it was of interest to obtain larger polysomes, and as there was a clear difference between the 'post-mitochondrial supernatant' from normal and diabetic rats (Fig. 3.3.2.7) the 'post-mitochondrial supernatant' was pelleted and the ribosomes analysed by analytical sucrose density gradient centrifugation in the hope of getting better resolution of individual peaks. However this re-analysis gave profiles of polysomes no larger than those from purified ribosomes (Fig. 3.3.2.8 - cf. with Fig. 3.2.1.3).

Thus it has not been possible to determine whether or not the extra polyA(+) RNA, associated with the ribosomal aggregates from diabetic rats, derives from messenger ribonucleoprotein particles.

Fig. 3.3.2.7 Sucrose density gradient analysis of 'post-mitochondrial supernatants' from skeletal muscle of normal and diabetic rats prepared at 350 mM KCl

The 'post-mitochondrial supernatant' (1.5 ml) prepared as described in section 2.3.3 from (a) normal or (b) diabetic rats, in medium containing 10 mM Tris-HCl (pH 7.6), 350 mM KCl, 10 mM $MgCl_2$, was applied to a 37 ml linear gradient of 15-40% (w/v) sucrose made in the same medium. They were centrifuged for 3 hr at 27,000 rpm (94,500 g) in a Beckman SW 27 rotor at $4^{o}C$, and analysed as described in section 2.5.2.

Fig. 3.3.2.7.

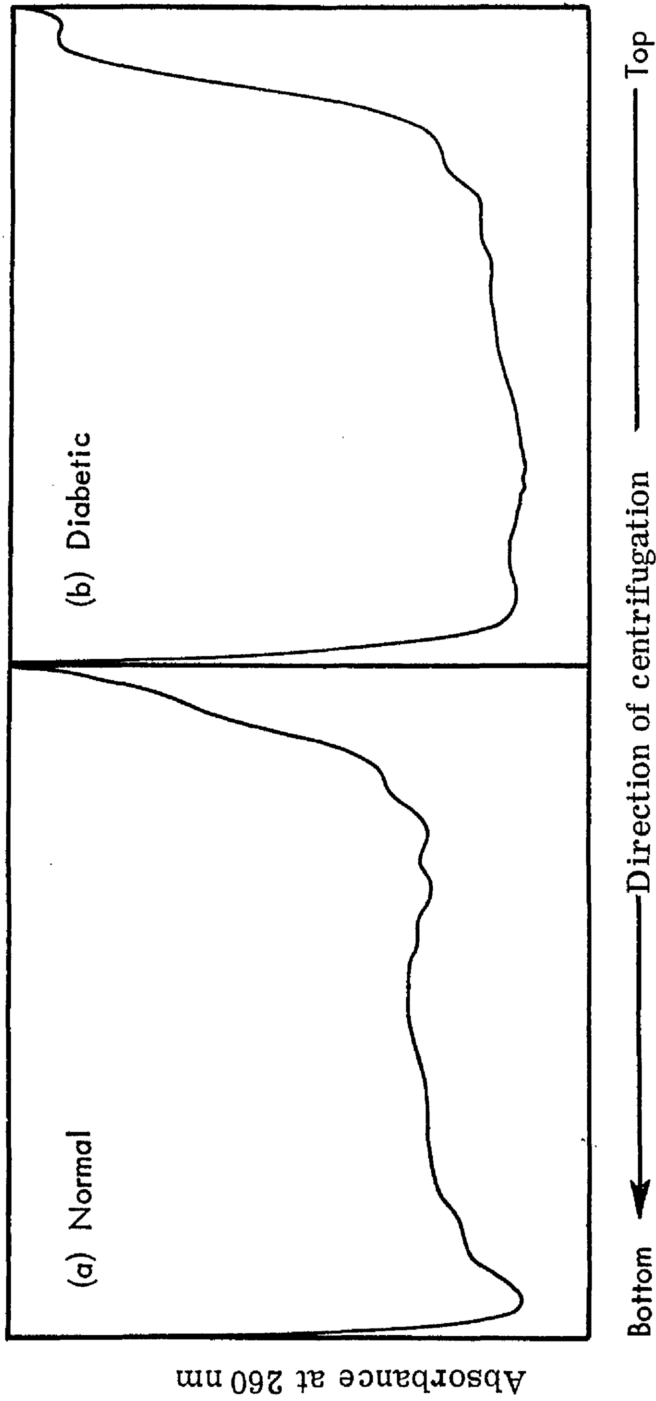
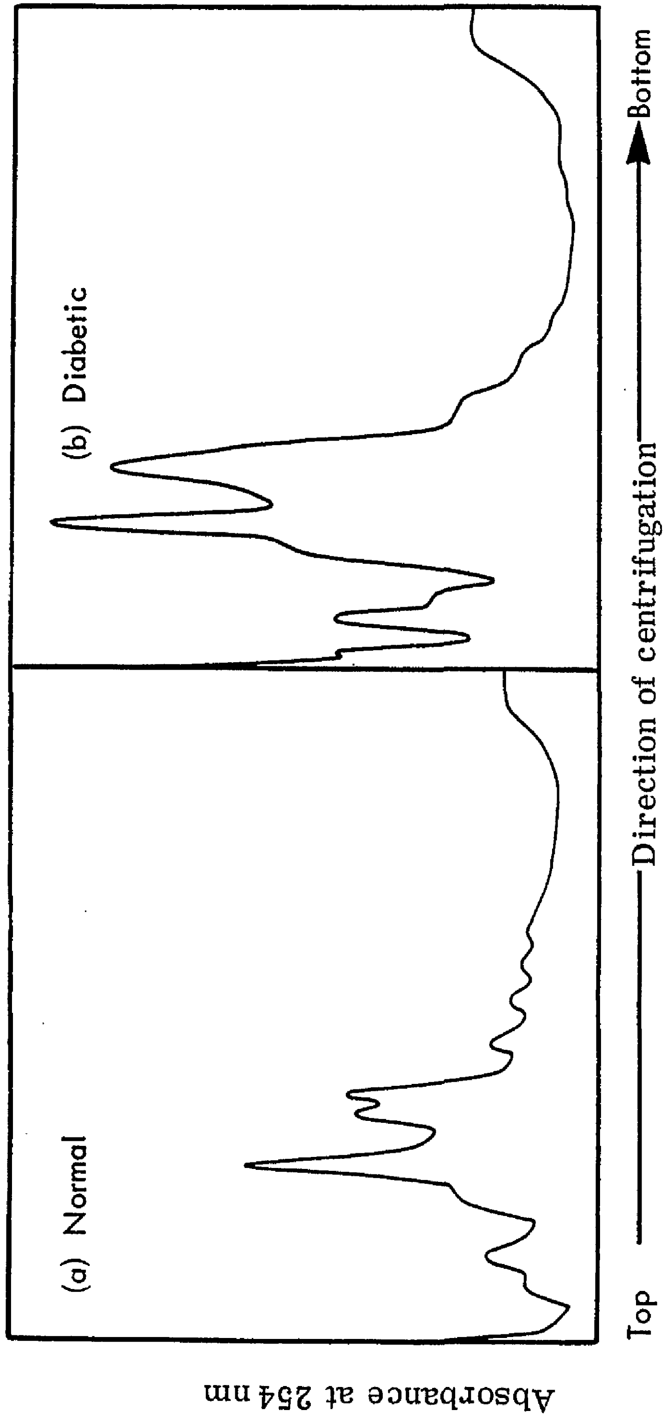


Fig. 3.3.2.8 Sedimentation profiles of ribosomes from rat skeletal muscle extracted at 350 mM KCl

The 'post-mitochondrial supernatant' from (a) normal, or (b) diabetic rats, described in the legend to Fig. 3.3.2.7, was layered on 5 ml of 500 mM sucrose containing 50 mM Tris-HCl (pH 7.6), 80 mM KCl, 12.5 mM $MgCl_2$, and centrifuged for 3 hr at 50,000 rpm (165,000 g) in a Beckman 50 Ti rotor at $4^{o}C$. The ribosome pellets were suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 200 mM KCl, 5 mM $MgCl_2$, and applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same medium. They were centrifuged for 35 min at 50,000 rpm (234,000 g) in a Beckman SW 50.1 rotor at $4^{o}C$, and analysed as described in section 2.5.1.

Fig. 3.3.2.8.



3.4 QUALITATIVE COMPARISONS OF POLYA(+)RNA FROM THE SKELETAL MUSCLE OF NORMAL AND DIABETIC RATS

3.4.1 Comparison of the size of polyA sequences in skeletal muscle polyribosomal polyA(+)RNA

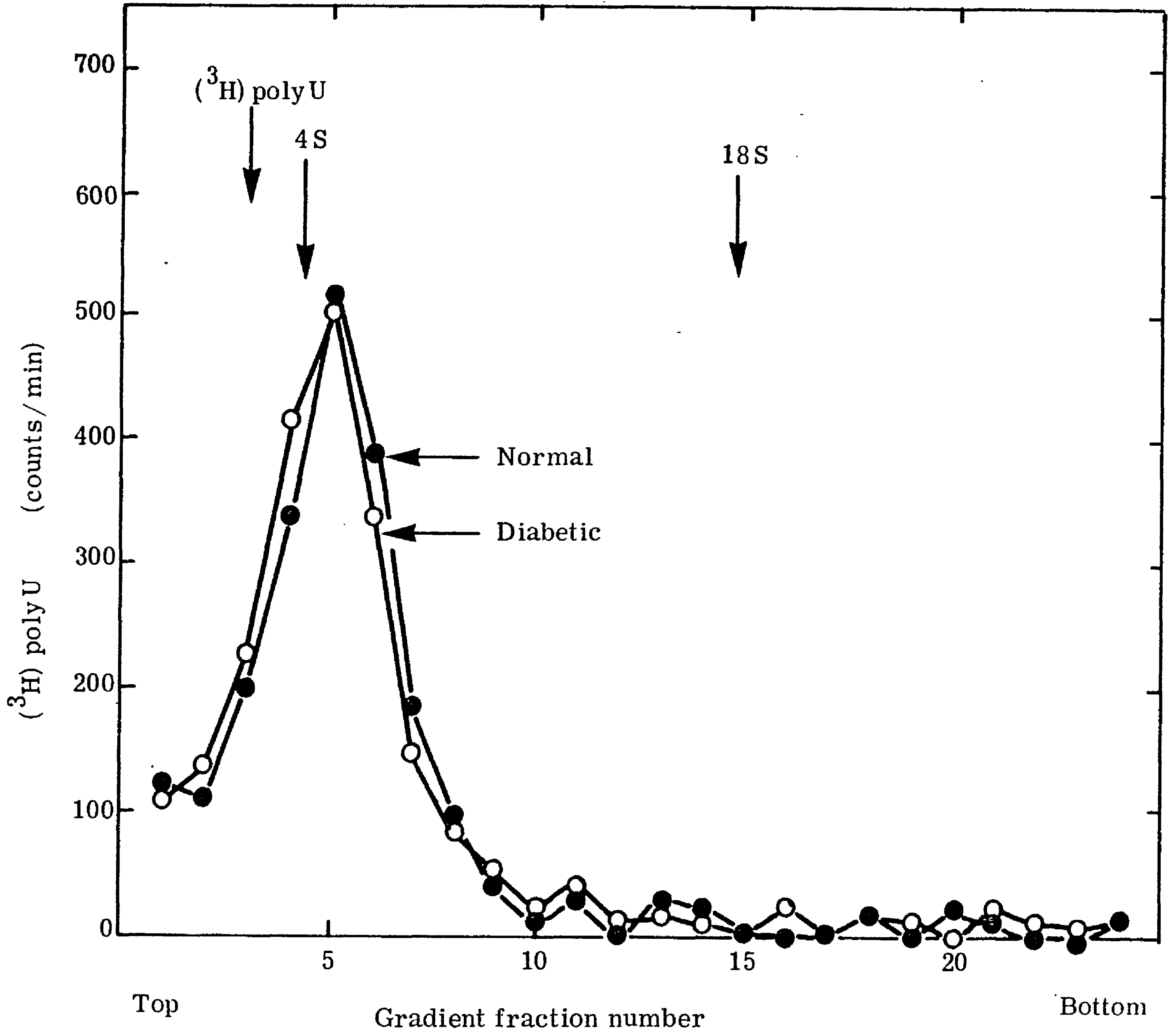
One important assumption in the quantitation of mRNA from the skeletal muscle of normal and diabetic rats by hybridization to (^{3}H) polyU is that the size of the polyA sequences is the same under both physiological conditions. However it has been found in mouse ascites cells (Mendecki, Lee and Brawerman, 1972) and in HeLa cells (Sheiness and Darnell, 1973) that the size of the polyA sequence decreases with the age of the mRNA. Thus it was important to see whether the size of the polyA sequence was the same in the mRNA from the skeletal muscle of normal and diabetic rats, to determine whether the quantitation in section 3.3. was in fact valid.

Two different approaches to this problem were adopted. The first, which was chosen for ease and simplicity, entailed hybridization of the polyribosomal RNA to (^{3}H) polyU, digestion with ribonuclease, fractionation by sucrose density gradient centrifugation and measurement of the radioactivity of different fractions (see section 2.5.3 and the legend to Fig. 3.4.1.1). Fig. 3.4.1.1 shows the sedimentation profiles of hybrids between (^{3}H) polyU and polyA sequences from skeletal muscle polyribosomal RNA which were resistant to pancreatic ribonuclease digestion. It is evident that the polyA sequences from the mRNA of the skeletal muscle of the diabetic rat were similar in size to those of the normal, with a peak at a sedimentation coefficient of about 5S.

Fig. 3.4.1.1 Sedimentation profiles of hybrids between (^{3}H) polyU and polyA tracts from rat skeletal muscle polyribosomal RNA

Polyribosomal RNA (50 μg) from normal (●—●—●) or streptozotocin-diabetic (O—O—O) rats was mixed with 0.01 μg (^{3}H) polyU (specific activity 512 μCi/μmole P) and the volume was adjusted to 0.2 ml in medium containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate. The mixtures were incubated at 50°C for 14 hr, cooled to 20°C and digested with pancreatic ribonuclease (section 2.6). Samples were then layered on a 5 ml linear gradient of 7-25% (w/v) sucrose in the medium mentioned above. The gradients were centrifuged for 4 hr at 45,000 rpm (189,000 g) in a Beckman SW 50.1 rotor at 20°C, analysed, fractionated and the radioactivity was measured as described in section 2.5.3. E.coli tRNA (4S) and rat skeletal muscle ribosomal RNA (18S) were used as markers on a parallel gradient. Limiting concentrations of (^{3}H) poly U were necessary in this and subsequent direct hybridization experiments to prevent the profiles being obscured by a large peak of unhybridized (^{3}H) polyU.

Fig. 3.4.1.1.



As the experiments described above compared the sedimentation of hybrids of the polyA sequences and (^{3}H) polyU, the value of the sedimentation coefficient obtained would be expected to be larger than that for the polyA sequence alone. It was conceivable that this might obscure a difference in the sizes of the polyA sequences. Therefore a second approach was adopted, in which the RNA was first digested, sedimented on sucrose density gradients and fractionated, before being hybridized with (^{3}H) polyU. Fig. 3.4.1.2 shows the sedimentation pattern of polyA sequences from normal and diabetic rats in two experiments. These experiments indicated that the sedimentation coefficient of the polyA sequence was about 4S in material from the mRNA of the muscle of both normal and diabetic rats. This sedimentation value was slightly lower than that obtained in the experiment in Fig. 3.4.1.1, as had been anticipated. In one experiment, (a), the polyA sequence from the diabetic rats appeared at a slightly higher sedimentation coefficient than that from the normal rats, the median value being displaced by about one fraction. However this difference was not seen in a second experiment, (b), where the median sedimentation coefficient for polyA from the diabetic rats was, if anything, somewhat lower than that from the normal.

Thus it was concluded that diabetes does not cause any significant alteration in the size of the polyA sequence at the 3'-end of skeletal muscle mRNA, and hence does not invalidate the basis of quantitation of mRNA used in this work.

3.4.2 Comparison of the size of polyA(+)RNA from the skeletal muscle of normal and diabetic rats

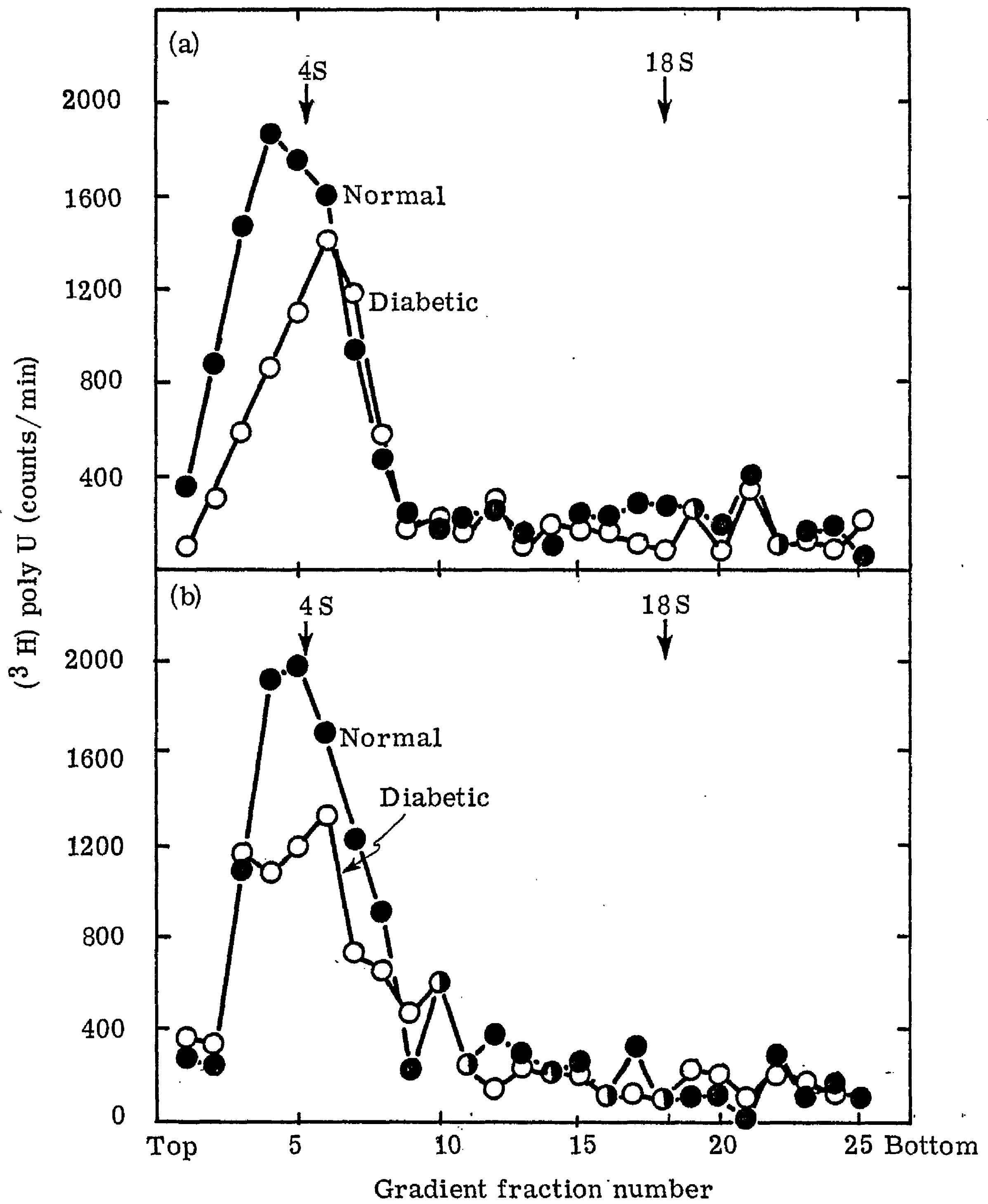
It was also of interest to compare the size of polyA(+)RNA extracted from the ribosome pellets from skeletal muscle of normal and diabetic rats.

Fig. 3.4.1.2 Sucrose density gradient sedimentation profiles of polyA tracts from rat skeletal muscle polyribosomal RNA

Polyribosomal RNA (50 μg) from normal (●—●—●) or streptozotocin - diabetic (O—O—O) rats was suspended in medium containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate and incubated with pancreatic ribonuclease (10 μg) and T_1 ribonuclease (10 μg) for 2 hr at 37°C. The mixtures were then extracted with phenol (section 2.4.2), precipitated with 95% ethanol at -20°C, resuspended in medium (0.2 ml) containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate, and applied to a 5 ml linear gradient of 7-25% (w/v) sucrose in the same medium. The gradients were centrifuged for 4 hr at 45,000 rpm (189,000 g) in a Beckman SW 50.1 rotor at 20°C, analysed and fractionated as described in section 2.5.3. (^{3}H) polyU (0.01 μg) was then added to each fraction, and the mixtures (0.5 ml in Medium H) were incubated at 50°C for 14 hr. The hybrids formed were digested with pancreatic ribonuclease, collected, and the radioactivity was measured as described in section 2.6.

(a) and (b) show the results of two separate experiments.

Fig. 3.4.1.2.



This was done by analogous methods to those used in section 3.4.1. Fig. 3.4.2.1 shows the sedimentation profiles of hybrids of (^{3}H) polyU with polyA(+)RNA from the skeletal muscle of normal and diabetic rats. The size distribution was similar in the two cases, the range of sedimentation coefficients being from about 5S-18S. In this experiment there appear to be two distinct peaks at about 6S and 8S, although it is not clear whether these really represent separate species.

The presence of large polyA(+)RNA species sedimenting faster than 18S under the conditions used is unlikely, since when the centrifugation time was decreased, no substantial radioactivity above the background could be detected (Fig. 3.4.2.2).

Similar results were obtained when the polyA(+)RNA was analysed by the second method, involving fractionation on sucrose density gradient followed by hybridization analysis of individual fractions (Fig. 3.4.2.3). The sedimentation distribution of the polyA(+)RNA was again in the range 4S-18S and was similar for RNA derived from the skeletal muscle of normal and diabetic rats with a median value around 9.5S.

As the most predominant polyA(+)RNA species from the skeletal muscle ribosomes of normal and diabetic rats were of small molecular size (Fig.3.4. 2.1), it was thought that this might possibly be due to degradation of the mRNA during isolation of ribosomes. Therefore polyA(+)RNA extracted from unfractionated skeletal muscle was subjected to sucrose density gradient analysis after having been hybridized to (^{3}H) polyU. However it is clear from Fig. 3.4.2.4 that the predominant polyA(+)RNA species were again of small molecular size.

Fig. 3.4.2.1 Sedimentation profiles of hybrids between (^3H) polyU and polyA(+)RNA from rat skeletal muscle polyribosomal RNA

Polyribosomal RNA (50 μg) from normal (●—●—●) or streptozotocin - diabetic (O—O—O) rats was mixed with 0.01 μg (^3H) polyU. The volume was adjusted to 0.2 ml in medium containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate, and applied to a 5 ml linear gradient of 7-25% (w/v) sucrose in the same medium. The gradients were centrifuged for 4 hr at 45,000 rpm (189,000 g) in a Beckman SW 50.1 rotor at 20°C, analysed, fractionated and the radioactivity was measured as described in section 2.5.3.

E.coli tRNA (4S) and rat skeletal muscle ribosomal RNA (18S) were used as markers on a parallel gradient.

Fig. 3.4.2.1.

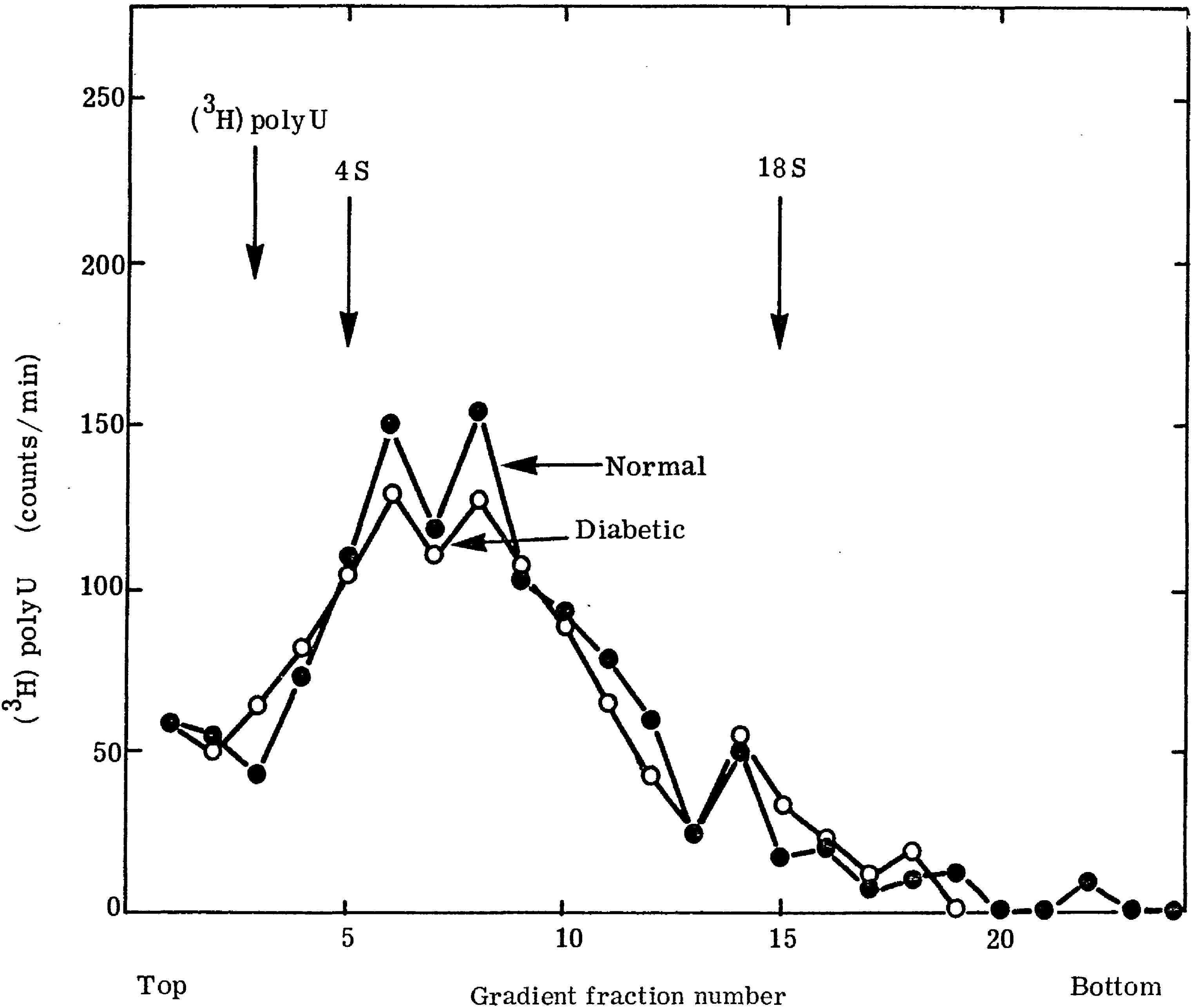


Fig. 3.4.2.2. Sedimentation profiles of hybrids between (^{3}H) polyU and polyA(+)RNA from rat skeletal muscle polyribosomal RNA, analysed under conditions allowing detection of larger species

Polyribosomal RNA (25 μg) from normal (●—●—●) or streptozotoein-diabetic (O—O—O) rats was mixed with 0.025 μg (^{3}H) polyU. The volume was adjusted to 0.2 ml in medium containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate, and applied to a 5 ml linear gradient of 7-25% (w/v) sucrose in the same medium. The gradients were centrifuged for 3 hr at 45,000 rpm (189,000 g) in a Beckman SW 50.1 rotor at 20°C, analysed, fractionated, and the radioactivity was measured as described in section 2.5.3.

E.coli tRNA (4S) and rat skeletal muscle ribosomal RNA (18S and 28S) were used as markers on a parallel gradient.

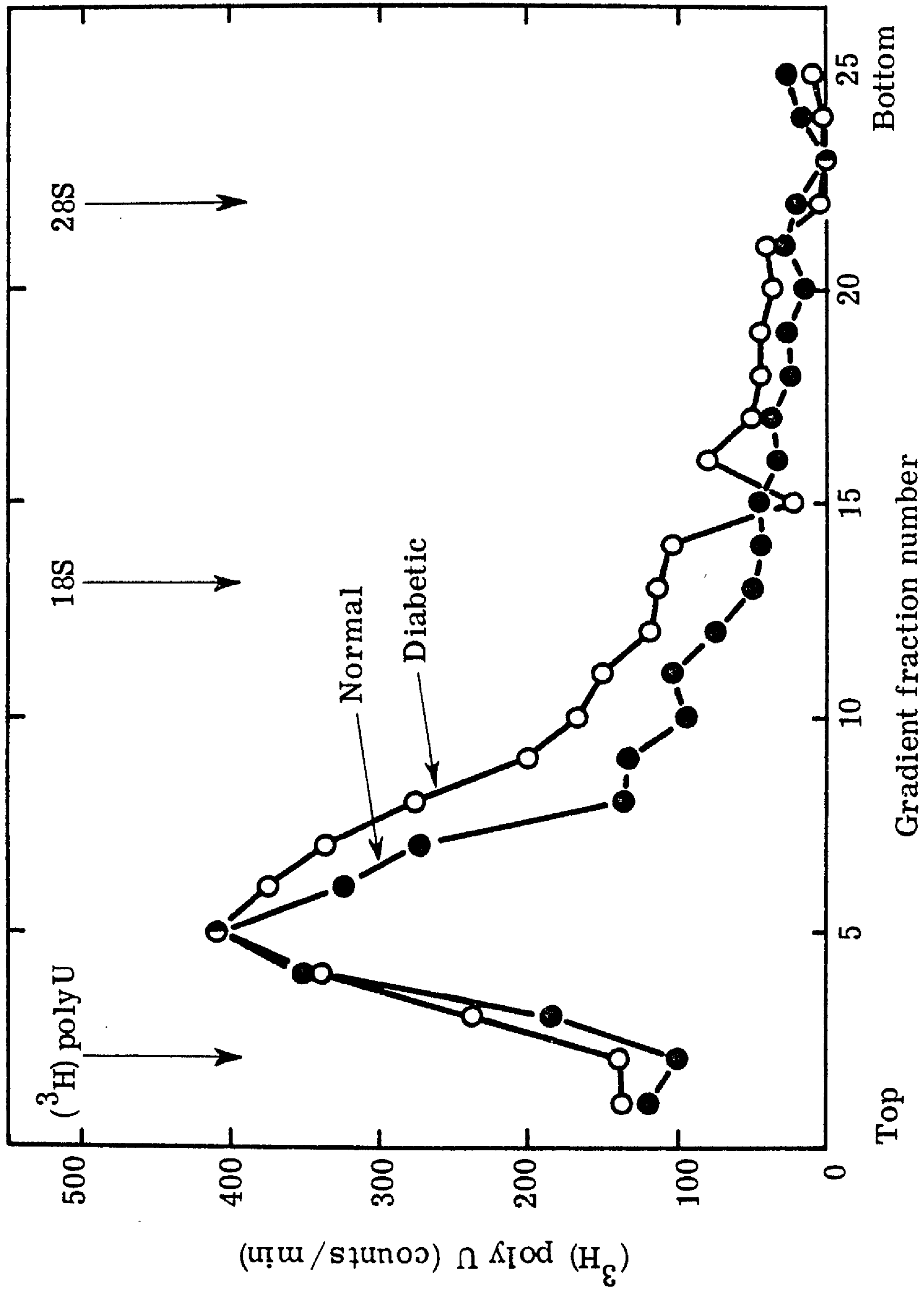


Fig. 3.4.2.2.

Fig. 3.4.2.3 Sucrose density gradient sedimentation profiles of polyA(+)RNA from rat skeletal muscle polyribosomal RNA

Polyribosomal RNA (50 μg) from normal (●—●—●) or streptozotocin-diabetic (O—O—O) rats was suspended in 0.2 ml of medium containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate, and applied to a 5 ml linear gradient of 7-25% (w/v) sucrose in the same medium. The gradients were centrifuged for 4 hr at 45,000 rpm (189,000 g) in a Beckman SW 50.1 rotor at 20°C, analysed and fractionated as described in section 2.5.3. (^{3}H) polyU (0.01 μg) was then added to each fraction, and the mixtures (0.5 ml in Medium H) were incubated at 50°C for 14 hr. The hybrids formed were digested with pancreatic ribonuclease, collected, and the radioactivity was measured as described in section 2.6.

Fig. 3.4.2.3.

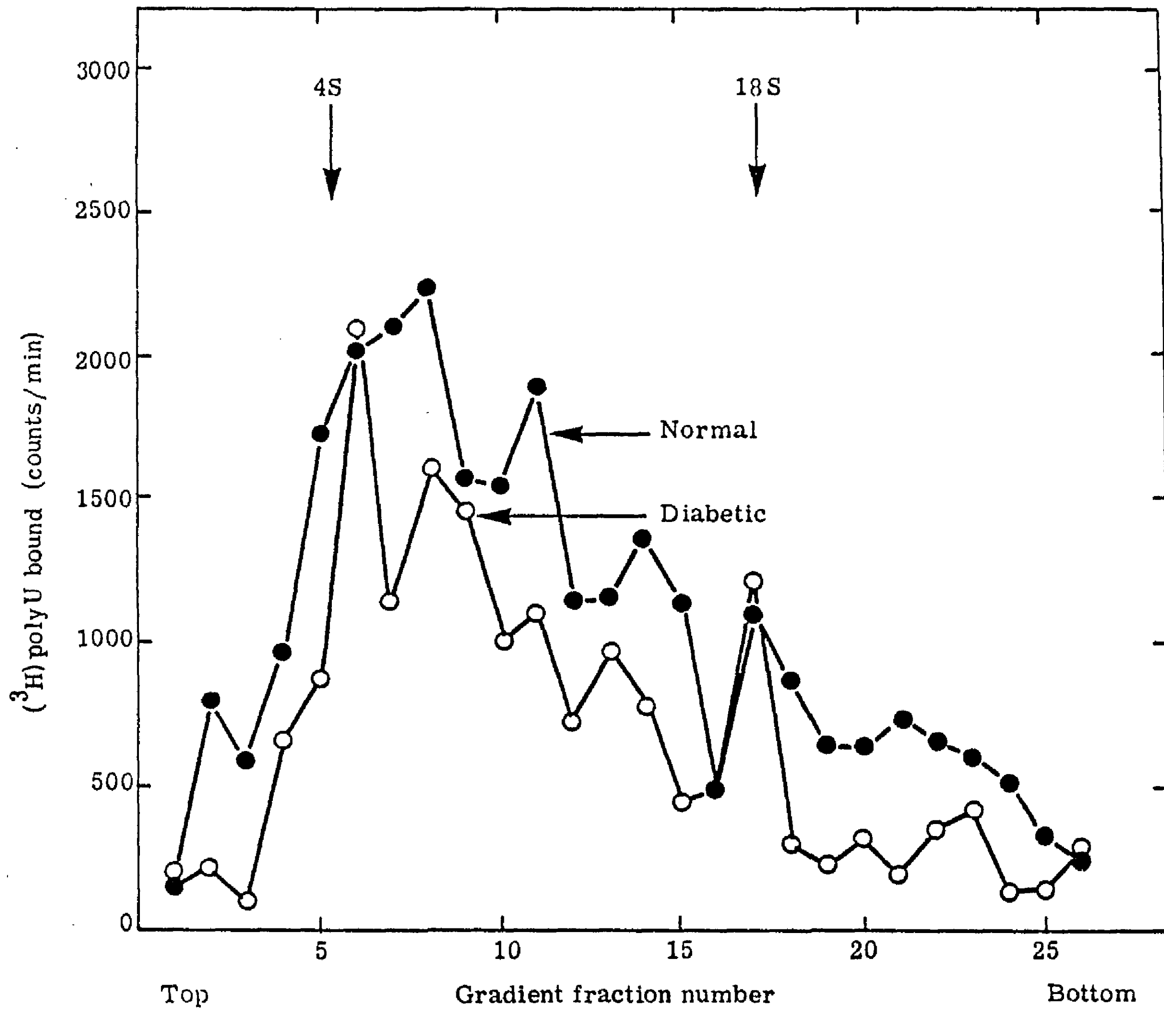
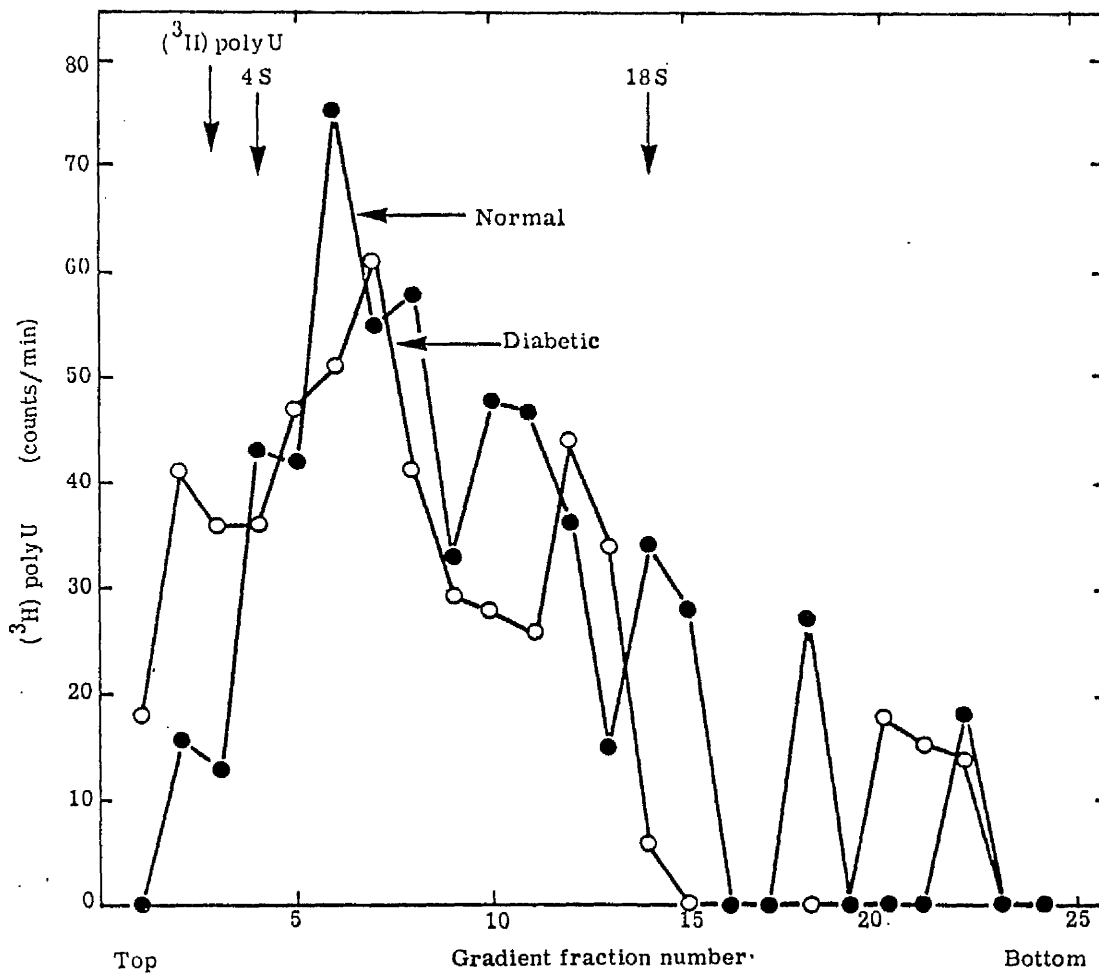


Fig. 3.4.2.4 Sedimentation profiles of hybrids between (^{3}H) polyU and polyA(+)RNA from unfractionated rat skeletal muscle

RNA (20 µg) was extracted from unfractionated muscle of normal (●—●—●) or streptozotocin-diabetic (O—O—O) rats as described in section 2.4.1., and mixed with 0.004 µg (^{3}H) polyU. The volume was adjusted to 0.2 ml in medium containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 0.5% sodium dodecyl sulphate, and applied to a 5 ml linear gradient of 7-25% (w/v) sucrose in the same medium. The gradients were centrifuged for 4 hr at 45,000 rpm (189,000 g) in a Beckman SW 50.1 rotor at 20°C, analysed, fractionated, and the radioactivity was measured as described in section 2.5.3.

E.coli tRNA (4S) and rat skeletal muscle ribosomal RNA (18S) were used as markers on a parallel gradient.

Fig. 3.4.2.4.



3.4.3 Comparison of template activity of polyA(+)RNA from the skeletal muscle of normal and diabetic rats

It was of interest to know whether the mRNA from the skeletal muscle of normal and diabetic rats had similar template activity. An attempt was therefore made to isolate the polyA(+)RNA, free from ribosomal RNA and to translate it in a cell-free system.

The mRNA was separated on polyU-Sepharose, as described in Experimental (section 2.4.3), and translated in a cell-free system from wheat germ (Experimental; section 2.7.2). It was found that polyA(+)RNA from the muscle of normal and diabetic rats exhibited a small but reproducible stimulation above the endogenous level of incorporation of radioactivity (Table 3.4.3.1). As far as can be judged, this stimulation would appear to be similar for the RNA from the muscle of normal and diabetic rats, although because of its relatively small size compared to the endogenous incorporation, the values obtained were not very consistent. Clearly it would be desirable to obtain better template activity of the polyA(+)RNA for valid comparison between that from normal and diabetic rats, and future work on this problem should include further experiments to optimize the conditions for isolation and translation of polyA(+)RNA.

Table 3.4.3.1 Translation of rat skeletal muscle polyA(+)RNA in a cell-free system from wheat germ

Reaction mixtures contained: 0.5 μg of polyA(+)RNA isolated as described in section 2.4.3, 15 μl of wheat germ S-30 fraction, 20 mM HEPES (pH 7.6), 2 mM dithiothreitol, 1 mM ATP (neutralized), 20 M GTP, 8 mM creatine phosphate, 0.5 μg creatine phosphokinase, 0.4 mM spermidine, 75 mM KCl, 1.5 mM μg acetate, 25 μCi of (^{35}S) methionine (1080 Ci/mmole), and water to 50 μl. Incubation was at 25°C for 2 hr, then 5 μl aliquots were removed for determination of radioactivity incorporated into protein as described in detail in section 2.7.2.

Table 3.4.3.1

Added RNA	(^{35}S) incorporation cpm	cpm above endogenous incorporation	% of normal
Experiment 1			
None	120,000	-	-
RNA from normal rats	149,000	29,000	100%
RNA from diabetic rats	177,000	57,000	197%
Experiment 2			
None	355,000	-	-
RNA from normal rats	437,000	82,000	100%
RNA from diabetic rats	420,000	65,000	79%

CHAPTER 4

DISCUSSION

4.1 CHARACTERIZATION OF mRNA FROM ADULT RAT SKELETAL MUSCLE

One original feature of the work described in this thesis is that it provides information concerning mRNA from the skeletal muscle of adult rats; previous studies on muscle mRNA having been almost exclusively concerned with embryonic tissue (see Introduction). The characteristics of this mRNA will therefore be considered before proceeding to a discussion of the effect of diabetes on the metabolism of the mRNA.

One aspect of the skeletal muscle mRNA described in this work is that it appears to be relatively small in size. Thus, sucrose density gradient analyses revealed polyA(+)RNA species with sedimentation coefficients in the approximate range 5-18S, the median value being 9.5S (calculated from data in Fig. 3.4.2.3). These values are small compared to those of the polyA(+)RNA of myoblasts, where a range of sedimentation coefficients of 6-32S has been observed (Buckingham, Cohen and Gros, 1976).

It is possible that the small size of this isolated mRNA does not represent the actual size of the mRNA in the cell. For example, it might have resulted from shearing during homogenization by the rapidly rotating blades of the Virtis "23" homogenizer. However, when the RNA was extracted directly from muscle that had not been homogenized, but frozen in liquid nitrogen and pulverized, the polyA(+)RNA species obtained had a similar range of sedimentation coefficients (Fig. 3.4.2.4), suggesting that shearing was not responsible for the small size of mRNA. Alternatively, the small size of mRNA could be due to nuclease action,

despite the precautions taken to avoid this (see section 3.1.3). Indeed, that portion of the polyA(+)RNA sedimenting at about 5S in Fig. 3.4.2.3 could well be mainly nuclease-resistant polyA tracts; for these latter sediment at only a slightly lower value of about 4S (Fig. 3.4.1.2). Nevertheless other results are consistent with a predominance of relatively small mRNAs.

Thus the polysomes from which muscle mRNA was extracted were of relatively small size (Fig. 3.1.1.1), the most predominant species being those containing three, four and five ribosomes respectively; although smaller peaks corresponding to polysomes bearing up to perhaps 15 ribosomes could sometimes be detected (Fig. 3.1.2.1). The size of these predominant polysome species is small compared to that in embryonic chick leg muscle (Heywood, and Rich, 1968), where polysomes containing 5-60 ribosomes were seen; those containing 15-25 ribosomes being most prevalent. The polysomes with 5-9 ribosomes were shown to be active in the synthesis of tropomyosin (molecular weight ca. 35,000), those with 15-25 ribosomes were shown to be active in the synthesis of actin (molecular weight ca. 43,000), and those with 50-60 ribosomes were shown to be active in the synthesis of the myosin heavy chain (molecular weight ca. 200,000). Further studies of embryonic chick skeletal muscle showed polysomes containing 4-6 ribosomes synthesizing myoglobin - molecular weight ca. 17,000 - (Thompson, Buzash and Heywood, 1973), and some with 4-9 ribosomes synthesizing the myosin light chains - molecular weight range ca. 17,000-30,000 - (Low, Vournakis and Rich, 1971).

The small size of polysomes could however be an artifact, perhaps due to limited ribonuclease activity. This could occur during isolation of ribosomes, either by the liberation of intracellular nucleases during homogenization, or by accidental exposure to ribonuclease from contaminated glassware during the preparation. However, isolation of ribosomes under sterile conditions, with heparin added to all solutions to inhibit ribonuclease (Palmiter, Christensen and Schimke, 1970), did not result in any increase in the size of polysomes (Fig. 3.1.3.4). Another possibility is that the small size of polysomes could be due to "run-off" of ribosomes from the mRNA. Inclusion of cycloheximide, however, did not increase the polysome size (Fig. 3.1.3.1 and 3.1.3.3). It is worth mentioning, however, that some heavy polysomes, might have sedimented to the bottom of the tube during centrifugation. The absence of large species of polyA(+)RNA (see Fig. 3.4.2.2 above) argues against this, but it is difficult to exclude the possibility that such hypothetical heavy polysomes carry exclusively polyA(-)mRNA, which would have escaped detection here.

The foregoing discussion has assumed that the size of the mRNA will be the main determinant of the size of the polysomes. However a changed rate of initiation of protein synthesis can alter the size of polysomes on a single mRNA (Lodish, 1971); thus the polysomes in skeletal muscle of adult rats might code for proteins larger than those of corresponding size in embryonic muscle; if the rate of initiation were slower in the

case of the adult muscle. This might allow for the synthesis of somewhat larger proteins (see below), but would hardly be expected to be of major significance, especially as no very large mRNA was detected.

When rat muscle polysomes were translated in a cell-free system in this investigation, the major products were polypeptides of 15,500 and, to a lesser extent, of 17,500 molecular weight; although others of larger size were detected after prolonged exposure of the autoradiograph (Fig. 3.2.1.5). Published values of sedimentation coefficients of mRNA species show a fairly good linear relationship to the logarithm of the molecular weight of the protein product (Fig. 4.1.1), allowing one to estimate that mRNA species of about 10S and 11S would be required to code for the major polypeptides described above. Although the median value of the muscle mRNA was only about 9.5S (corresponding to a protein of molecular weight 12,500), these sizes of 10S and 11S for the mRNA can be accommodated within the range of sizes of polyA(+)RNA observed (Fig. 3.4.2.3). Again, it is possible to use published data to estimate that proteins of molecular weight 15,500-17,500 would be made on polysomes containing 5-7 ribosomes and hence could be made on the polysomes observed here (Fig. 4.1.2).

Although this predominantly low molecular weight exhibited by the cell-free products of translation would support the other two lines of evidence suggesting that the muscle mRNA is, in fact, small, this piece of evidence, like those discussed above, is unfortunately equivocal. While the cell-free translation system from wheat-germ is efficient in synthesizing proteins of small molecular weight, it has been shown with

Fig. 4.1.1 Comparison of the molecular weights of some proteins with the sedimentation coefficients of their mRNAs

This graph includes published data for the sedimentation coefficients of some mRNAs and the molecular weights of their protein products.

(1) Histone H4 from sea urchin embryos (Grunstein *et al.*,1973).

(2) α-globin from rabbit reticulocyte (Gould and Hamlyn, 1973).

(3) β-globin from rabbit reticulocyte (Gould and Hamlyn, 1973).

(4) Immunoglobulin light chain from mouse myeloma (Brownlee *et al.*, 1973).

(5) αA_2-crystallin from calf lens (Berns, Janssen and Bloemendal, 1974).

(6) Ovalbumin from chick oviduct (Haines, Carey and Palmiter, 1974).

(7) δ-crystallin from calf lens (Zelenka and Piatigorsky, 1974).

(8) Immunoglobulin heavy chain from mouse myeloma (Stevens and Williamson, 1973).

(9) Collagen from embryonic chick tendons (Harwood *et al.*, 1974).

(10) Myosin heavy chain from embryonic chick muscle (Heywood and Nwagwu, 1969).

Fig. 4.1.1.

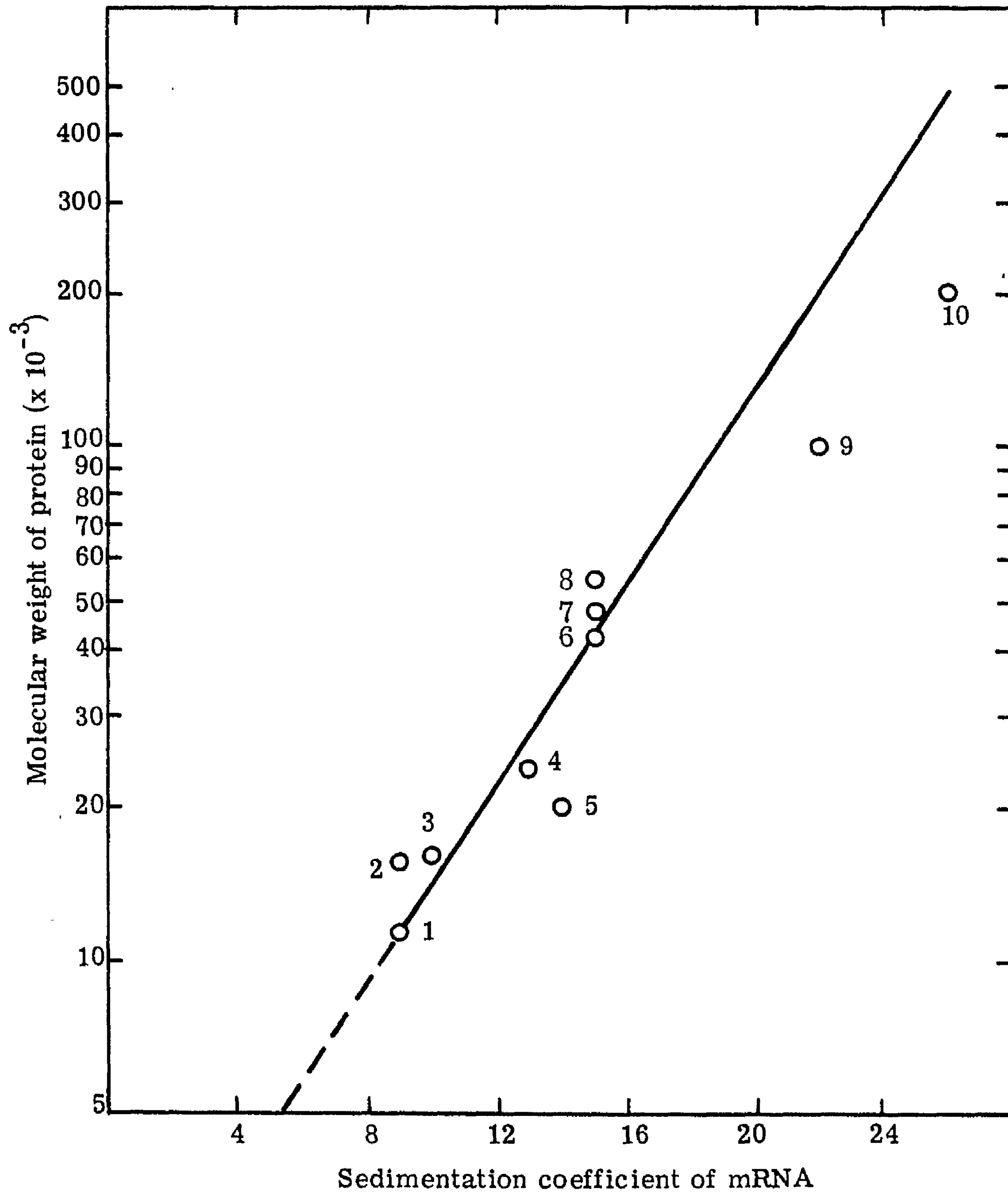


Fig. 4.1.2 Comparison of the molecular weights of some proteins with the number of ribosomes comprising their polysomes

This graph includes published data for the molecular weights of some proteins and the number of ribosomes comprising their polysomes.

(1) Haemoglobin from rabbit reticulocyte (Warner, Rich and Hall, 1962).

(2) Myoglobin from embryonic chick muscle (Thompson, Buzash and Heywood, 1973).

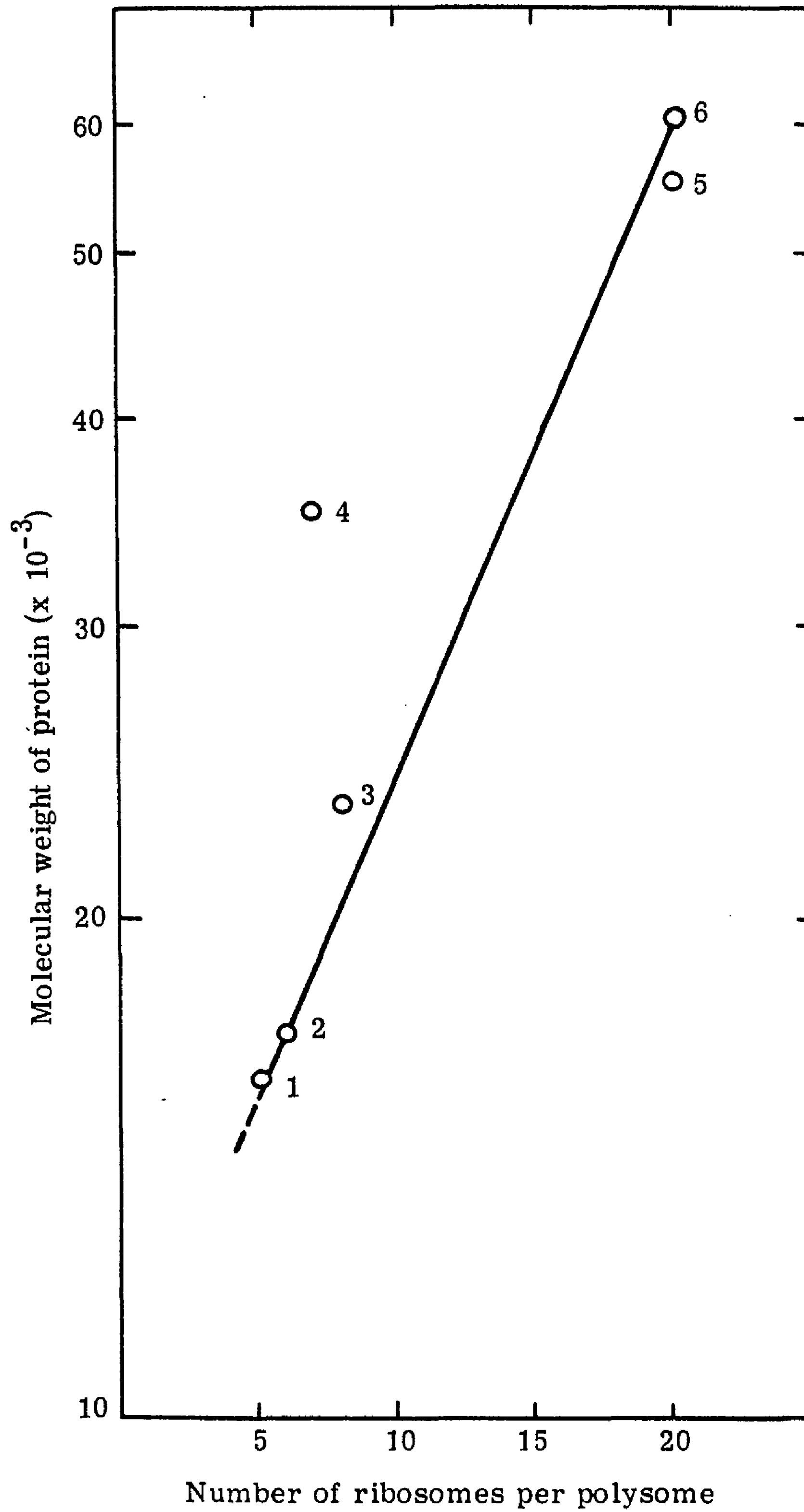
(3) Immunoglobulin light chain from mouse myeloma (Becker, Ralph and Rich, 1970)

(4) Tropomyosin from embryonic chick muscle (Heywood, and Rich, 1968).

(5) Immunoglobulin heavy chain from mouse myeloma (Becker, Ralph and Rich, 1970).

(6) Actin from embryonic chick muscle (Heywood, and Rich, 1968).

Fig. 4.1.2.



other mRNAs to synthesize fewer higher molecular weight proteins than the intact cells (see for example, Prives et al., 1974). Thus the cell-free products of low molecular weight in this study might be due to premature termination of nascent polypeptide chains.

Nevertheless, if it is assumed that the adult muscle mRNA really is small, can one reconcile such an apparent small mRNA with the large proteins which characterize the skeletal muscle? This is possible, in the context of the proposal (Zak, Ratkitzis and Rabinowitz, 1971) that in the muscle of adult rats the rate of turnover of the structural proteins of the myofibrils (which include most of the large proteins) is slower than that of the soluble proteins, which tend to be small. Consistent with this idea, Velick (1956) reported that in rabbit skeletal muscle the half-life of the structural protein, myosin, was 80 days, while that of the soluble protein, aldolase, was 50 days; and Swick and Song (1974) found a similar difference in rat skeletal muscle, although they obtained different absolute values (myosin: 35 ± 10 days; and aldolase: 18 ± 3 days). However measurements on a much wider range of other proteins (especially the soluble proteins) are clearly required before one can accept this idea. In fact recent investigations indicate that in skeletal muscle the various myofibrillar proteins may not be synthesized and degraded as a unit, but that they may turn over separately (Millward, 1970; Funabiki and Cassens, 1972; Koizumi, 1974; Zak et al., 1977). However it is still possible that the larger proteins are more stable (and would have less mRNA), as the large myosin heavy chain (molecular weight ca. 200,000) appears quite stable by comparison with the much smaller tropomyosin chain (molecular weight ca. 35,000) or the even smaller myosin light chain - molecular

weight about 20,000 (Low and Goldberg, 1973). However Dice et al (1978) have found large proteins to be generally less stable (i.e. to turn over more rapidly) than small proteins.

Assuming that the mRNA of adult skeletal muscle codes predominantly for soluble proteins, it is of interest to compare the size of the subunits of such proteins with that of the cell-free products of the polysomes, as seen in Fig. 3.2.1.5. Some of the proteins which are most abundant in skeletal muscle (depending, to some extent, on the proportion of red and white muscle fibres) are aldolase (molecular weight of the subunits ca. 40,000), α-glycerophosphate dehydrogenase (molecular weight of the subunits ca. 40,000), glyceraldehyde-3-phosphate dehydrogenase (molecular weight of the subunits ca. 37,000), enolase (molecular weight of the subunits ca. 41,000), pyruvate kinase (molecular weight of the subunits ca. 57,000), lactate dehydrogenase (molecular weight of the subunits ca. 35,000) and creatine kinase (molecular weight of the subunits ca. 40,000). Some of these protein subunits might clearly correspond to the well-defined bands at molecular weights of about 41,000 and 34,000 (Fig. 3.2.1.5). Also from Fig.4.1.1, it can be seen that the molecular weight 40,000 corresponds to a value of about 15S, which is not inconsistent with the range of sizes of polyA(+)RNA observed (Fig. 3.4.2.3). As myoglobin has a molecular weight of about 17,000, and as the major proteins synthesized in the cell-free system have molecular weights of about 15,500 and 17,500 (Fig. 3.2.1.5), it is possible that one of these proteins is myoglobin. This is feasible as the rat gastrocnemius muscle is composed of, approximately, 50% fast white, 40% fast red (rich in myoglobin), and 10% slow red fibres (Baldwin et al., 1972). However further more accurate determination of the molecular weights are required (see legend to Fig. 2.7.3.1.).

It was of interest in this study to estimate the percentage of skeletal muscle RNA that is mRNA. To do this it was necessary to estimate the size of the mRNA and that of the polyA tract. It has been shown that, under non-denaturing conditions, there is no accurate relationship between the sedimentation coefficients and the molecular weights of RNA species (Boedtker, 1968), and that mRNAs exhibit anomalous sedimentation coefficients relative to ribosomal RNA on aqueous sucrose gradients (Macleod, 1975). It was not, however, possible during the course of this study to analyse the skeletal muscle mRNA under the denaturing conditions of 70% formamide sucrose gradients (Suzuki, Gage and Brown, 1972), as this would have interfered with the hybridization of the polyA tract of the mRNA to (^{3}H) polyU. Therefore it was necessary to use the results obtained in non-denaturing sucrose density gradients and compare these with published values for other mRNAs, the size of which had also been determined by independent means (Fig. 4.1.3). When the median value (9.5S) of the sedimentation coefficient of rat skeletal muscle mRNA (Fig. 3.4.2.3) is compared with that of other classes of well characterized mRNAs, it is found that this sedimentation coefficient corresponds to a size of about six hundred nucleotides.

The polyA tracts obtained from skeletal muscle mRNA by ribonuclease digestion (see legend to Fig. 3.4.1.2) had, on average, a slightly lower sedimentation coefficient than that of E.coli tRNA (known to be 4S). Although the average size of E.coli tRNA corresponds to about 80 nucleotides (Dayhoff, 1969), one can not conclude that the polyA tracts contain less than 80 nucleotides because of differences in the

Fig. 4.1.3 Comparison of the number of nucleotides of some mRNAs with the sedimentation coefficients of the mRNAs

This graph includes published data for the number of nucleotides of some mRNAs and the sedimentation coefficients of their mRNAs.

(1) Histone H4 mRNA from sea urchin embryos (Grunstein *et al.*, 1973).

(2) α-globin mRNA from rabbit reticulocyte (Gould and Hamlyn, 1973).

(3) β-globin mRNA from rabbit reticulocyte (Gould and Hamlyn, 1973).

(4) Immunoglobulin light chain mRNA from mouse myeloma (Brownlee *et al.*, 1973).

(5) αA_2-crystallin mRNA from calf lens (Berns, Janssen and Bloemendal, 1974).

(6) Immunoglobulin heavy chain from mouse myeloma (Stevens and Williamson, 1973).

(7) δ-crystallin mRNA from calf lens (Zelenka and Piatigorsky, 1974).

(8) Ovalbumin mRNA from chick oviduct (McReynolds, *et al.*, 1978).

(9) Collagen mRNA from embryonic chick tendons (Harwood *et al.*, 1974).

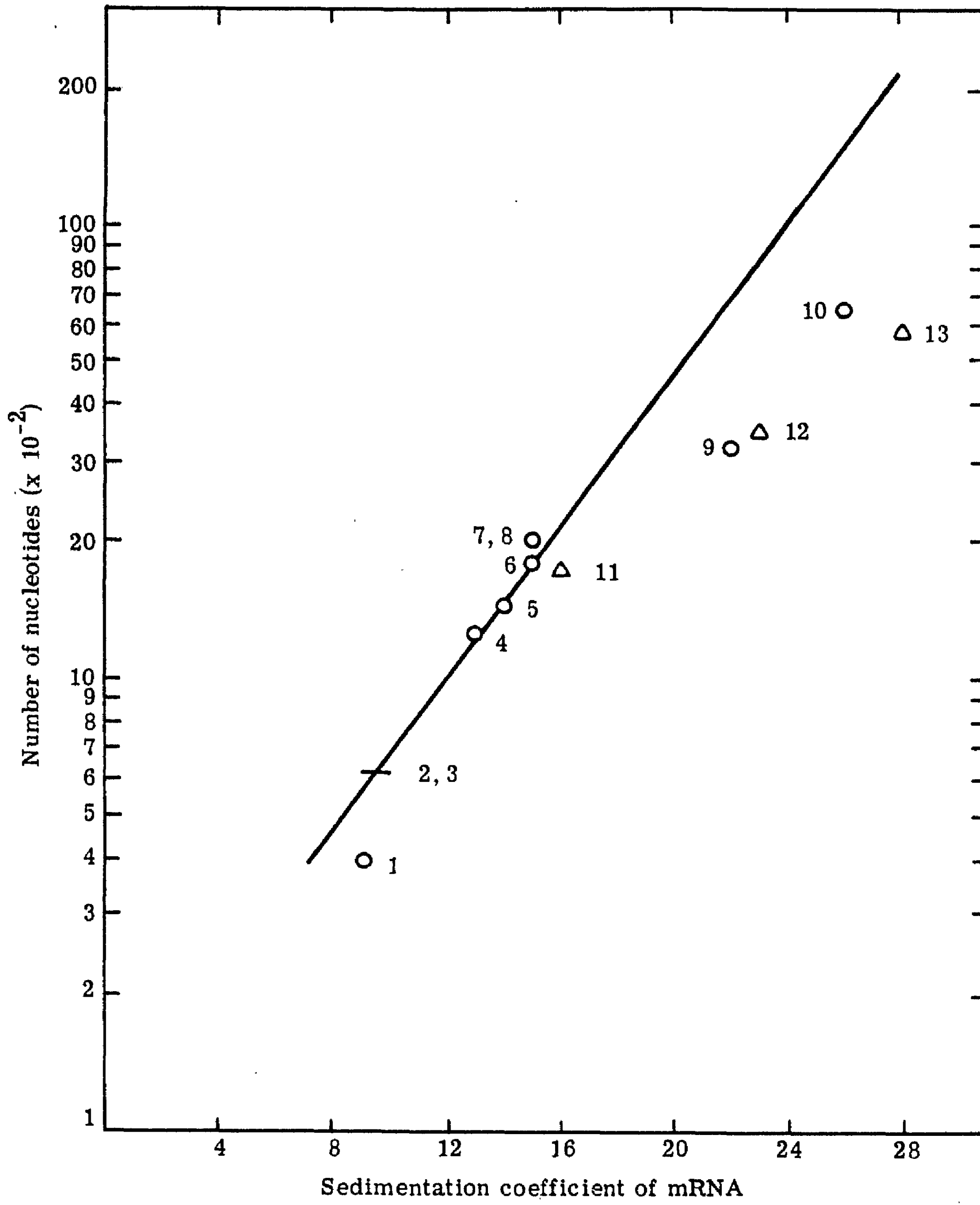
(10) Myosin heavy chain mRNA from embryonic chick muscle (Mondal *et al.*, 1974).

(11) 16S rRNA from *E.coli* (Spohr *et al.*, 1976).

(12) 23S rRNA from *E.coli* (Spohr *et al.*, 1976).

(13) 28S rRNA from HeLa cells (Spohr *et al.*, 1976).

Fig. 4.1.3.



sedimentation properties of homo- and heteropolynucleotides, and in molecules with different degrees of secondary structures. The average sedimentation coefficient of total cytoplasmic polyA from mouse sarcoma ascites cells was rather similar to that described here, at 3.4S (Brawerman, 1976), and when converted into nucleotide size using the data of Fresco and Doty (1957), a value of about 120 nucleotides was obtained. This value is consistent with that reported for myoblast polyadenylated mRNA, where the polyA moiety comprises 90-110 nucleotides (Paterson and Bishop, 1977). Therefore it seems more reasonable to assume a value of 120 nucleotides for the polyA tract in the present work.

Thus using the value of 120 nucleotides for the average length of polyA tracts in rat skeletal muscle, and 600 nucleotides for the average length of the mRNA, one can conclude that the polyA tract accounts for 20% of the length of skeletal muscle mRNA of adult rats. From the hybridization assay described in section 3.3.1, it was found that about 0.100% of the muscle polyribosomal RNA consists of regions of polyA - seven experiments giving an average value of 0.108% (Table 3.3.1.1) - and hence the proportion of mRNA to polyribosomal RNA in rat skeletal muscle is 0.54%. This value appears to be relatively small compared with that of 3% reported by Rudland _et al._ (1975) for BALB/c 3T3 cultured mouse fibroblasts (where it was assumed that 10% of the length of mRNA is polyA), or that of 3% reported by Whalen and Gros (1978) for cytoplasmic polyA(+)RNA in calf myoblasts. However, it should be pointed out that, if the size of mRNA determined in this study is smaller than the actual size (as discussed above), then this would

decrease the proportion of polyA to mRNA and accordingly the percentage of mRNA would be increased. Another possible source of underestimation of the proportion of mRNA could arise if the skeletal muscle of adult rats contained an unusually large proportion of mRNA lacking polyA, which would not have been detected in this work.

The proportion of polyA(+)RNA may also be estimated from the results of the polyU-Sepharose affinity chromatography described in Experimental (section 2.4.3). The amount of RNA eluted as polyA(+)RNA was on average 4% of the total applied. This value is higher than that obtained above, but it is possible that the eluted polyA(+)RNA still contains some ribosomal RNA, non-specifically bound to the polyU-Sepharose.

Implicit in the foregoing discussion is the assumption that polyA(+)RNA is mRNA. There is every reason to think that this assumption is valid (see Introduction), but ultimate proof that an RNA is a mRNA rests on its translation in a cell-free system. Thus, attempts were made to translate the skeletal muscle polyA(+)RNA (isolated from polyribosomal RNA by polyU-Sepharose affinity chromatography) in a cell-free system from wheat germ. However only a small stimulation above the endogenous level of incorporation of radioactivity was obtained (Table 3.4.3.1). This might, again, be due to degradation of mRNA. However the optimum conditions adopted for translation vary with different mRNAs and systematic experiments are required to try to find the correct conditions in this case. Such experiments would be of high priority in any future development of this work. Another possible explanation for the poor

translation in the wheat germ cell-free system is that specific muscle initiation factors are required to translate muscle mRNA. However the bulk of evidence does not favour this idea (see Introduction).

The supposition that the skeletal muscle polyA(+)RNA is mRNA is supported by its distribution during cell fractionation (Fig. 3.3.2.1; Table 3.3.2.2), which will now be considered in a little more detail. First it should be pointed out that the 'mitochondrial pellet' fraction contains much more of the total RNA and polyA(+)RNA than would be at first expected. However this is due to the fact that at 80 mM KCl (Medium A), this fraction contains a large amount of trapped ribosomes, which can be partially recovered (Table 3.1.1.1) at 250 mM KCl (Medium C). It is also striking that the percentage of polyA in this fraction is higher than in purified ribosomes. This can be explained by the presence in this fraction of the nuclei which are rich in heterogeneous polyA(+)RNA species, but contain relatively little rRNA and tRNA precursor species. The majority of the remainder of the polyA(+)RNA (ca. 70%) is in the 'ribosome pellet' fraction, as expected. However some polyA(+)RNA (ca. 15%) is present in the 'post-microsomal supernatant'. This might represent free mRNA, similar to that found in the 'post-ribosomal supernatant' by Bonanou-Tzedaki, Pragnell and Arnstein (1972); or mRNA in the form of mRNP particles of sedimentation coefficient less than 40S, as found by Jacobs-Lorena and Baglioni (1972) in the 'post-ribosomal supernatant' of rabbit reticulocyte lysates. The proportion of polyA to total RNA is low in this fraction (compared to other fractions) and this is consistent with the expectation that the major species of RNA here would be tRNA. Finally it should be noted that some polyA(+)RNA (ca. 15%) is found in the 'post-ribosomal supernatant'.

This is presumably released from the polysomes during resedimentation, and a similar occurrence may explain the high proportion of polyA in the slowest sedimenting fraction of sucrose density gradients on which the purified polysomes were analysed. (Fig. 3.3.2.3; Table 3.3.2.5).

4.2. METABOLISM OF SKELETAL MUSCLE mRNA IN DIABETES

The main objective of this investigation was to determine the quantitative and qualitative effects of diabetes on mRNA metabolism; in particular to see whether insulin modulates protein synthesis by stimulating the translation of pre-existing mRNA.

In the present work it was found that diabetes decreased the total amount of RNA in skeletal muscle, as others had found previously (Pain and Garlick, 1974). The new finding however, was that there was a similar reduction in the amount of polyA(+)RNA. Thus the concentrations of different RNA species were decreased in concert. This extends earlier work, in heart muscle, showing that the concentrations of rRNA and tRNA were reduced to the same extent during diabetes (Wool, Stirewalt and Moyer, 1968), and implies that in diabetes there is a co-ordinated decrease in the concentrations of all the RNA species involved in muscle protein synthesis. It would be of interest to know whether the concentration of the various protein species involved in protein synthesis (e.g. initiation and elongation factors) are also reduced proportionally in diabetes.

The major result of this study was that the relative proportion of polyA(+)RNA in muscle from diabetic rats is similar to that in muscle from diabetic rats treated with insulin. This implies that insulin stimulates protein synthesis in these circumstances by causing the recruitment of pre-existing mRNA into polysomes, and not by inducing the synthesis of new mRNA. Thus insulin would appear to regulate protein synthesis in the skeletal muscle of diabetic rats through translational control. In the light of this finding it is worth referring to the two

models for the polysomes from the skeletal muscle of diabetic rats, put forward in the Introduction (Fig. 1.2.1). Model (i) implied less mRNA in the polysomes, whereas Model (ii) implied an equal amount of mRNA, the number of ribosomes being decreased. It is clear from the analysis of the ratio of polyA(+)RNA to total RNA in the different regions of the sucrose density gradients (Fig. 3.3.2.3; Table 3.3.2.5), that Model (ii) is not tenable, and that the results are more consistent with Model (i).

Both these foregoing conclusions rely on one assumption which, though reasonable, lacks experimental basis. That is that the polyA(+)RNA is representative of the total muscle mRNA (polyA(+)RNA and polyA(-)RNA) in normal and in diabetic rats. Clearly, if the concentration of polyA(-)RNA were affected by diabetes in a different way to other RNA species, the conclusion above regarding co-ordinated changes in RNA concentrations would need modifying. It is probable, however, that most muscle mRNA is polyA(+)RNA, as Wahlen and Gros (1978) found that the great majority of mRNA from either prefusion or postfusion cultures of calf myoblasts was polyA(+)RNA. Of course, this situation need not necessarily be similar in the skeletal muscle of adult rats, especially given the different size distribution of mRNA species (see section 4.1). Even if the proportion of polyA(+)mRNA to polyA(-)mRNA did alter in diabetes, the results still show that insulin controls the translation of polyA(+)RNA.

These results imply that there is in the skeletal muscle of the diabetic rats a pool of potentially active 'masked' mRNA. There is a

precedent for inactive mRNA in muscle during development of myoblasts, and in this case it is found in messenger ribonucleoprotein particles of sedimentation coefficients 70-90S (Heywood, Kennedy and Bester, 1975). It was therefore of interest to try to determine whether the mRNA stored in the skeletal muscle of diabetic rats was also in the form of mRNP particles, as might be anticipated. Unfortunately this problem could not be solved due to the aggregated form in which the polyA(+)RNA was found in ribosomes during the sucrose gradient analyses. Further attempts are clearly required to overcome this problem of aggregation.

It is important to know whether the structure of mRNA in the muscle of diabetic rats differs from that of mRNA in the muscle of normal rats. For example, differences in the non-coding sequences of the mRNA might be related to the control of the translation of muscle mRNA. If the skeletal muscle mRNA is stored in an inactive form in the muscle of diabetic rats, one might expect that this mRNA would have a longer half-life than that in polysomes, and thus than the majority of the mRNA in the skeletal muscle of normal rats. As the polyA tract in mRNA becomes gradually shorter as the RNA ages in the cytoplasm (see Introduction), one would anticipate that the longer half-life might be reflected in a decrease in the length of the polyA tract. Moreover, it has been found that the decrease in polyA size with age occurs whether or not the mRNA is associated with polysomes (Brawerman, 1973), and that it is independent of the translation process (Sheiness and Darnell, 1973). In this study, the sedimentation coefficient of the polyA tract was found to be similar in muscle from normal and diabetic rats (section 3.4.1). This may mean that - contrary to expectation - the stored mRNA

is turning over at a similar rate to that mRNA which is being translated.

Another possible difference in the non-coding sequences of the mRNA might be in the 'cap' at the 5'-end (see Introduction). The 'cap' structure might be eliminated in mRNA from the muscle of diabetic animals resulting in the inactivation of the mRNA and its transfer to the stored pool. Alternatively, a difference in the number of penultimate 2'-O-methylated nucleotides in the two types of mRNAs might exist. Such a difference as this latter was found for α-and β-globin mRNA (Lockard, 1978), and may possibly explain the fact that each molecule of β-globin mRNA initiates protein synthesis at a rate 30% faster than α-globin mRNA (Lodish and Jacobsen, 1972; Lodish, 1974).

More subtle chemical modifications of the structure of the muscle mRNA during diabetes can not be ruled out. These might be, for example, in the internal methyl^{6}adenine or methyl^{5}cytosine that is present in some eukaryotic cellular mRNAs (see Introduction). It would be of interest to know whether there is a change in the proportion of such modified residues.

Another question of interest is whether there is a qualitative difference between the proteins coded by the mRNAs stored during diabetes and the mRNAs which are translated. Such a difference would be expected to be reflected in different products synthesized by ribosomes of normal and diabetic rats; but from Fig. 3.2.1.5 it appears that the polypeptides synthesized in a cell-free system by the ribosomes from diabetic rats were similar to those synthesized by ribosomes from normal rats. Although some polypeptides of higher molecular weight are visible in the products

of ribosomes from normal rats, this difference was not reproducible. This similarity between the proteins synthesized by the muscle of normal and diabetic rats is consistent with earlier observations using discontinuous electrophoresis on polyacrylamide gels (Kurihara and Wool, 1968), but more extensive investigations are required on this point. Further analysis using the improved resolution of two-dimensional gel electrophoresis (O'Farrell, 1975; Whalen, Butler-Browne and Gros, 1976) would be desirable. It would also be interesting to analyse the mRNA from skeletal muscle of normal and diabetic rats for possible differences by preparing complementary DNAs from these and comparing their hybridization to the two populations of mRNAs.

4.3 NUCLEASE ACTIVITIES IN THE MUSCLES OF DIABETIC RATS

One minor and unexpected observation in this study was that insulin administered to rats, made diabetic with alloxan, did not lead to the reformation of polysomes when ribosomes were isolated in a medium of high ionic strength (section 2.3.2). Reformation of polysomes did occur if the rats had been made diabetic with streptozotocin, or if the ribosomes were isolated at low ionic strength, and it was concluded that alloxan - but not streptozotocin - causes the induction or activation of a nuclease, which is only extracted into the 'post-mitochondrial supernatant' at high ionic strength. This causes the breakdown of polysomes which have been reassembled *in vivo* in response to insulin.

Although this interpretation of the results seems virtually inescapable, no independent evidence was obtained for the existence of the putative ribonuclease. The assays used in the unsuccessful attempts to detect the nuclease relied on measurements of the acid-soluble degradation products of RNA. Thus it is possible that the putative nuclease induced by alloxan is a relatively specific endonuclease releasing large oligonucleotides which are insoluble in acid. One could perhaps detect such an activity by analysing the products of hydrolysis of (^{32}P)-labelled rRNA using gel electrophoresis.

These results imply that alloxan-diabetes is more severe, and produces more side effects than streptozotocin-diabetes. This is consistent

with previous claims that streptozotocin-diabetic rats — unlike alloxan-diabetic rats — have normal levels of blood ketones and plasma free fatty acids, and of heart glycogen (Mansford and Opie, 1968). It is also consistent with the observation that insulin did not restore normal blood glucose concentrations in alloxan-diabetes, although it did so in streptozotocin-diabetes (Table 2.2.2.1).

The results of the present work emphasize the need for careful assessment of studies on protein synthesis in rats made diabetic with alloxan. Specifically, where such studies have used a high ionic strength for preparation of ribosomes, their results should not be accepted unless control experiments with insulin indicate otherwise. Most of the experiments from Wool's laboratory cited in the Introduction (Chapter 1) are immune for this criticism, as ribosomes were prepared at low ionic strength. However two late pieces of work were performed with extraction at high ionic strength and without such insulin controls. Thus one must have strong reservation whether there is a defect in the 60S subunits of ribosomes from alloxan-diabetic rats which affects their ability to form 80S couples and to allow 80S ribosomes to be dissociated by initiation factor-3 (Wettenhall, Nakaya and Wool, 1974; Nakaya, Ranu and Wool, 1974).

In view of the net breakdown of RNA during diabetes it might have been expected that the measurements of ribonuclease made in this work (for the reasons indicated above) would have detected an increase in the activity of acid ribonuclease during diabetes, similar to that seen in dystrophic muscle (Abdullah and Pennington, 1968). However, no increase was found in the activity of acid ribonuclease present in unfractionated

homogenates of skeletal muscle (Table 3.2.2.4). However the measurements made were probably of total nuclease activity, some of which is 'latent' in lysosomes of the intact cells (Rannels, Kao and Morgan, 1975). Careful measurements of 'free' ribonuclease would be required to determine whether there is an increase in ribonuclease responsible for the decreased concentration of RNA in rat skeletal muscle during diabetes. Only if such experiments gave negative results could one conclude that the loss of skeletal muscle RNA during diabetes is caused solely by a decrease in the rate of RNA synthesis, rather than an increase in the rate of RNA degradation. If this were the case, the situation would be analogous to that established for the control of the total protein of skeletal muscle under a variety of conditions. (Millward et al., 1976).

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